Advances in Experimental Medicine and Biology 814

Lubo Zhang
Charles A. Ducsay *Editors*

Advances in Fetal and Neonatal Physiology

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Lubo Zhang • Charles A. Ducsay
Editors

Advances in Fetal and Neonatal Physiology

Proceedings of the Center for Perinatal Biology 40th Anniversary Symposium

Springer

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ISSN 0065-2598 ISSN 2214-8019 (electronic)
ISBN 978-1-4939-1030-4 ISBN 978-1-4939-1031-1 (eBook)
DOI 10.1007/978-1-4939-1031-1
Springer New York Heidelberg Dordrecht London

Library of Congress Control Number: 2014941729

Foreword

Personal Reflections of Our Perinatal Research Group in the Early Years

Lubo has asked me to share some of my memories about the early days of the Center. As more formal reports of the Center's work will be provided in the following chapters, I have decided to write a few of my remembrances related only to the early days of our group. In hopes of conveying a more humanistic aspect of our beginnings, I have included mention of some of the foibles of which we were part of.

The idea for a perinatal group at Loma Linda grew slowly. It was conceived by Larry D. Longo and the dean at that time, David Hinshaw, who convinced Larry to come west in 1968. Larry had trained at the University of Pennsylvania with Robert E. Forster, II, a noted respiratory physiologist. He came to Loma Linda to find very modest circumstances: one lab with one technician. He had high hopes to give a serious push to research at LLU, previously known mostly for its training of medical missionaries.

About a year later Larry recruited me as I was finishing a 3-year tour of duty in the Army Medical Corps, Natick, MA. The Vietnam War was at its peak. I turned down faculty appointments at Harvard and UCLA to come here to LLU. My wife approved of the location because the palm trees reminded her of growing up in Cuba. As a member of the Society of Friends (Quaker), I openly communicated that some aspects of my life did not fit the mold of an ideal Adventist faculty member. The Board of Trustees accepted me as I was.

For financial support, I applied for a Research Career Development Award from the NIH and Donald H. Barron came to site-visit the lab and me. Barron was a pioneer in the field of developmental physiology, having developed the chronic sheep preparation enabling fetal sheep to be studied unanaesthetized in utero. Barron landed at a small airport in San Bernardino on a hot, dry, windy day. Larry and I went to meet him. Dr. Barron got off the plane, sniffed the dry desert air, and looked at the mountains. He sniffed again, took a deep breath and said, "This is where young investigators come to die" (sniff-sniff). I didn't get the award at that time. I did later, however. Larry later dedicated a book he had co-edited to Dr. Barron.

The first project I undertook after arriving at Loma Linda was to test the belief that maternal blood pressure in the placenta squeezed on fetal vessels. I had the idea that near term, when a pregnant woman lay down on her back, the gravid uterus would flop back and partially occlude the inferior vena cava,

dam up her blood into the placenta, thus increasing the pressure in the fetal vessels to inhibit umbilical blood flow, compromising oxygen delivery to the fetus. Larry and I did some experiments with sheep that supported this "sluice" flow concept. There was considerable ado and national recognition of this concept, not surprising since there were implications that women should deliver squatting, rather than delivering in the supine position flat on her back. However two other labs did not find evidence of a sluice or waterfall flow (although their experiments were done in circumstances that gave the least chance of seeing such flow), and the idea gradually died. To this day I firmly believe, especially in humans where the placenta has a lake of maternal blood that surrounds fetal capillaries, that the concept holds true. After all, what could prevent the fetal capillaries from collapsing? We needed someone clever enough to do these studies in humans.

About 3 years later, in the early 1970s we recruited Ray Gilbert. He came from Johns Hopkins and had earlier obtained his Ph.D. with Sid Cassin in Florida. Ray worked on control of fetal cardiac output, and showed there was a plateau in heart function that could not be exceeded. He had magnificent surgical skills, if you can imagine placing a catheter in a beating left atrium of a fetal sheep. A reviewer looking over one of Ray's grant applications said, "Ray, what you propose is impossible" and turned the proposal down. In fact, Ray had already done the "impossible" and received the award at the next review cycle. I mention this because it cautions us against a tendency to become all knowing, once granted the title of reviewer.

Larry, Ray, and I had common interests in pursing whole-animal fetal physiology. Surgeries were performed in lab B (the surgical lights are still there), and the schedule was quite busy with two surgeries in parallel, and sometimes several surgeries each week. We freely shared money, lab space, equipment, and supplies. I doubt NIH would have approved of this enlightened communism had they known, but it worked well for us with our common interests.

Larry had a particular weakness for spending more money than the School had allocated for the year's work, to help us support our research, which chiefly was supported by the NIH. The reputation of our perinatal group became progressively worse each year, and one year things were especially bad. The Dean (Gordon G. Hadley, at the time) called us into the conference room and we knew we were on the carpet and would have to account for every penny. The dean greeted us with tears welling up in his eyes. He said, "How bad is it this time? Larry, don't tell me the details, just tell me how bad it is, and the School will pay whatever it is." Talk about unwavering support.

Bob A. Brace brought a bioengineering approach to the group after training with Arthur Guyton. He studied fetal body fluids—blood, lymph, amniotic fluid—with great energy and purpose. In one of our faculty meetings Bob asked Larry for $2,000 so that he could complete an ongoing study. Larry went to the Dean's office to see if he could obtain the funding and he was successful. Several weeks later, Bob ran into the Dean's Financial Officer, Ida Winzel, in the hallway. She asked Bob what he did with the $10,000 that the Dean provided. Gulp. Bob quickly replied that he had finished the study and was preparing it for publication! Years later after Bob had left Loma Linda, he became one of the best-funded perinatal investigators in the country.

Our work took on a mathematical approach in the 1970s. This included, for example, Esther Hill's mathematical modeling of the kinetics of respiratory gas transfer in the placenta and the lungs. Another is the work of John Wilbur, who I mention because of his distinguished career after leaving LLU. John obtained a Ph.D. in pure mathematics from the University of California at Davis and then came to Loma Linda. As a medical student he worked mostly with me to set up a mathematical model of placental water and solute flow between the blood of mother and a fetus. This involved solving a set of more than 70 coupled differential equations on a new fast computer system to which the lab had access. I think it was among the best theoretical descriptions of a biologic process ever written. Later in his career as an NIH scientist, John developed the PubMed algorithms that produce search results and allow "fuzzy phrase" matching. These PubMed searches are now used thousands of times a day by investigators all over the world.

As the years passed the theoretical phase of our group slowed down and some people thought it was about time. Nicholas Assali at UCLA said to me "So much for that theoretical crap, Gordon. You've got to stop all that nonsense and get back to some real experiments."

About this time a stroke of good luck came to us. Everett Koop was Surgeon General and wanted to show the dangers of a mother's smoking on the fetus and newborns. He contacted officials at the Child Health Institute, and they in turn asked Larry for help, knowing he had written about carbon monoxide effects during pregnancy. (In fact, Larry and my first collaboration used carbon monoxide to study placental function.) In response, Larry then largely wrote the section in the Surgeon General's report about smoking and pregnancy that led to cigarette package labeling, and so forth. The officials at NICHD were deeply grateful to Larry for his help. Thereafter, Larry's grant applications had a way of sliding through a bit more smoothly than they might have otherwise. The NIH largess extended for decades after that.

One year I met Geoffrey Thorburn of Australia at a meeting. He was in failing health. I said, "Geoff, how are you enjoying the meeting?" He said, "Well, you know these meetings are a drag." I said, "Yeah, I understand." He said "But, you know, the reason I come to the meetings is to talk about ideas, ideas about how the body works." That piece of wisdom has stuck with me.

In the early 1970s Howard Hughes was making war-related electronics for the Vietnam War effort. As many of you know he had established the Howard Hughes Research Foundation that funded biomedical investigators around the country. Larry had a brainstorm—why not get Hughes to establish the Howard Hughes Perinatal Biology Center at LLU? We thought this was reasonable because the Foundation's funding decisions were made by three trustees, one of whom drove hundreds of miles to have her teeth fixed at the Loma Linda School of Dentistry. We thought she would be very partial to LLU. So, after her dental visit, Larry arranged for her to come over and talk with us. Of course, we didn't know quite what to expect, we did know trustees operated with CIA-like secrecy, had complete autonomy, sought to protect Hughes' reputation, and that if she were favorably disposed millions of dollars might be provided. She arrived, a buxom, middle-aged woman accompanied by two bodyguards. They wore dark suits and had suspicious bulges

in their left armpits. Larry and I waxed eloquent about how reproductive biology would add new dimensions to Hughes' reputation. She seemed responsive and said she wanted to help Loma Linda. Then I said, "Well, would you like to see our lab, see where we work?" The entourage came into the lab; there on the wall was a giant poster saying, "Make love, not war" and there were other anti-Vietnam war posters around the lab too. A certain iciness descended on the room. The trustee and her bodyguards backed slowly out the door and we never heard further from the Hughes foundation.

I'd like to say something about Steve Dale as a remembrance. Steve had been working as a manager in a Radio Shack before Ray Gilbert recruited him to our perinatal group. His first task was to set up the computer networks and data collection systems for our new group. He single-handedly wrote a spreadsheet application and data analysis program that rivaled Excel. He was an amazing self-taught programmer who wrote in C and Fortran. He worked with Tim McNaughton trying to figure out the source of the maternal fetal electrical potential difference. He was a brilliant statistician and mathematician.

In September 1988, Steve and Tim attempted to ascend Lone Pine Peak in the high Sierras. The ascent was more difficult than they had expected, and they turned back late in the day. A Sierra storm rolled in and their descent was cold, dark, wet, and extremely difficult. They were exposed along the ridge line. In a tragic irony, Steve, who studied the physiology of hypoxia, developed hypothermia and acute mountain sickness, beginning with headache and vomiting and progressing to mental changes and ataxia as the hypothermia progressed. He died just as the sun was rising and he had nearly reached base camp. His loss was a huge blow to the perinatal group, both in morale because Steve was so much admired, and because of the loss of such a brilliant mind.

One year Larry was driving back from a meeting in San Francisco and while passing the Sierras looked up at the mountains. He thought the high altitude would provide a natural hypoxic chamber to study the effects of low oxygen tension on fetal sheep. Thus the White Mountain project was born. A Program Project Grant application was submitted, approved, and has now continued for 26 years. It has provided cohesion and funding for the perinatal group.

Physically, the Research Wing of Loma Linda University looks and feels now very much as it did 40 years ago, aside from increased bureaucracy and the fact that surgeries have moved down to the basement or to a neighboring farm due to a rampant fear of Q-fever.

The perinatal group itself has grown and changed. New investigators joined the group with energy and divergent interests. Like most of the field of physiological research, the interests have shifted particularly toward cell and molecular biology. Some loss in focus of the Center's work has occurred. There has been a growing disconnect between molecular biology and clinical medicine within the Center and nationally. The emphasis on whole animal physiology has diminished while its importance has remained central to an integrated understanding of medicine. Thus there remains a need for a reconnection between molecular biology and clinical medicine. I firmly believe whole animal physiology is desperately needed to bring about this reconnection and the Center is uniquely positioned to fulfill this role.

The free sharing of money and resources that we supported has been largely lost, and replaced with rugged individualism. This is not surprising given the low approval rates of NIH grant applications, and the need for young faculty to be funded to survive. Through it all, the good will among the faculty has been largely preserved and we have usually been able to ignore the more irritating quirks and idiosyncrasies of each other.

Postdoctoral Fellows have come from around the world for training and returned to their native countries to have enhanced academic careers. Medical students have come, done a summer project, and gone on to residencies at big name institutions on the East Coast. The group has been promoted to a "Division" and then to a "Center," becoming a centerpiece of research at LLU. Something over 4,000 sheep have been studied. The support of the administration has never wavered; although officials have not always understood what those "sheep doctors" were doing down in the basement.

It is my wish that those of you who are reading this will find an institution that lets you pursue your interests with the freedom that I have been granted for the past 45 years.

Loma Linda, CA, USA Gordon G. Power, M.D.

Center for Perinatal Biology: The Early Years

Top, left to right:
Gordon G. Power, M.D.
Lawrence D. Longo, M.D.
Hobe Schroeder, M.D.
Raymond D. Gilbert, Ph.D.
Robert A. Brace, Ph.D.
Dianne McClure
Sylvia Hixson
Charles Hewitt

Preface

The planning of this book evolved from serendipity and history. The Center for Perinatal Biology was turning 40 and we wanted to celebrate. On February 11, 2013, some of the most illustrious names in the field of Perinatal Biology as well as a Nobel Laureate and researchers and friends from around the globe gathered at Loma Linda University, including six past presidents of the Society of Gynecologic Investigation. This 40th Anniversary Celebration of the Center for Perinatal Biology was actually a tribute to the founder of the Center, Lawrence D. Longo, M.D. Through his visionary leadership and tireless efforts the Center not only came into existence, but more importantly, evolved into a world renowned research hub. An integral part of the Center's success is that it has served as an international "incubator" for students, fellows, colleagues, and collaborators for over four decades. This volume represents contributions from individuals who in some way or other were influenced by Dr. Longo. As will become obvious, it is a mix of a wide range of topics, illustrating the diversity of thinking and scientific interests. We hope that this book serves not only to honor Dr. Longo, but also to inspire a new generation of curious individuals who will make their mark in the field of perinatal biology and beyond.

Loma Linda, CA, USA

Charles A. Ducsay, Ph.D.
Lubo Zhang, Ph.D.

Center for Perinatal Biology
40th Anniversary Symposium

Top Row, left to right:
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Raymond D. Gilbert, Ph.D.
Charles A. Ducsay, Ph.D.
Lawrence D. Longo, M.D.
Peter W. Nathanielsz, M.D., D.Phil.
Frederick Naftolin, M.D., Ph.D.
Kent L.R. Thornburg, Ph.D.

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Steven M. Yellon, Ph.D.
Roy M. Pitkin, M.D.

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John R.G. Challis, Ph.D., D.Sc.

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Syuji Ueda, M.D., Ph.D.

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David J. Baylink, M.D.
J. Jacek Kraczkowski, M.D., Ph.D.

SGI Past-Presidents

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Frederick Naftolin, M.D., D.Phil.—1992
Leslie Myatt, Ph.D.—2010

Bottom Row from Left:
John R.G. Challis, Ph.D., D.Sc.—2003
Roy M. Pitkin, M.D.—1986
Lawrence D. Longo, M.D.—1983

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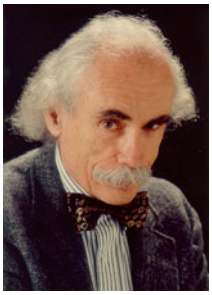

"Surprised by Joy"*: Four Decades of Contributions to Developmental Physiology

1

Lawrence D. Longo

Abstract

Presented at the 40th Anniversary Celebration of the Center for Perinatal Biology of Loma Linda University School of Medicine, honoring Dr. Longo for his 40 years of extraordinary leadership and service, February 11, 2013.

Keywords

Center for Perinatal Biology • Loma Linda University • Biomedical research • Academic medicine • Developmental biology

President Hart, Dean Hadley, Professor Murad, colleagues, and dear friends. What can one say? I am speechless. To note that this is all rather breathtaking is the understatement of the week. You are the Pantheon of the Great in Fetal and Neonatal Biology. No words can express my feeling of being overwhelmed by your affection, laudation, and honor.

It was a bit more than four decades ago that Gordon G. Power, with whom I had collaborated at the University of Pennsylvania under the mentorship of Robert E. Forester II, elected to join me at Loma Linda. Gordon had finished his tour of duty at the U.S. Army Research Laboratory at Natick, MA, and after looking over the prospects here, we with Dean David B. Hinshaw decided that the school needed a good Quaker who could challenge people's assumptions and their thinking. Shortly thereafter, I received a call (no e-mail or texting then) from Saul Permitt at Johns Hopkins University. Saul exuded that one of the best postdocs he had ever had in cardiovascular physiology was starting to look for a position, and that perhaps a good Southern Baptist would help keep us on the straight and narrow. Thus, a month or so later at the Federation for Experimental Biology meeting, Gordon and I met Raymond D. Gilbert. Following his visit, Ray agreed that life might be good here, especially when he saw the ski slopes on the nearby mountains! Then, just a few years later Dean Hinshaw called Gordon, Ray, and I into his office. At that time, the Dean was serving on an Accrediting Committee for the American Association of Medical Colleges. He had returned

*With apologies to William Wordsworth (1770–1850) and Clive Staples (C.S.) Lewis (1898–1963).

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L. Zhang and C.A. Ducsay (eds.), *Advances in Fetal and Neonatal Physiology*, Advances in Experimental Medicine and Biology 814, DOI 10.1007/978-1-4939-1031-1_1,

recently from site visits to several schools of medicine where he saw some unique patterns of organization. Dr. Hinshaw was aware that the clinical departments with which we were associated knew that our hearts were not in clinical work, and the basic science departments believed that we were only marginally competent investigators. He suggested, therefore, that we should develop an independent Center that would report directly to him. Several years later, the subsequent visionary Dean, G. Gordon Hadley, over a period of a decade, allowed us to recruit a number of bright young investigators. These included: Robert A. Brace, Cecelia Y. Cheung, Brian J. Koos, Steven M. Yellon, Charles A. Ducsay, and William J. Pearce. So we were off and running. In helping to recruit outstanding young scientists, Dean Hadley liked to use the metaphor of the farmer who must insure an adequate supply of "seed corn" as an investment to grow and mature a bumper crop in future years. Indeed, that is exactly what he did!

This, of course, is neither the time nor the place to present a detailed history of the Center. In his introductory remarks, Lubo Zhang has outlined some of that. However, several have asked about my philosophy and goals in promoting the Center and helping it to advance. To be honest, it was all fairly simple and straightforward. My overriding objective was to attract the finest, most promising, and creative young scientists possible, to establish an environment in which they could be productive; to do everything possible to help them flourish both on their own and in collaborations with other investigators and, rather than play the role of *éminence grise*, to just stay in the background out of everyone's way.

To my mind, academic medicine is one of the highest callings that one can pursue in life. To help develop a major research group shares that high calling, and serving as Director of this Center has been one of the great privileges of my life. It has made my days endlessly interesting, energizing, and inspiring. Of course, our years together have been marked by some major challenges as well as great opportunities. In the late 1960s through the 1980s, the "Golden Era" of biomedical research, funding from the National Institutes of Health (NIH) or other agencies was a given, provided one kept his head on straight and was productive. As most of you are acutely aware, however, the past decade has been painfully challenging. To date, through everyone's hard work and ingenuity, we have weathered the storm and, hopefully, have become a case study in how excellence can be achieved and maintained in an era of fiscal stringency.

A century ago, the founders of this institution were inspired and driven by the belief that to educate dedicated, committed physicians to work as a "Holy Calling" could change the world. To us they bequeathed an ambitious vision, that by diligence and dedication we could participate in an intellectual transformation to fulfill the motto of the University "To Make Man Whole". As the founders dreamed, during these four decades together we have attempted, in some small way, to make a difference in the world. We have touched the lives of several thousand medical students, and a large number of graduate students, as well as several hundred postdoctoral fellows from academic centers around the world. In part, this has demonstrated our responsibility to expand the scientific knowledge base for a health care-based ministry.

Let me be clear. Without question, the foundation of the Center's success has been the intense dedication, imagination, and creativity of our faculty. In our undying devotion to the discovery of new basic and clinically relevant knowledge in developmental biology, and its application to improved clinical care, our zeal has been to "catch lightning in a bottle" so to speak. (Professor Murad and many of the rest of you know what that is all about). Beyond stratospheric IQs, these fellow faculty have far above average CQs (curiosity quotients) and PQs (passion quotients). That is what helps to make them so great. Also a critical factor, each faculty member has a unique background, possessing different and unusual scientific and technological gifts. In addition to first-rate science and laboratories, this has allowed us to develop outstanding core facilities for cell and molecular biology, for advanced imaging microscopy, and for microsurgery.

During my tenure as Center Director a major satisfaction has been to follow the steep career ascents of these young, zealous, "hot-shot" Center faculty. Each has developed into a celebrated investigator and doyen of young scientists, illustrious, innovative and creative researcher, distinguished world authority in the reproductive sciences, outstanding role model, and friend.

As a model of research success at Loma Linda University, a question one might ask is what does it take to be a first-rate scientist, and how can we achieve that distinction? Obviously, one must have some degree of intelligence, but need not necessarily qualify for Mensa. Also one must stay focused, not overreaching or galloping off in too many directions. And one must be lucky; as picking the right hypothesis to test can be chancy. Members of the Center have displayed these characteristics and more. In many ways their great gift is creativity. Each reads widely and thinks about a problem from several perspectives. Their minds are constantly exploring, working to identify vital questions and dreaming of hypotheses to test. Disciplined fantasies are the fountainhead of all creative thinking. Sir Isaac Newton (1642–1727) dreamed, Albert Einstein (1879–1955) dreamed. Each Center member is a dreamer, encircling a problem and with creative insights, and contemplating the solution so as to advance the frontiers. What an unspeakable gift. Without exception, each has become a leader in his or her field, serving on study sections for the NIH, review groups of the American Heart Association and other societies, and holding office in national and international organizations. To a great extent, it has been each member's total commitment, dedication, passion, and perseverance that has helped the Center to experience its success. Of particular significance, the Center group works together as a close-knit family; friends, contributing their best in collegiality to make a difference in the world.

A term, I believe, that encapsulates the life of each of the Center faculty is mentor. As you recall from Homer's *The Odyssey*, when he left for the Trojan War, Odysseus chose his friend Mentor to be in charge of his son Telemachus. Like Mentor of Greek mythology, Center faculty have given their careers to mentoring young physiology and biochemistry graduate students and postdoctoral fellows. In educating and transforming the lives and careers of a generation of young basic scientists and physician-scientists, their mentorship has helped to mold them into true and productive academicians. An added joy has been to see almost every one of our graduate students and postdoctoral fellows, many of whom have joined us today, continue their diligence on the path of productivity as leaders in their academic centers and universities throughout the world.

An additional contribution to the intellectual life of the Center and Loma Linda University, has been the Visiting Scientist program, in which many of you present today have participated. Supported, in part, by our long-funded NIH P01 Program Project Grant, we have brought over 200 notable scientists and dignitaries to the campus. Not only have these visiting scientists presented Seminars and Grand Rounds, but have interacted with, inspired, and developed collaborations with faculty, and stimulated postdoctoral fellows and graduate students.

And that raises the point, truly the community of developmental scientists is global. From its genesis in the "big bang" of investigation that, under the leadership of Geoffrey S. Dawes commenced in the mid-twentieth century at the Nuffield Institute at Oxford University, a closely interconnected chain of investigators forms an "invisible college" that stretches across America and Canada and around the globe. This day, we witness that brotherhood of collaboration in mind and spirit.

Recently, a friend asked what lessons I have learned from this journey. Although in the "prime of my semi-senility", if I may reflect a bit, I am struck with the complexity of life and of biomedical science. Despite all that we know, there exists a vast sea of the unknown. We have so much yet to learn and even more to understand. For our edification, an historical perspective can fill us with a sense of humility. Especially humility in appreciating how much we owe to those who have gone before. Indeed, as Sir Isaac Newton and others have noted in searching the great

unknown that lies before us, "we each stand upon the shoulders of giants".

Also, I have been asked what I foresee in the next four decades in the life of the Center and advances in the field. Unfortunately, I find it impossible to put on a Janus-like mask and see the future as well as the past. As has been noted by others, "It is difficult to predict, especially the future." For each of us, I believe, it is sobering to reflect upon the degree of our limited vision. When you think about it, just one (much less several) decade(s) ago, who of us could have predicted life today. Almost daily we witness remarkable advances in technology, bioinformatics, and science. It all is beyond imagination. We only can know that every day presents new challenges and fresh opportunities. As we continue to advance the frontiers, and remain on the cutting edge of biomedical science, in our passion to stay *au courrant*, we must continually reinvent ourselves. Our studies must range in a hierarchical and reductionist manner from organ/tissue, to cell, subcell, and molecule, and then back again integrating it all in an attempt to understand function at the organ/systems level. Also in our passion to contribute to life, we must not forget our responsibilities in helping to educate and develop bright, innovative students and postdoctoral fellows, the future of developmental science.

In conclusion, to serve in a locus of intellectual, international standing, to focus on the pursuit of truth wherever it may lead, to promote scholarship, to contribute to betterment of the human condition, to keep in mind the considerable social relevance of our work, this then is our inspiration, our responsibility, and our legacy for the future.

Nothing in my life has prepared me for the kindness, enthusiasm, and generosity of each of you here today. It is humbling and inspiring, and as I noted earlier, quite overwhelming.

Thank you, thank you, thank you, and let us all Persevere!

sGC-cGMP Signaling: Target for Anticancer Therapy

2

Ka Bian and Ferid Murad

Abstract

The biologic endogenous production of cGMP was reported in the 1960s and followed by the demonstration of guanylyl cyclase activity and the isoforms of soluble and membrane-bound guanylyl cyclases. During the same period, cGMP specific phosphodiesterases also was discovered. Murad's lab established link between the endothelium derived relaxation factor (EDRF) and elevated cGMP concentration in the vascular system. October 12, 1998, the Nobel Assembly awarded the Nobel Prize in Medicine or Physiology to scientists Robert Furchgott, Louis Ignarro, and Ferid Murad for their discoveries concerning nitric oxide (NO) as a signaling molecule in the cardiovascular system. In contrast with the short research history of the enzymatic synthesis of NO, the introduction of nitrate-containing compounds for medicinal purposes marked its 150th anniversary in 1997. Glyceryl trinitrate (nitroglycerin; GTN) is the first compound of this category. Alfred Nobel (the founder of the Nobel Prize) himself had suffered from angina pectoris and was prescribed nitroglycerin for his chest pain while he refused to take due to the induction of headaches. Almost a century after its first chemical use, research in the nitric oxide and 3′,5′-cyclic guanosine monophosphate (NO/cGMP) pathway has dramatically expanded and the role of NO/cGMP in physiology and pathology has been extensively studied. Soluble guanylyl cyclase (sGC) is the receptor for NO. The α1β1 heterodimer is the predominant isoform of sGC that is obligatory for catalytic activity. NO binds to the ferrous (Fe^{2+}) heme at histidine 105 of the β1 subunit and leads to an increase in sGC activity and cGMP production of at least 200-fold.

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L. Zhang and C.A. Ducsay (eds.), *Advances in Fetal and Neonatal Physiology*, Advances in Experimental Medicine and Biology 814, DOI 10.1007/978-1-4939-1031-1_2,

In this chapter, we reviewed the studies of sGC-cGMP signaling in cell proliferation; introduced our work of targeting sGC-cGMP signaling for cancer therapy; and explored the role of sGC-cGMP signaling in the chromatin-microenvironment.

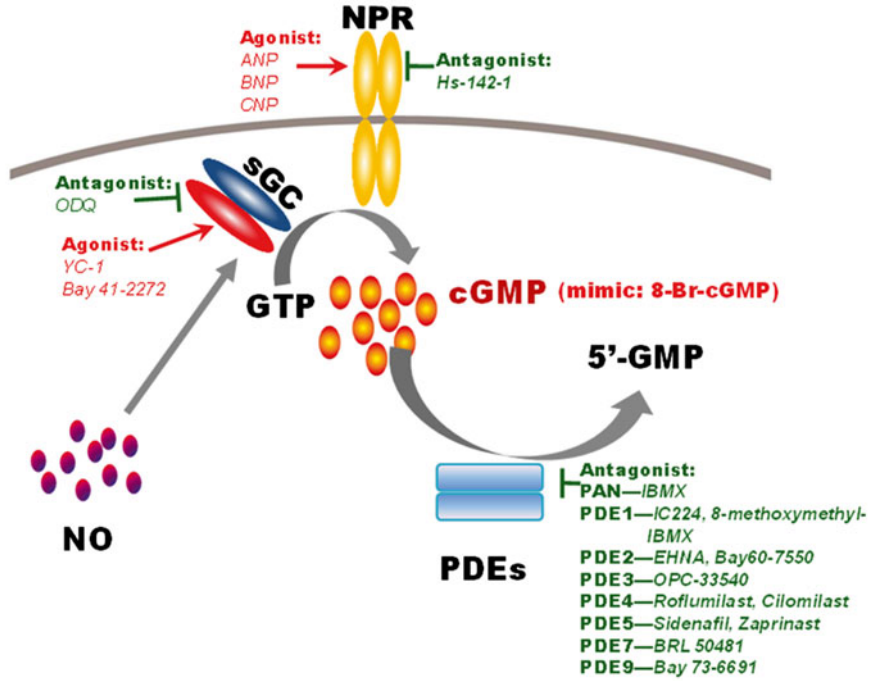
Keywords

Nitric oxide synthase (NOS) • Cyclic guanosine monophosphate (cGMP) • Soluble guanylate cyclase (sGC) • Particulate guanylyl cyclase (pGC) • Natriuretic peptide • Phosphodiesterase (PDE) • cGMP-dependent protein kinase (PKG)

1 sGC-cGMP Signaling and Cell Proliferation

NO research has been enormously extended in the past 30 years and the role of nitric oxide (NO) in physiology and pathology has been extensively studied [1–3]. The utilization of intact cell cultures, tissues and cell-free preparations along with the use of pharmacological, biochemical and molecular biological approaches to characterize, purify and reconstitute these regulatory pathways could lead to the development of new therapies for various pathological conditions which are characterized by altered production of NO [4–6]. The steady-state concentration and the biological effects of NO and cyclic guanosine monophosphate (cGMP) are critically determined not only by its rate of formation, but also by its rate of decomposition. Biotransformation of NO and cGMP occurs via different metabolic routes within the body and present another attractive field for our research as well as for the venture of drug discovery. The NO can be generated through enzymatic dependent or enzymatic independent mechanisms. The first nitric oxide synthase (NOS) isoform to be purified was the neuronal or brain NOS or type I NOS (nNOS or NOS-1); [7]. This was followed shortly thereafter by inducible NOS, also known as type II NOS (iNOS or NOS-2); [8, 9], and then by endothelial NOS or type III NOS (eNOS or NOS-3); [10–12]. On the other hand, NOS-independent NO generation can be derived from organic nitrates (RONO2s), *S*-nitrosothiols (RSNO), and NONOates (diazeniumdiolates). The nitrite and nitrate have been considered as alternative source of NO production in the body (see our review for further information [13]). NO has been shown to impact perinatal biology through various aspects which will not be the focus of this chapter but can be found in the studies of other's [14–16].

The endogenous production of cGMP was reported in 1960s [17, 18] and followed by the demonstration of guanylyl cyclase activity [19–22] and the isoforms of soluble and membrane-bound guanylyl cyclases [23, 24]. During the same period, cGMP specific phosphodiesterases also was discovered [25–27]. Murad's lab established the link between the endothelium derived relaxation factor (EDRF) [28] and elevated cGMP concentration in vascular system [29] which also resulted an increase of protein phosphorylation [30]. In Scheme 2.1, we outline the major members in cGMP signaling. Soluble guanylyl cyclase (sGC) is a heme-containing NO receptor which consists of four isoforms: α1, α2, β1 and β2. It seems that only α1/β1 and α2/β1 heterodimers are activated by NO [31]. α1/β1 has been considered as a major player in daily cGMP-dependent physiological functions since a markedly attenuated relaxing effects of major vasodilators such as acetylcholine, NO, YC-1 and BAY 41-2272 have been observed in the α1 sGC knockout mice of both genders [32]. The biological synthesis of cGMP also can be stimulated by natriuretic peptide receptor (NPR) which is also known as particulate guanylyl cyclase (pGC) type A [33], pGC type B [34], and pGC type C [35]. As a well-accepted model, cGMP acts



Scheme 2.1 The NO/sGC/cGMP signaling molecules and reagents which can regulate the signaling function. Thus, characterization of the NO/sGC/cGMP signaling molecules serves an important base for tumor therapy

through downstream effectors such as the family of cGMP-dependent protein kinases, cyclic nucleotide-gated channels, and cGMP-regulated phosphodiesterases [13, 36, 37]. The phosphodiesterases (PDEs) are intracellular enzymes that hydrolyze cAMP and/or cGMP to the inactive metabolites AMP and GMP. Among the 11 PDE isoforms, PDE5, 6, and 9 selectively catalyze the breakdown of cGMP [38]. cGMP-dependent protein kinase (PKG) are serine/threonine kinases [39, 40] which derived from two different genes coding for PKG type I (PKG-I) and type II (PKG-II) respectively. The PKG-I is encoded by two alternatively spliced exons that specify for the PKG-Iα and PKG-Iβ isoforms [41].

The role of cyclic nucleotides in the regulation of cell proliferation and tumor growth was noted as early as in the 1960s [42]. cAMP and cGMP were thought to act as biological antagonists in the regulation of cell growth at the beginning [43], and elevated cGMP levels have been observed in certain human tumor tissues [44–46]. Increased urinary cGMP in rats with hepatoma or renal tumor implants was also detected by Murad et al. [47, 48]. Inhibition of proliferation by cGMP was initially observed in vascular smooth muscle cells [49], and then extend to endothelial cells [50] and the cells from nervous system, e.g. cerebellar glial [51] and neuroblastoma cells [52]. The major constituent of green tea, (−)-epigallocatechin-3-*O*-gallate (EGCG), has been shown to have cancer-preventive and therapeutic activities without clear mechanism to be elucidated. In a recent issue of the JCI, Kumazoe et al. [53] reported that EGCG activates 67-kDa laminin receptor (67LR) to elevate intracellular cGMP levels, and then induces cancer cell apoptosis. A phosphodiesterase 5 inhibitor, vardenafil, synergizes with EGCG to induce cancer cell death. Frattola et al. [54] reported a lower activity of guanylyl cyclase in human neurinomas and glioblastomas. Our recent study demonstrated an altered expression of sGC in malignant brain tumors, and suggests a therapeutic approach by manipulating sGC/cGMP signaling either pharmacologically or genetically. Stable clones with

the overexpressed heterodimer $\alpha1\beta1^{Cys\text{-}105}$ sGC resulted in elevated cGMP levels and inhibited colony formation *in vitro* and extended survival of tumor-bearing mice *in vivo* [55].

2 Targeting sGC-cGMP Signaling Molecules for Cancer Therapy

2.1 Restoring sGC-cGMP Signaling for Glioma Treatment

Gliomas account for almost 75 % of primary malignant brain tumors. Of 10,000 Americans diagnosed each year with malignant gliomas, only half will live beyond 1 year after diagnosis, and the others will be dead within 2–3 years. Despite compelling advances in diagnostic imaging, surgery, radiation and/or antineoplastic agents, the prognosis for people with glioma has remained largely unchanged [56]. Thus, new concepts in glioma etiology, therapy and clinical management are needed. We have used the data base of the Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information (NCBI) and analyzed the expression of sGC genes in human glioma of different grades. The data clearly indicate a marked decrease of the expression of sGCα1 and sGCβ1 in astrocytoma, oligodendrocytoma, and glioblastoma multiforme when compared with normal brain tissues. To verify whether restoring sGC function through cGMP elevation may influence proliferation of glioma cells, we investigated the effect of 8-bromo-cyclic guanosine monophosphate (8-bromo-cGMP), a cell membrane-permeable cGMP analog resistant to PDE hydrolysis, on glioma cell growth. The cGMP analog significantly reduced proliferation of glioma cells in a concentration dependent manner [55]. Based on NPR expression profile in glioma cell lines, we tested the hypothesis that elevating endogenous cGMP through NPR stimulation should have similar effects as 8-bromo-cGMP on tumor proliferation. As expected, ANP did inhibit proliferation of U87 glioma cells in a concentration-dependent manner [55]. Inhibiting PDE activity is another approach to elevate intracellular cGMP. Considering the fact that multiple PDE isoforms are expressed in U87 cells, we utilized a non-selective PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX) and showed that IBMX concentration-dependently suppressed glioma cell proliferation. Our data also indicated that U87 cells express higher levels of PDE5. We then treated the cells with a PDE5 specific inhibitor zaprinast which resulted in a significant inhibition of cell proliferation. To further confirm our observations, we treated U87 cells with an NPR agonist in combination with a PDE inhibitor. The combined treatment concentration-dependently inhibited tumor cell growth for up to 6 days [55]. To verify the effects of these pharmacological agents on the levels of intracellular cGMP, we have assayed cGMP levels and demonstrated that 24-h treatment with ANP or IBMX markedly increased cGMP accumulation in U87 glioma cells. To further rule out possible cAMP involvement, we measured cAMP accumulation in U87 glioma cells under stimulation of the cGMP-promoting agents. In contrast to the magnitude of forskolin-stimulated cAMP, the reagents such as IBMX, ANP, and BNP failed to significantly influence the levels of cAMP [55]. Thus, glioma cell proliferation was significantly inhibited upon pharmacological restoration of sGC function through increasing intracellular cGMP levels.

To provide direct evidence that restoring sGC expression blocks the aggressive course of glioma, we stably cloned U87 cells with overexpression of sGCα1 and a $sGC\beta1^{Cys\text{-}105}$ mutant in which the His-105 residue is substituted with Cys-105. The $sGC\alpha1\beta1^{Cys\text{-}105}$ is a heme-independent enzyme with constitutively elevated activity, this allowed observing the effect of sGC with less inference from endogenous NO [57]. Basal and NO-stimulated wild-type and mutant $\alpha1\beta1^{Cys\text{-}105}$ sGC did not produce significant amount of cAMP [55, 57]. To verify the effect of genetically restored sGC activity on glioma cell growth, we have performed the MTT assay and colony formation experiments. In both assays, $\alpha\beta^{Cys\text{-}105}$ sGC expressing U87 cells demonstrated a decreased ability for growth [55]. Orthotopic xenograftment of glioma cells with a $sGC\alpha1\beta1^{cys\text{-}105}$ stable clone

in athymic mice increased the survival time by fourfold over the control, this effect was more significant than the treatment with avastin/temozolomide, a popular combination of VEGF-A antibody with alkylating agent for glioma treatment [58]. In summary, we have provided genetic, proteomic, and functional evidence from *in vitro* and *in vivo* experiments supporting a role for sGC/cGMP in the diagnosis and therapy of human malignant intracranial tumors. The NO pathways may pave the way for developing new protocols with activation or inhibition of iNOS, sGC, particulate GC, PKG and PDE to treat human malignant tumors by altering NO and cGMP signaling.

2.2 Distinct Role of sGC-cGMP Signaling in Lung Adenocarcinoma

According to American Cancer Society (Cancer Facts and Figures 2012), the number of deaths due to lung cancer has increased approximately 4.3 % from 1999 to 2008. However, the rate of new lung cancer cases (incidence) over the past 33 years has dropped for men (22 % decrease), while it has risen for women (106 % increase). Lung cancer takes more lives than breast, prostate and colon cancers combined; it accounts for 27 % of all cancer deaths. Non-small cell lung cancers account for about 80 % of lung cancers, and of these, roughly 30 % are squamous cell carcinoma which is strongly associated with smoking. The squamous cell carcinomas, also called epidermoid carcinomas, tend to remain localized longer than other types and are generally more responsive to treatment. Adenocarcinoma is the most common type of lung cancer in lifelong non-smokers [59]. Its incidence has been increasing in many developed Western nations in the past few decades and has become the most common type of lung cancer in both smokers and nonsmokers. Adenocarcinoma accounts for approximately 40 % of lung cancers [60].

Recently, we have evaluated the impact of the NO-cGMP signaling pathway on lung cancer patients' overall survival rate by using the "Kaplan-Meier Plotter" database [61]. The database of KM plotter was established by using gene expression data and survival information of the patients from the National Center for Biotechnology Information Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/; Affymetrix HGU133A and HGU133+2 microarrays), and is capable of assessing the effect of 22,277 genes on survival in 1,715 lung cancer patients [62]. We have found there is a large difference in survival probability depending on gene expression levels of cGMP producing enzymes in adenocarcinoma when compared to squamous cell carcinoma. Our data indicated that adenocarcinoma patients with high levels of sGCα1 and/or sGCβ1 expression were shown to have a markedly higher probability (three times more) of surviving at the time points of 20, 40, and 60 months. The same pattern was also observed with NPR1and NPR2 genes. However, the overall survival rate of squamous cell lung cancer patients was not influenced by the expression levels of sGCα1, sGCβ1, NPR1and NPR2 genes. We tested the hypothesis that elevating endogenous cGMP through NPR stimulation could reverse the proliferation of lung adenocarcinoma. We established the colony formation assay with the A549 lung adenocarcinoma cell line and demonstrated that treatment with BNP produced a significant inhibitory effect on the colony formation.

3 Role of sGC-cGMP Signaling in Chromatin-Microenvironment

Our previously published data [55] shows that glioma xenografts of U-87 control or U-87 with the empty vector (pcDNA or pMG) exhibited the morphology of cancer cells with a hypertrophic nucleolus, decreased cytoplasmic/nuclear ratio, and shrunken cytoplasm. It is notable that xenografts expressing $\alpha 1\beta 1^{Cys\text{-}105}$ sGC had heterogeneous populations of tumor cells with a large population of cells resembling the morphology of the normal cells. We propose that sGC/cGMP signaling normalizes glioma cellular architecture through a pro-differentiation mechanism. It is generally accepted that tumor malignancy correlates with undifferentiated (anaplastic) status.

Our group has previously reported low levels of sGCα1 and β1 expression in undifferentiated embryonic stem cells from both human and mouse, and the embryonic stem cells regain sGC expression while entering the differentiation stage [63, 64]. Tumorigenesis and organogenesis are similar in many respects, and many types of cancers (including brain tumors) contain cancer stem-like cells (CSC) [65, 66]. Thus, we isolated a population of CD133-positive cancer stem-like cells from human glioma and did not detect sGCα1 expression while the expression of sGCβ1 mRNA was very low [55]. Together with our findings that restoring sGC function inhibits glioma growth and normalizes cellular architecture, we suggest that involvement of a pro-differentiation mechanism in sGC-targeted therapy may be an alternative or complementary approach to toxic treatments such as chemotherapy and radiation.

There is extremely limited information concerning the mechanisms how NO and cGMP influence the chromatin-microenvironment. By using U937 cells, a human monoblastoid line that lacks soluble guanylate cyclase, NO was found to regulate 110 transcripts that annotated disproportionately to the cell cycle and cell proliferation (47/110, 43 %). Also, more frequently than expected the cells contained AU-rich, post-transcriptional regulatory elements (ARE) [67]. On the other hand, cGMP-regulated transcription factors include the cAMP-response element binding protein (CREB), the serum response factor (SRF), the nuclear factor of activated T cells (NF/AT), the multi-functional transcription factor TFII-I, and possibly the nuclear factor-kappaB (NF-kappaB) [68]. Our histological analysis [55] showed that the human glioma tumor tissue from $\alpha1\beta1^{Cys\text{-}105}$ sGC-transfected cells-xenografted mice had significantly fewer microvessels as assessed by CD31 staining. Immunostaining for Ki-67, a marker associated with cell proliferation, also demonstrated a marked reduction in Ki-67 positive cells in double sGC transfectant-derived tumors.

Despite lack of the action mechanism, the presence of NO and cGMP in nucleus has been well documented. Recently, we have discovered that sGCβ1, but not sGCα1, can migrate into the nucleus of cancer cells. Overexpression of sGC β1, $\beta1^{Cys\text{-}105}$ mutant, and $\alpha1\beta1^{Cys\text{-}105}$ all promoted β1 accumulation in the nuclear fraction which was also observed with sGCβ1 immunohistochemistry confocal studies showing a high density of the subunit in the nucleus. In contrast, nuclear localization of sGCα1 was undetected even with stable sGCα1 overexpression (Bian K un-published observation). Similar to our findings, cGMP-dependent protein kinase (G-kinase) was reported to translocate to the nucleus in response to the increase of cGMP [69].

Very recently, Thomas and co-workers [70] discovered that nitric oxide plays an important role in epigenetics by inhibiting the activity of the demethylase KDM3A through forming a nitrosyl-iron complex in the catalytic pocket. The authors suggested three distinct mechanisms whereby (•) NO can affect histone methylation as follows: direct inhibition of Jumonji C demethylase activity, reduction in iron cofactor availability, and regulation of expression of methyl-modifying enzymes. Our un-published observations indicate that sGCβ1 expression is epigenetically regulated. To summarize our results: sGCβ1 is regulated epigenetically by histone acetylation in breast and lung cancers cell lines. Treatment with HDAC inhibitors LBH-589 (Panobinostat), MS-275 (Entinostat) and Trichostatin-A were able to increase expression levels of sGCβ1 in breast and lung cancer cell lines. On the other hand, the treatment of cells with the histone lysine demethylase inhibitor (BIX-0192); the histone lysine demethylase inhibitor (trans-2-PCPA), and DNA methylation inhibitors (5-aza-2′-deoxycytidine and chlorogenic acid had no effect on sGCβ1 expression. Thus, modulation of HDAC and HAT activity plays an influential role in regulating sGCβ1 expression, while other epigenetic processes such as DNA methylation, and histone methylation do not.

4 Summary

The role of NO and cGMP signaling in tumor biology has been extensively studied during the past three decades. The earliest studies with NO/cGMP in tumors and other tissues were done in

our lab in the 1970s and 1980s. Simple applications of NO or cGMP-regulating reagents to various cancer cell lines or animal models has generated controversial results. Whether the pathway is beneficial or detrimental in cancer is still open to question. We suggest several reasons for this ambiguity: first, although NO participates in normal signaling (e.g., vasodilation and neurotransmission), NO is also a cytotoxic or apoptotic molecule when produced at high concentrations by inducible nitric-oxide synthase (iNOS or NOS-2). In addition, the cGMP-dependent (NO/sGC/cGMP pathway) and cGMP-independent (NO oxidative pathway) components may vary among different tissues and cell types. Furthermore, solid tumors contain two compartments: the parenchyma (neoplastic cells) and the stroma (nonmalignant supporting tissues including connective tissue, blood vessels, and inflammatory cells) with different NO biology. Thus, the NO/sGC/cGMP signaling molecules in tumors as well as the surrounding tissue must be further characterized before targeting this signaling pathway for tumor therapy. Our preliminary data indicate that sGC-cGMP pathway cab be utilized to develop some novel tumor therapy products with some specific tumors. Whether those data can be extended to other tumor remains to be seen.

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Lawrence D. Longo: From Chronic Fetal Hypoxia to Proteomic Predictors of Fetal Distress Syndrome – A Life Devoted to Research and Mentoring Based on Virtue-Ethics

3

Justo G. Alonso

Abstract

The present chapter presents the experience of the author during his fellowship granted by the Fogarty Foundation of the NIH in the Division of Perinatal Biology, Loma Linda University, from 1989 to 1991. Experiments on maternal and fetal responses to long-term hypoxemia (including high-altitude) were performed successfully in pregnant sheep and their fetuses.

Cardiovascular, hormonal and blood flow distribution responses were studied under a strict experimental protocol. As result of this research, four papers were accepted for publication in major scientific journals, and have served as basis for further research.

Most of all, the leadership, virtue-based ethics, perseverance and continuous stimulus of Lawrence D. Longo is presented as an example to follow for future generations.

Keywords

Long-term fetal hypoxia • Basic research • High altitude fetal adaptation

1 Introduction

I met Dr. Lawrence D. Longo on January 1989 at the Division of Perinatal Biology, Loma Linda University, on the first day of my Research Fellowship granted by the Fogarty Foundation of the NIH. At first sight I realized that I was in front of a great man and mentor.

On the previous year I had worked intensely on the research project to be presented to the Fogarty Foundation under the guidance of my previous mentor and friend, the late Roberto Caldeyro-Barcia, in Montevideo, Uruguay, with the assistance of Larry from California.

I had worked with Caldeyro-Barcia and his team since 1972 in research on fetal responses to acute hypoxia with the sheep model and was excited by this new direction in my academic formation.

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L. Zhang and C.A. Ducsay (eds.), *Advances in Fetal and Neonatal Physiology*, Advances in Experimental Medicine and Biology 814, DOI 10.1007/978-1-4939-1031-1_3,

Uruguay is one of the smallest countries in America (only three million inhabitants), but has played a key role in the development of modern Perinatal Medicine, under the leadership of Caldeyro-Barcia and his team, in which I was proudly included. Uterine activity is measured to this day worldwide in Montevideo Units.

Roberto Caldeyro-Barcia and Lawrence D. Longo are the two personalities that had the most important influence in my academic formation. Both of them agreed, in my younger years, that great benefit would arouse from a 2 year fellowship in Loma Linda University, and arranged the appropriate means to fund my travel and weekly allowance to move with my family to beautiful California and start my work at the Division of Perinatal Biology in research in chronic fetal hypoxia in the sheep model.

I spent two immensely productive years.

2 Previous Research

Prior to my arrival in Loma Linda, the Division of Perinatal Biology under the guidance of Dr. Longo had produced a series of original papers studying maternal and fetal responses to long term hypoxemia.

I was introduced to this work by my good friend, Dr. Takashi Kitanaka, who guided me in my first weeks in the research facilities of the Division and the work methodology. I was familiar with basic research involving fetal sheep and cardiovascular responses to acute hypoxemia due to my prior work with Caldeyro-Barcia [1–8].

I felt at home since the first day, and realized that a great team was working coordinately pursuing the major goal of producing first level knowledge in biological sciences.

Doctors Takashi Kitanaka and Raymond Gilbert were key figures in mi insertion in the dynamics of research in Loma Linda. They had been working previously in chronic fetal hypoxia in the fetal sheep model, using the method of keeping the ewes in an hypoxic chamber for up to 3 weeks, and had already submitted a paper that was later published in the American Journal of Physiology focusing on maternal responses to long-term hypoxemia in sheep [9] (Fig. 3.1).

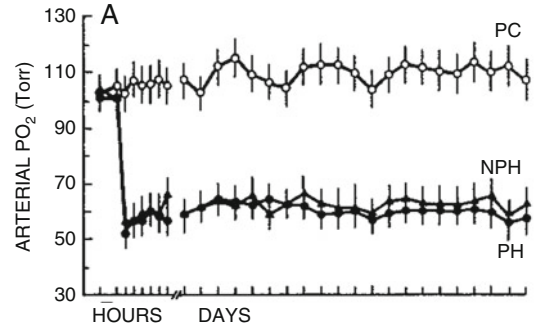

Fig. 3.1 (**a**) arterial O_2 tensions in hypoxemic (*closed circles*) or control (*open circles*) sheep. Values are means ±95 % confidence intervals of means. NPH: Non Pregnant Hypoxemic. PH: Pregnant Hypoxemic. In this figure and subsequent, abscissa shows initial period of 6 h after onset, followed by subsequent 21 days of hypoxia. From Kitanaka et al. [9], with permission

3 Fetal Responses to Long Term Hypoxemia

After I arrived in Loma Linda, chronic hypoxemia was induced in pregnant sheep by the method described by Gleed [10] by a tracheal catheter that infused Nitrogen continuously, displacing the Oxygen from the air breathed by the ewe, obtaining a maternal PO_2 of about 60 Torr for up to 3 weeks. Summarily, the results of this work are shown on Figs. 3.2, 3.3, 3.4, 3.5, and 3.6: maternal PO_2 decreased from approximately 100 to 60 Torr for 3 weeks, while in a few minutes fetal arterial PO_2 decreased from control value of 29.7 ± 2.1 to 19.1 ± 2.1 Torr, where it remained throughout the study. Hemoglobin increased from 10.0 ± 1.0 to 12.9 ± 1.9 g/dl by day 7, where it remained. This was associated with an increase of Erythropoietin from 22.8 ± 2.2 to 144 ± 37 mU/ml within 24 h, but by day 7 it had returned to levels slightly above normal. Epinephrine also increased moderately and remained elevated

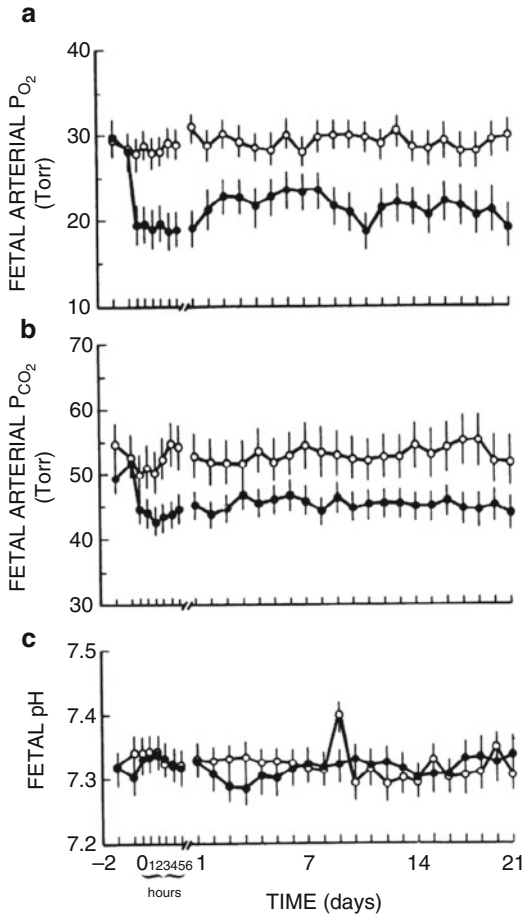

Fig. 3.2 (**a**) arterial O_2 tensions in hypoxemic (*closed circles*) or control (*open circles*) fetuses. Values are means ±95 % confidence intervals of means. (**b**) fetal arterial CO_2, tensions. (**c**) arterial pH values. From Kitanaka et al. [11], with permission

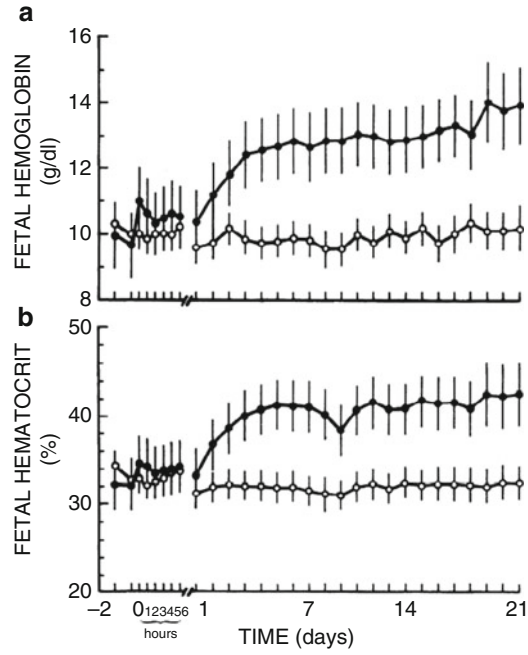

Fig. 3.3 Hemoglobin concentration (**a**) and hematocrit (**b**) in two study groups. From Kitanaka et al. [11], with permission

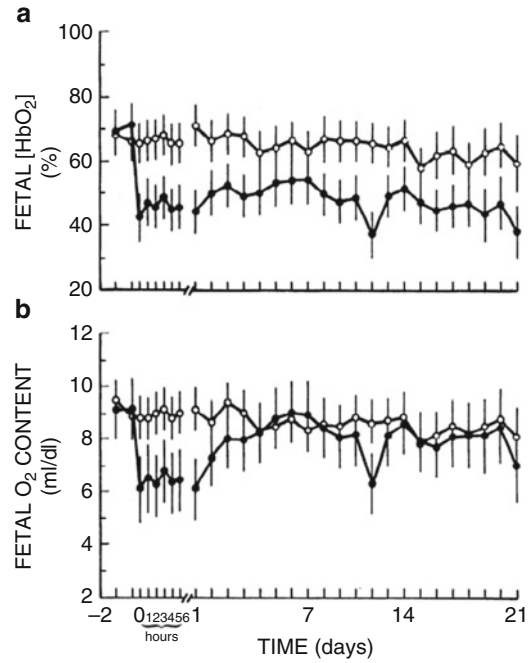

Fig. 3.4 Arterial oxyhemoglobin saturation (**a**) and arterial O_2 content (**b**) in hypoxemic and control fetuses. From Kitanaka et al. [11], with permission

throughout the study. However, values of mean arterial pressure and heart rate did not differ from controls.

3.1 Fetal Compensation

Perhaps surprisingly, these fetuses were able to compensate so that at term their body weights were normal, 3.77 ± 0.2 kg. Figure 3.2 depicts fetal responses. In the hypoxemic group following

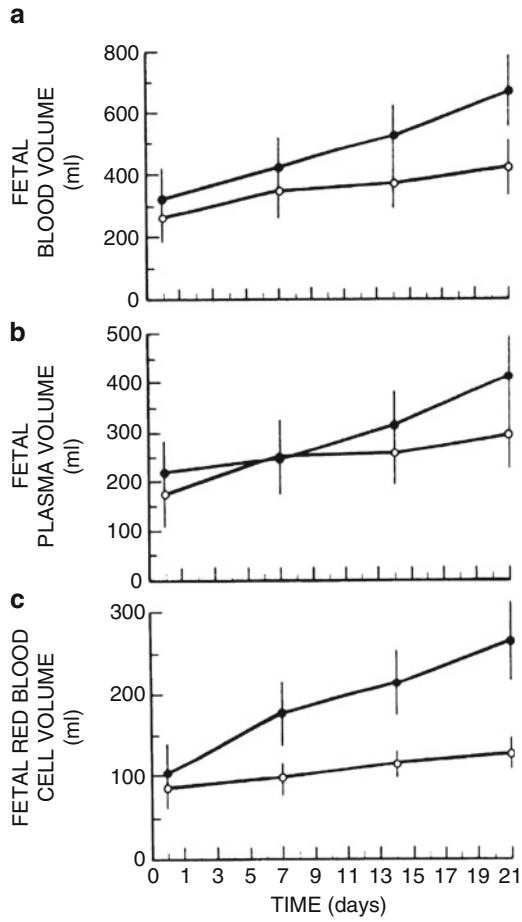

Fig. 3.5 Whole blood volume (**a**), plasma volume (**b**) and erythrocyte mass (**c**) in hypoxemic and control fetuses during course of the study. From Kitanaka et al. [11], with permission

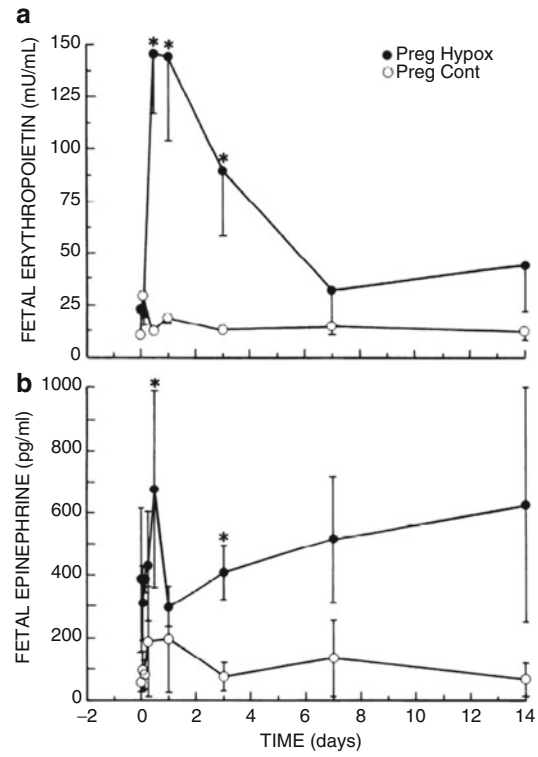

Fig. 3.6 Concentrations of Erythropoietin (**a**) and Epinephrine (**b**) in hypoxic and control fetuses. From Kitanaka et al. [11], with permission

the onset of lowered maternal inspired O_2 fraction (FIO_2), fetal arterial PO_2 dropped 36 % from 29.7 ± 2.1 to 19.1 ± 2.1 Torr. During the remainder of the study the fetal PO_2 tended to increase slowly, as did that of the ewe. To maintain these values relatively constant we periodically lowered the O_2 concentration of the breathed air down to 12 % on day 14. Arterial PO_2 values did not change significantly in the control group. Again, in the hypoxemic fetuses, arterial PCO_2 decreased from 49.4 ± 2.0 to 45.3 ± 2.0 Torr in association with the decrease in maternal arterial PCO_2. The arterial pH values did not change significantly during the experimental period in either group. In the hypoxemic fetuses oxyhemoglobin saturation decreased −36 % from 69.1 ± 6.6 to 44.3 ± 6.6 %, where it remained during the course of the study. Control fetuses showed no significant changes.

Figure 3.4 shows the arterial O_2 content, which in the hypoxemic group initially decreased −34 % from 9.2 ± 1.1 to 6.1 ± 1.1 ml/dl. However, during the course of the study fetal arterial O_2 content increased to near control values in concert with its increase in hemoglobin concentration. These variables showed no significant change in the control group. Hemoglobin, hematocrit, and blood volume. Figure 3.3 shows hemoglobin concentration and hematocrit in the hypoxemic and control fetuses. In contrast to the ewe, by day 7 of hypoxemia fetal hemoglobin concentration increased 29 % from 10.0 ± 1.0 to 12.9 ± 1.0 g/dl, rising slightly further to 14.0 ± 1.2 g/dl by day 21. The fetal hematocrit (Fig. 3.3) reflected the hemoglobin increase. Again, these variables did not change significantly in the control animals.

Fetal 2,3-diphosphoglycerate (2,3-DPG) concentration was 0.21 ± 0.02 mol/mol of hemoglobin in the control period and showed no significant change in either the hypoxic or control animals. In both hypoxemic and control fetuses whole blood volume increased during the course of the study. For the hypoxemic sheep this increase equaled ~112 %, from a control value of 320 ± 90 to 680 ± 0.46 and 1.75 ± 0.11 kg in the control and hypoxemia groups, respectively. At the end of the experiment (138.8 ± 1.6 days gestation) the hypoxemic fetuses weighed 3.34 ± 0.30 kg, whereas the controls weighed 3.17 ± 0.41 kg, values not significantly different. Fetal arterial glucose concentrations decreased slightly from 27.1 ± 3.7 to 23.2 ± 3.7 mg/dl (NS) in the hypoxemic animals.

3.2 Respiratory Blood Gases

In the hypoxemic group following the onset of lowered maternal inspired O_2 fraction (FIN,), fetal arterial POT rapidly dropped −36 % from 29.7 ± 2.1 to 19.1 ± 2.1 Torr. During the remainder of the study the fetal PO_2 tended to increase slowly, as did that of the ewe [11]. To maintain these values relatively constant we periodically lowered the chamber O_2 concentration, e.g., from 13 to 12.5 % on day 7 and to 12 % on day 14. Arterial PO_2 values did not change significantly in the control group. Again, in the hypoxemic fetuses, arterial PCO_2 decreased from 49.4 ± 2.0 to 45.3 ± 2.0 Torr.

3.3 Hormone Concentrations

Fetal plasma Erythropoietin concentration increased from 22.8 ± 2.2 to 145 ± 27 mU/ml at 12 h and 144 ± 37 mU/ml at 24 h, on day 1, remaining near that level until day 21 of hypoxemia. Again, the control fetuses showed no significant changes. Fetal plasma Epinephrine concentration increased from a control value of 222 ± 120 to 675 ± 316 pg/ml at 12 h and was 408 ± 87 pg/ml at 3 days. At 7 and 14 days, the values were 517 ± 203 and 623 ± 375 pg/ml, respectively. Norepinephrine concentration also increased from 310 ± 76 to 779 ± 136 pg/ml ($p < 0.05$) at 6 h and was 729 ± 194 pg/ml ($p < 0.05$) at 3 days. Although it appeared to remain elevated throughout the study, the increase was not significant. Fetal cortisol concentrations showed no consistent change during the hypoxic period.

4 Fetal Cardiovascular Responses to Chronic Fetal Hypoxia

4.1 The Basis of the Experimental Preparation

The next step of the project included measurement of fetal cardiovascular responses to long-term fetal hypoxemia. For this purpose we surgically instrumented pregnant ewes and their fetuses at 105–115 days gestation, placing arterial and venous catheters, as well as an electromagnetic flow-probe on the trunk of the fetal pulmonary artery in order to measure right ventricular output. We also implanted a tracheal catheter to the ewe to continuously infuse N_2 to displace oxygen to obtain a maternal PO_2 of 60 Torr for up to 14 days. After a recovery period of 5–7 days, the instrumented ewe and fetus were placed in a hypoxic state by infusing nitrogen continuously to the ewe's trachea as described above.

In normoxic conditions we confirmed that fetal right ventricular output responds to increases in ventricular pressure following Frank-Starling's curve, although moving on the flat part of it (Fig. 3.7). Our experiments confirmed that an increase in fetal blood pressure (afterload) was associated with a decrease in ventricular output (Fig. 3.8) in the sheep fetus. This behavior is called "right ventricular pressure sensitivity".

This study was published in the American Journal of Physiology [12].

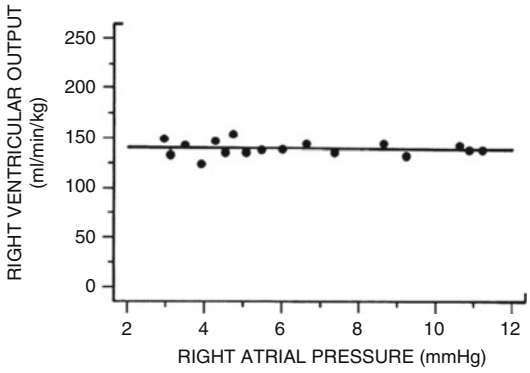

Fig. 3.7 Individual fetal cardiac function curve (control fetuses) during infusion of 30 ml of a 5 % (wt/vol) dextrose solution after autonomic blockade. Slope is not significantly different from 0. Mean value of these points represents cardiac function at a given afterload. From Alonso et al. [12], with permission

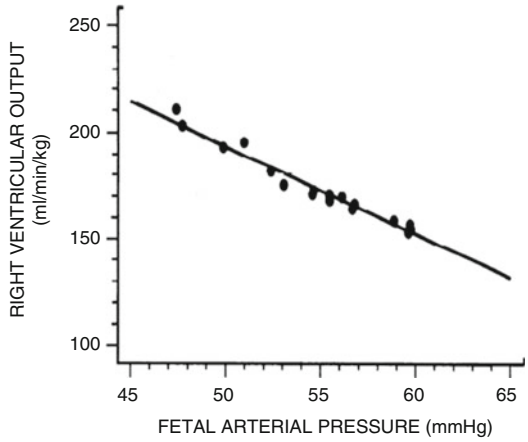

Fig. 3.8 Fetal right ventricular output as a function of fetal arterial pressure during administration of methoxamine in one animal. Heart rate was stable. Afterload effect is represented by slope of best-fitting linear correlation and is used to indicate right ventricular pressure sensitivity. From Alonso et al. [12], with permission

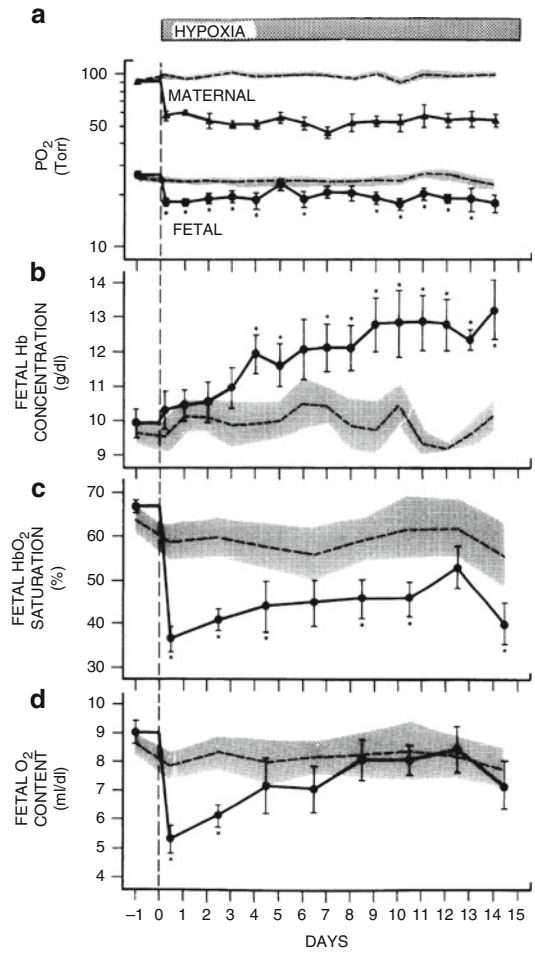

Fig. 3.9 Blood gases and hemoglobin (Hb) values. *Dashed area* represent means ± SE for control animals. Maternal (*triangles*) and fetal (*circles*) represent means ± SE for hypoxemic animals. N_2 administration to mother started at time 0. (**a**) Maternal and fetal PO_2 values. Maternal PO_2 values were at all times statistically different from controls. (**b**) Fetal Hb concentration. (**c**) Fetal oxyhemoglobin (HBO_2) saturation. (**d**) Fetal O_2 content (ml/dl). Points in **c** and **d** represent 2-day means. *$P < 0.05$ compared with control and own base line. From Alonso et al. [12], with permission

4.2 Fetal Cardiovascular Responses

4.2.1 Blood Gases and Hemoglobin

In the hypoxic group, maternal arterial PO_2 was reduced throughout the experiment from 90.7 ± 2.7 to 53.9 ± 4.2 Torr, and fetal arterial PO_2 was reduced from base-line values of 26.2 ± 1.3 to 19.5 ± 1.6 Torr (overall means for the hypoxic period). During the hypoxic period, fetal PO_2 showed a tendency to recover (with minimal increases in maternal PO_2), and on several occasions after the fifth day, it was not statistically different from the corresponding values of the control group (Fig. 3.9).

Control overall mean maternal and fetal PO_2 values were 96.6 ± 0.7 and 26.9 ± 0.3 Torr, respectively. The maternal to fetal arterial PO_2 difference was reduced 50 % from 73.1 ± 1.0 in the control group to 34.7 ± 1.0 Torr in the hypoxic group. No changes in this difference were evident during the course of the hypoxic period. No differences were found between groups in maternal or fetal pH and PCO_2, although fetal pH was somewhat lower in the hypoxemic group. In the hypoxic fetuses, the hemoglobin concentration rose steadily from basal values of 9.9 ± 0.4 to 13.2 ± 0.8 g/dl on day 14. No significant changes were observed in the control group during the study period, in which fetal hemoglobin concentration averaged 10.0 ± 0.4 g/dl. On day 4 of hypoxia, fetal blood hemoglobin concentration became statistically higher than its own base-line values and those of the control group. Maternal hemoglobin concentrations showed similar variations as those of the fetus in both groups.

Fetal oxyhemoglobin saturation remained relatively constant in the control group (58.7 ± 1.2 %) and was not different from prehypoxic values in the hypoxemic group (64.0 ± 1.3 %). In the hypoxic fetuses, oxyhemoglobin saturation fell to −40 % during the first day, thereafter rising slightly. It was at all times lower than in the control animals, although on several days this difference did not achieve statistical significance.

4.2.2 Fetal Blood O_2 Content

Fetal blood O_2 content also did not change significantly during the study period in controls value in the hypoxic group (9.34 ± 0.39 ml/dl). In the latter, it fell −45 % to 5.12 ± 0.41 ml/dl within 15 min of the onset of hypoxia and remained lower than control values until day 4. Thereafter, it recovered and was not statistically different from controls.

4.2.3 Heart Rate and Blood Pressure

Maternal heart rate showed a slight but significant Values are means t SE. increase during the first days of hypoxemia, from 98 ± 4 to 114 ± 2 beats/min ($p < 0.05$), and then returned to values not different from control. Base-line maternal arterial pressure was not different in the hypoxic group (88.0 ± 4.9 mmHg) as compared with controls (91.6 ± 2.4 mmHg) and did not change during the course of hypoxia.

Resting FHR decreased significantly in both groups with advancing gestational age from 188 ± 9 (control) and 176 ± 7 (hypoxia) beats/min on day −1 to 162 ± 6 (control) and 149 ± 6 (hypoxia) beats/min at day 14. FHR was at all times lower in the hypoxic than in the control group but only significantly so on day 3 (control, 187 ± 6; hypoxia, 168 ± 5 beats/min; $p < 0.05$). After autonomic blockade, FHR during the entire study averaged 174 ± 2 beats/min for the control group and 163 ± 2 beats/min for the hypoxic group (NS). On any experimental day autonomic blockade caused FHR to approach a value not different from these mean values. The result was an almost constant FHR at all ages at which cardiac function was studied.

In hypoxic fetuses arterial pressure (fAP) rose from base-line (day −1) values of 39.9 ± 1.6 to 51.8 ± 2.5 mmHg by day 14, becoming significantly higher after day 3.

Fetal arterial pressure in the hypoxic group was also significantly higher than that of the control group from day 3 onward. In the control animals, fAP, rose slightly, but not significantly, with advancing gestational age. At any age, administration of atropine and propranolol increased fAP, in hypoxic fetuses by 8–11 mmHg. In control animals, autonomic blockade was also associated with an increase in arterial pressure, although significantly less than in the hypoxic group.

Resting fetal central venous pressure was stable in the control group throughout the experimental period. During the hypoxic period it was somewhat higher, particularly on day 3 (control, 3.2 ± 0.3; hypoxia, 5.2 ± 1.4 mmHg; NS) (Fig. 3.10). Administration of atropine and propranolol produced a 1- to 2-mmHg increase in average venous pressure in both groups.

4.2.4 Fetal Cardiac Function

In both groups of animals the fetal heart worked at all times on the plateau of its function curve at any given afterload, with Q_{RV} remaining constant

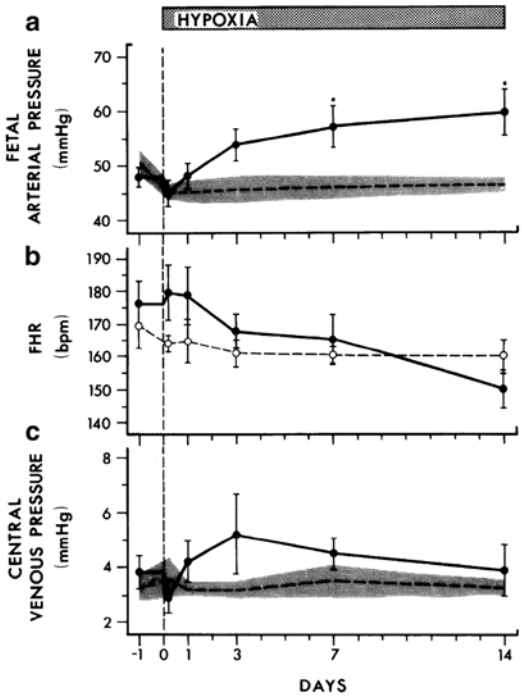

Fig. 3.10 Fetal hemodynamic variables during long-term hypoxemia. *Dashed lines* and *shaded areas* represent means ± SE for control group. (**a**) Arterial pressure after administration of atropine and propranolol. (**b**) Heart rate in hypoxic animals before and after administration of atropine and propranolol. (**c**) Central venous pressure at resting values before autonomic blockade.*p < 00.5 compared with control and own base line. *FHR* fetal heart rate. Symbols are as in Fig. 3.9. From Alonso et al. [12], with permission

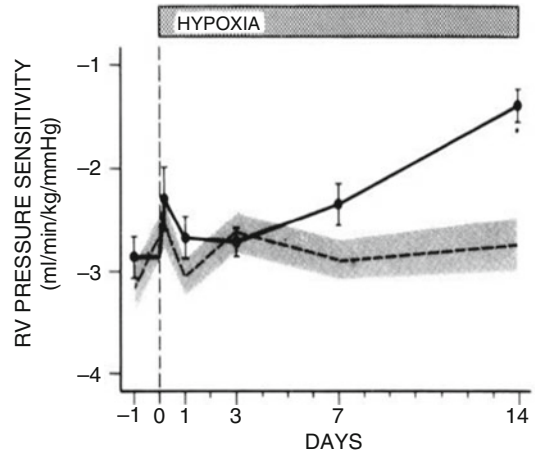

Fig. 3.11 Fetal right ventricular (RV) pressure sensitivity obtained by multiple regression analysis of afterload slopes as shown in Fig. 3.8 (representing relationship between right ventricular output (Q_{RV}) and arterial pressure) for hypoxemic and control groups. Symbols are as in Fig. 3.9. From Alonso et al. [12], with permission

at values of right atrial pressure between 3 and 20 mmHg. After fitting a linear correlation to the right ventricular function curves (values of Q_{RV} vs. right atrial pressure), the slope was 0.17 ± 0.26 for controls (n = 80 curves) and 0.27 ± 0.44 ml/min/kg/mmHg for hypoxic animals (n = 86 curves). (Fig. 3.11).

These values are not statistically different from zero nor from each other. Also, no differences were found comparing groups for each time period. In control fetuses Q_{RV} (expressed by the average of the values obtained during the cardiac function curve) was fairly constant during the 2-week study period (broken line of Fig. 3.12), starting at 183 ± 42 ml/min/kg^{-1} at day −1 and averaging 197 ± 34 ml/min/kg^{-1}. for the entire period.

Administration of atropine and propranolol before the first cardiac function curve induced small, but insignificant, changes of Q_{RV} in both groups. Before the administration of these drugs in the control group, Q_{RV} was 204.5 ± 36.8, 213.5 ± 37.8, and 200.5 ± 33.9 ml/min/kg^{-1} on days −1, 3, and 14, respectively. After atropine and propranolol, it was 183.4 ± 41.7, 203.5 ± 34.1, and 218.0 ± 46.6 ml/min/kg^{-1} on the same days. In the hypoxic group, the corresponding values were 204.3 ± 33.1, 135.9 ± 10.5, and 180.8 ± 30.1 ml/min/kg^{-1} before and 175.9 ± 35.3, 124.0 ± 8.2, and 203.5 ± 34.1 ml/min/kg^{-1} after administration of the drugs on days −1, 3, and 14, respectively. In the hypoxic fetuses no important changes in Q_{RV} were evident during the first 2 days. However, 3 days after starting hypoxia, Q_{RV} fell 30 % to 123.9 ± 8.2 ml/min/kg^{-1} from its beginning value of 176 ± 35 ml/min/kg^{-1}. This value was statistically different both from day −1 levels and the control group. The depression in Q_{RV} was maintained on day 7 but recovered to almost normal values by day 14. This depression in cardiac function was not caused by changes in FHR, since the latter was very constant in each animal after autonomic blockade.

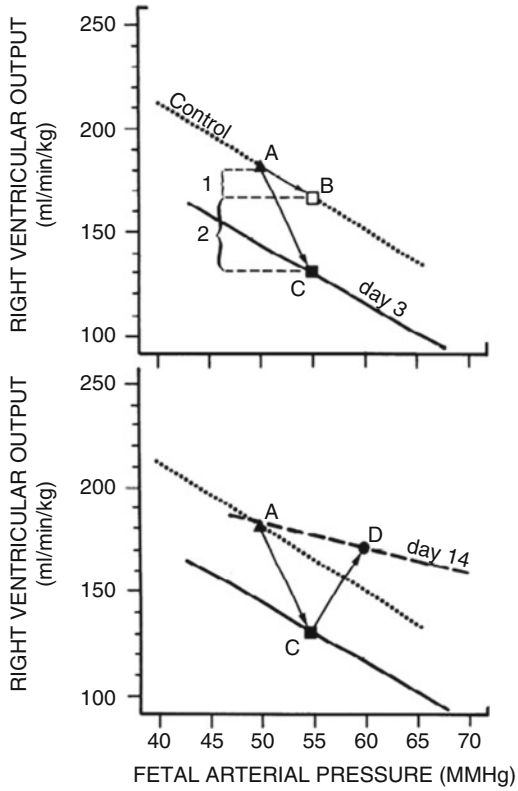

Fig. 3.12 Schematic representation of right ventricular output (Q_{RV}) and pressure sensitivity during long-term hypoxia. Each point represents coordinates of mean Q_{Rv} and fetal arterial pressure (P_{fa}) at days −1, 3 and 14 of study for hypoxic group. *Lines* represent afterload slopes forced through these coordinates. Control group behaved at all times similarly to hypoxic group on day 1, which is represented as a *dotted line* and *closed triangles* (*A*). *Top*: on day 3, control cases maintain all parameters, but in hypoxic group, Q_{RV} is shifted downward (*closed squares*, *C*) while maintaining same afterload slope (*solid line*). Observed increase in P_{fa}, does not suffice to explain all the decrease in Q_{RV}. *B* (*open squares*) represents expected Q_{RV} if this depression had been solely dependent on increased afterload (decrease depicted as *1*); *C* (m) represents actual value (additional decrease depicted as *2*). *Bottom*: on day 14 (*D*, *long-dashed line* and *1*), Q_{RV} has recovered in hypoxic group, and this is accompanied by a decreased pressure sensitivity. This decreased response to afterload may be an important factor for Q_{RV} recovery. See text for details. Alonso et al. [12], with permission

SV during the cardiac function curve in the control group was very stable throughout the study, averaging overall 1.142 + 0.042 ml/kg. On day 3 in the hypoxic fetuses SV decreased 30 % from its initial value of 1.037 + 0.169 to 0.766 + 0.054 ml/kg ($p < 0.05$ compared with day −1 and the control group). This depression was maintained to day 7 but recovered to normal values by day 14. This recovery in Q_{RV} and SV on day 14 in hypoxic fetuses was associated with a lower response of the right ventricle to increases in afterload, i.e., decreased pressure sensitivity, as illustrated in Fig. 3.12. In the control group the slope of the relationship between Q_{RV} and arterial pressure remained constant throughout the study. However, in the hypoxic group the slope became significantly less negative on day 14 (−1.40 ± 0.15 ml/min/kg/mmHg) compared with day −1 (−2.86 ± 0.26 ml/min/kg/mmHg), indicating Q_{RV} decreased less with increased afterload. Interestingly, on day 3 when cardiac function was most depressed (see Fig. 3.12), the heart was no more sensitive to increases in afterload than on day −1, i.e., the depression in Q_{RV} during day 3 corresponded to a downshift of the cardiac function curve with no change in the afterload slope (Fig. 3.12). Only small and insignificant increases were found in plasma activities of CK, LD, and AST in the hypoxic group.

Overall mean values for CK, LD, and AST in the control group were 14.6 ± 1.1, 394 ± 14, and 20.8 ± 1.8 U/l, respectively, and did not change with advancing gestational age. During the hypoxic period in the hypoxic group, values were 17.5 ± 1.4 (CK), 424 ± 15 (LD), and 26.8 ± 2.2 U/l (AST) and also did not change with time.

After the recovery of the fetal blood O_2 content to near-control level, right ventricular function, Q_{RV}, and SV began to recover on day 7 and by day 14 had returned to near-normal values despite a persistently high Pf. To achieve this, the pressure sensitivity of the right ventricle decreased (Fig. 3.12), and the afterload slope became more similar to that reported for the normal fetal left ventricle. Again, the mechanisms accounting for the decreased sensitivity to afterload are unknown. Cardiac hypertrophy was not observed, however it is possible that the number of contractile elements in myocardial cells increased.

The reduction of maternal PO_2 to −60 Torr, fetal PO_2 falls −7 Torr and is maintained at this level for 2 weeks, although it tends to recover at

the end of the period. Fetal O_2 content initially fell but by day 7 returned to normal values because of an increase in hemoglobin concentration.

Fetal cardiac function and Q_{RV} were maintained for several days but were significantly reduced on days 3 and 7, returning to normal by day 14. Day 14 probably represented the achievement of a new steady state, in which blood O_2 content, Q_{RV}, SV, and FHR had returned to normal values, in concert with normal fetal growth. In this new steady state, the fetal heart developed the ability to pump against an increased afterload, resulting from the polycythemia and the elevated arterial pressure.

5 The White Mountain Project

5.1 Further Experiments

After this experiments at the Division of Perinatal Biology at sea level, the next step was to study fetal responses to high altitude hypoxemia. For this purpose, a new project was started with the University of California that provided the facilities of the Barcroft Research Station at White Mountain (3,830 m). For that purpose a first flock of six pregnant sheep was transferred to this station together with an acid-base analyzer by Al Van Varick and me on a truck.

We sought to determine the effects of long-term hypoxemia on fetal cardiac output and flow distribution. On the second trip to White Mountain Research Station Al and me went to pick up the sheep back to Loma Linda after a period of 105 days in high altitude.

After we had fit the animals in the truck we started our descent, which was hazardous: a snow blizzard struck us in the middle of our descent to the point where the truck was unable to advance since we received more than 2 ft of snow in less than 1 h. This happened before the massive use of cell phones, so we had no way of getting help. We decided to abandon the truck and started walking downhill for a couple of hours under the snowstorm and we were lucky enough to find a shelter (constructed and supported by the Ranger Corps) with food and a radio which allowed us to communicate and get that a big four metal wheel tow truck helped us out of our compromised situation.

After a trip of more than 10 h, including this huge snow storm, we arrived in Loma Linda with all six ewes and their fetuses, as well as ourselves, alive.

5.2 Study Design

We exposed six pregnant sheep to high altitude (3,820 m) hypoxia from 30 to 135 days gestation (term 146 days). We then transferred the ewes to Loma Linda and instrumented them surgically as described above. Ten to 14 days after surgery we determined fetal cardiac output and organ blood flows by means of the radiolabeled microsphere technique during a baseline period and also during an additional 30-min period of more severe added acute hypoxemia. At this point doctors Takashi Okai and Masato Kamitomo, from Japan, had joined our team. It is with great pride that I consider Takashi Okai as one of my best friends to this day. Doctors Kamitomo and Okai continued the project and published the following paper [13].

5.3 Results

Baseline maternal arterial PO_2 was 60.7 ± 1.7 Torr and fell to 35.1 ± 3.0 Torr during the added acute hypoxemia. Fetal arterial PO_2 decreased from 18.5 ± 1.1 to 11.4 ± 1.5 Torr during added acute hypoxemia. Baseline fetal cardiac output was 351 ± 55 ml/min/kg^{-1}, which was significantly lower than previously reported values in low-altitude fetuses. Blood flow to critical organs such as the heart and brain was maintained at levels found in low-altitude fetuses, but flow to the carcass was significantly lower (−49 %) than the mean value reported in the literature for low-altitude fetuses. Oxygen delivery was also maintained at normal levels to the brain and heart but was reduced in the kidneys (−31 %), gastrointestinal tract (51 %), and carcass (−58 %). During added acute hypoxemia cardiac output did not change significantly;

however, blood flow to the brain, heart, and adrenal glands increased 112 %, 135 %, and 156 % ($p<0.05$), respectively.

We concluded that during long-term high-altitude hypoxemia redistribution of fetal cardiac output is maintained favoring the brain and heart [13].

6 Further Studies

After my return to Montevideo, I continued to work on perinatal medicine, although on a clinical perspective, and my research was focused on fetal diagnosis and therapy, biochemical genetical disposition to prematurity, placental adaptations to hypoxemia, and several other projects.

At Loma Linda, the team headed by Larry and Raymond Gilbert continued the study at high altitude, keeping a flock of several pregnant sheep at the Barcroft Research Station (Fig. 3.13), and after studying thoroughly fetal adaptations published several papers on the subject [14, 15].

7 Persevere

Everyone that knows Larry is familiar with the word that he always uses at the end of his letters. Since my return to Montevideo I had been regularly in touch with Larry, who has stimulated me constantly on keeping research in my main interest. Perhaps a sign I read on my last visit to the Center of Perinatal Biology (authorship unknown) reflects one of the principal late-motifs of this team:

IF YOU THINK MEDICAL RESEARCH IS EXPENSIVE—TRY DISEASE

With Dr. Ravi Goyal and Larry we developed a new research project including epigenetics: "Epigenetic Profiles in Placenta and Biomarkers in Maternal Blood: Severe Preeclampsia and Intrauterine Growth Restriction", investigators: Justo Alonso, Ravi Goyal, Lawrence D. Longo. The working hypothesis is: Changes in the genomic expression of the placental cytotrophoblast and syncytiotrophoblast cells in preeclampsia (with and without IUGR) is associated with

Fig. 3.13 Pregnant sheep kept during most of gestation at the Barcroft Research Station, University of California, White Mountain (3,830 m above sea level)

alterations in epigenomic transcriptional (DNA methylation and histone modifications) and post-transcriptional (microRNA) regulators. At first, this project was planned to start in 2013, but it is still in the phase of fund raising.

I would like to end this chapter quoting Professor Lawrence D. Longo:

> The challenges that now confront the practice of medicine can be addressed successfully only to the extent that physicians promote virtue-ethics, act collectively In the public interest, and render service that clearly transcends their own self-interests. [16]

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4

Pregnancy Programming and Preeclampsia: Identifying a Human Endothelial Model to Study Pregnancy-Adapted Endothelial Function and Endothelial Adaptive Failure in Preeclamptic Subjects

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Abstract

We have previously reported that the increase in vasodilator production in an ovine model pregnancy is underpinned by an increase in connexin 43 (Cx43) gap junction function, so allowing more uterine artery endothelial cells to produce a more sustained Ca^{2+} burst response to agonist stimulation. Since activation of endothelial nitric oxide synthase (eNOS) requires elevated $[Ca^{2+}]i$, it follows that the direct result of enhanced bursting in turn is an increase in nitric oxide (NO) production per cell from more cells, and for a longer period of time. Preeclampsia (PE) is associated with endothelial vasodilatory dysfunction, and the endocrine profile of women with PE includes an increase in a number of factors found in wound sites. The common action of these growth factors and cytokines in wound sites

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L. Zhang and C.A. Ducsay (eds.), *Advances in Fetal and Neonatal Physiology*, Advances in Experimental Medicine and Biology 814, DOI 10.1007/978-1-4939-1031-1_4,

is to mediate Cx43 dysfunction through kinase phosphorylation and closure. Translational studies are now needed to establish if inhibitory phosphorylation of Cx43 in human endothelium is the cause of endothelial dysfunction in PE subjects and if so, to identify the kinase pathways best targeted for therapy in PE subjects. Consistent with this we have already shown endothelial Ca^{2+} and NO responses of human umbilical vein from normal subjects are similar to that of ovine pregnant uterine artery, and that those same responses in cords from PE subjects are blunted to levels more typical of nonpregnant uterine artery. In this review we summarize the further evidence that growth factors and cytokines may indeed mediate endothelial dysfunction in PE subjects through closure of Cx43 gap junctions. We also consider how we may clinically translate our studies to humans by using intact umbilical vein and isolated HUVEC in primary culture for an initial screen of drugs to prevent deleterious Cx43 phosphorylation, with the ultimate goal of reversing PE-related endothelial dysfunction.

Keywords
Endothelial • Nitric oxide • Ca^{2+} • Programming • Connexin • Preeclampsia • Wounding

1 Introduction

1.1 Pregnancy Adaptation of Endothelial Function and the Nature of Adaptive Failure

Pregnancy is associated with a need to expand blood volume and increase cardiac output to allow preferential redistribution of blood flow to the uterus. Normal maternal vascular adaptation involves enhanced vasodilation, including enhanced activation of endothelial nitric oxide synthase (eNOS) to make the vasodilator nitric oxide (NO). While the greatest effect is seen in the uterus, enhanced vasodilation is also seen in the systemic circulation. In preeclampsia (PE), a condition occurring in 8 % of pregnancies, this adaptive vasodilatory response fails and hypertension results [1, 2]. Clinical treatments are currently limited to antihypertensive agents targeted at the vascular smooth muscle, along with $MgSO_4$ to prevent preterm birth. Such approaches have only limited success. Given pregnancy-adapted endothelial function is known to have failed, a new treatment aimed at restoring endothelial function could be of considerable value. But to get there we need to know two things: (1) How is pregnancy-adapted endothelial vasodilatory function achieved in the first place, and (2) Could knowing the molecular cause of endothelial failure also help identify a potential drug target to restore function in human disease?

A number of studies in intact rat and sheep uterine artery have answered the first question, revealing that it is pregnancy-specific augmentation of sustained endothelial Ca^{2+} signaling that underlies pregnancy-enhanced NO production and corresponding increases in vasodilation [3–6]. Studies in both ovine uterine artery vessels and cells in culture have further shown that pregnancy-enhanced Ca^{2+} signaling occurs under the permissive control of pregnancy-enhanced connexin (Cx) 43 gap junction function [7]. The resulting increases in sustained Ca^{2+} signaling (burst number and cell number responding) are causally related to dramatically enhanced eNOS activation [5]. Furthermore, any strategy that blocks Cx43 function or the resulting Ca^{2+} response in turn also results in an equally blunted NO output down to nonadapted levels [4–7].

Growth factors and cytokines found in wound sites are already known to converge though signaling kinases that phosphorylate and so close Cx43 gap junctions [8], and we have recently commented that the altered endocrine profile of PE subjects includes the same factors found in wound sites [9, 10]. It is this realization that provides a framework for approaching the second question; i.e., given Cx43 gap junction closure by phosphorylation in response to these many factors is indeed a cause of the loss of pregnancy-adapted endothelial function, can targeting the signaling kinases responsible provide a way to prevent PE-associated endothelial dysfunction? The purpose of this review is to consider this question further, and so propose a way to move forward with human translational therapeutic studies for the first time.

2 Background

Before we consider the cause of endothelial adaptive failure in PE subjects, we first need to review in more mechanistic terms what pregnancy adaptation of endothelial function is, and how it couples to increased vasodilation. To that end, it is easiest to work backwards from what signals are responsible for greater and more sustained activation of eNOS, and how changes in those signals may result from altered Cx43 function.

2.1 eNOS Activation Through Ca^{2+} vs. Phosphorylation

We have chosen to focus this review on pregnancy adaptation of endothelial function at the level of eNOS. The advantage of this approach is that NO is generated by a simple, single step enzyme pathway and the added availability of NO-sensitive dyes has allowed us to study changes in both Ca^{2+} and NO in real time on a single cell level. Nonetheless, cytosolic phospholipase A2 (cPLA2) activation which liberates arachidonic acid for prostaglandin biosynthesis is also $[Ca^{2+}]i$ sensitive, and so more sustained or amplified Ca^{2+} responses may equally support pregnancy-enhanced prostacyclin output by vascular endothelium [1].

Many other reviews have detailed the 'life cycle' of eNOS as it undocks from the caveolae, dimerizes, and associates with proteins such as Hsp90 in the cytosol [11], and it is not our intent to repeat that here. Suffice it to say that eNOS is also specifically phosphorylated at a number of well-defined sites, some of which are classically considered inhibitory (positions 116, 495) or stimulatory (positions 617, 635, 1177) [11]. Pregnancy further increases the extent of eNOS phosphorylation in response to ATP in ovine uterine artery endothelial cells (UAEC) [12, 13], but not so clearly with vascular endothelial growth factor (VEGF) stimulation [14]. In reality, the assignment of the terms "inhibitory" and "stimulatory" to these sites is misleading, and more direct analysis of this question in pregnant UAEC has shown that changes in eNOS activation do not always parallel the state of phosphorylation [12]. Use of agents such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in P-UAEC can even induce 617, 635, and 1177 phosphorylation without substantially changing 497 phosphorylation and still not cause any detectable eNOS activation. TPA is not, of course, a stimulator of a Ca^{2+} response in P-UAEC, and recently it has become clear the 'activating' phosphorylations serve to stabilize Ca^{2+}/calmodulin binding to the dimer, as well as increasing transfer efficiency of electrons between complimentary eNOS domains [11]. Lin et al. [15] have shown that when eNOS is activated by Ca^{2+} mobilizing agonists, it is the sustained capacitative Ca^{2+} entry (CCE) phase of the Ca^{2+} response that is most responsible for activation. More recently Tran et al. [16] confirmed that optimal phosphorylation of eNOS at positions 617 and 1177 causes an increase in sensitivity of the eNOS complex to $[Ca^{2+}]i$ in the physiologic range. Thus Ca^{2+} is in fact a necessary *trigger* for activation, and only then can optimal phosphorylation regulate the gain in terms of NO output as $[Ca^{2+}]i$ rises through the nanomolar range. It is for these reasons that an understanding of the effect of pregnancy on Ca^{2+} signaling in

response to vasodilatory stimuli is critically important, as it is both an essential trigger and dose-dependent regulator of eNOS activation in vascular endothelium.

2.2 Pregnancy Adaptation of Ca^{2+} Signaling in Uterine Artery Endothelium

The establishment of the UAEC model has been important for dissecting the molecular mechanisms behind pregnancy adaptation of Ca^{2+} signaling in uterine artery endothelium. One of the earliest observations was that although passage 4 UAEC from luteal phase nonpregnant (NP) and late pregnant (P) ewes have equal expression levels of key proteins involved in both Ca^{2+} signaling and NO production, P-UAEC consistently show greater CCE and higher levels of NO production in response to stimulation with both physiologic agonists such as ATP and nonphysiologic receptor-independent agonists such as thapsigargin [9, 17]. Subsequent experiments revealed that the pregnancy-adapted CCE phase of $[Ca^{2+}]i$ elevation is causally related to pregnancy-enhanced NO production, and is mediated through a pronounced increase of TRPC3 association with inositol 1,4,5-trisphosphate (IP3) receptor IP3R2 that is not otherwise observed in NP-UAEC [18].

On an individual cell level, the Ca^{2+} response is biphasic in nature, consisting of an immediate initial $[Ca^{2+}]i$ peak occurring immediately upon agonist addition, followed by a sustained phase of $[Ca^{2+}]i$ elevation in the form of multiple periodic $[Ca^{2+}]i$ bursts. Of note, the duration of repeated bursting and number of total bursts in 30 min is clearly greater in P-UAEC than in NP-UAEC [7]. The enhanced Ca^{2+}/NO response seen in P-UAEC is also inherently dependent upon the ability of the cells to communicate with each other. In cell density studies, increasing the likelihood of communication between UAEC by cell contact (increasing cell culture density) resulted in a pregnancy-specific enhancement of Ca^{2+} bursting. Conversely, if P-UAEC were grown to low density, such that they rarely touch, this resulted in fewer cells bursting and fewer observed bursts per cell to a level more typical of the response seen in NP-UAEC. Another observation made during high-density studies was that coordination/synchronization of Ca^{2+} bursts among P-UAEC that was far greater than in NP-UAEC. The timing of bursts in the CCE phase was synchronized across multiple cells in P-UAEC, and a higher percentage of cells (P > NP) showed bursts that would initiate at the same time as others in the observable field. As coordinated cells were not necessarily immediate neighbors, it was inferred that cells may be transferring IP3 or even undertaking a form of electrical communication. Further studies in the intact endothelium of freshly isolated vessels (UA Endo) confirmed that such pregnancy enhancement of CCE burst frequency and synchronization was observed in intact vessels, and was associated with dramatically increased NO production [4, 5].

Endothelial cell communication with its neighboring cells occurs extensively through gap junctions, which exist as several isoforms. It has recently been shown that in UA Endo both Cx43 and Cx37 expression are locally elevated in the endothelium of the uterine artery most proximal to the site of implantation during pregnancy [6], but it is Cx43 that is most widely recognized as a facilitator of Ca^{2+} and possibly IP3 exchange between cells [19, 7]. Consistent with this, application of GAP27 inhibitory mimetic peptide (specific to Cx43) resulted in a complete loss of the pregnancy-adapted Ca^{2+} burst phase that was paralleled by inhibition of NO production in P-UAEC down to the levels of NP-UAEC [7]. Similar observations were also made in preparations of intact P-UA Endo treated with GAP27 [6]. Of note, the application of GAP peptides to human subcutaneous resistance arteries verified it is Cx43 that also mediates bradykinin (BK)-stimulated vasodilation in pregnant human subjects [20].

In summary, while it is still not completely understood how Cx43 may alter the environment in which TRPC3 and IP3R2 interact, it seems clear that changes in Cx43 function underlie

pregnancy adaptation of Ca^{2+} bursting and so NO output by uterine arteries. The three specific ways in which Cx43-driven P-UAEC function is enhanced are through enhanced number of Ca^{2+} bursts observed per cell, greater synchronization in the timing of the Ca^{2+} bursts across multiple UAEC, and functional recruitment of additional cells to respond.

2.3 Wounding and Associated Inflammation as a Negative Regulator of Cx43

Given the entire process of pregnancy adaptation of endothelial NO output depends on the enhanced function of just one molecule (namely Cx43), then it follows that any interference with that activation process could cause pregnancy-adapted vasodilation to fail [9, 10]. Some indication that this may indeed occur is already clear from animal and cell studies of wound sites.

Perturbations in gap junction function occur in the epidermis during the inflammatory process of wound repair. Healing of the epidermis requires cell migration, proliferation and differentiation, all of which involve gap junctions [21]. In human epidermal keratinocytes, Cx43 is the most abundant form and is expressed in all layers, although is most concentrated in the more differentiated upper stratum spinosum and lowest in the proliferative stratum basale region [22]. After wounding, the most immediate response is to stop cell–cell communication through local elevations of growth factors and cytokines that promote inhibitory phosphorylation events on Cx43 [21, 23]. Expression of Cx43 is then reduced at the wound margin in both humans and rodents despite differences in Cx distribution within the epidermis [22, 24]. Restoration of Cx43 expression and function only occurs after complete re-epithelialization of the wound [25]. This prolonged period of imposed Cx43 dysfunction is necessary to allow cells time to proliferate and migrate to the new position. Once in place, and as healing allows the return of local growth factors and cytokines to basal levels, normal Cx43 function resumes.

In chronic wounding states, cytokines and growth factors can remain elevated and the resulting erroneous endocrine signaling can actively prevent reestablishment of correct tissue structure and function. Excessive elevation of a number of cytokines including tumor necrosis factor (TNF)-alpha and interleukin (IL)-1beta can occur through mutual reinforcement, and the wound site can end up with substantial increases in TNF-alpha, IL-1, and IL-6 in the continued presence of growth factors including VEGF, basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) [21, 23]. Many cell studies, including those of wound sites, have helped further elucidate the role of these individual growth factors and cytokines in regulating Cx43 function through multiple signaling kinases, and these are reviewed in detail elsewhere [8, 26–28]. Of relevance here, even when the expression level of Cx43 total protein appears to be normal, these growth factors and cytokines can still negatively regulate Cx43 through their common ability to promote Cx43 inhibitory phosphorylation at position s368 (by protein kinase C), at positions s279/282 (by ERK) and at sites including position y265 (by Src kinase). Differences in the action of each endocrine factor, alone or combined, dictate the overall balance of activation of these three signaling kinase pathways and the duration of activation of each pathway. Chronic activation of these pathways above normal levels can result in a sustained inhibition of functional gap junctions, as well as altered Cx protein translocation, gap junction assembly and disassembly, and channel transport properties [29–31]. This begs the question of whether the lack of pregnancy-adapted vasodilatory function in PE is indeed due to the generalized elevation of the same endocrine factors identified at chronic wound sites. We also ask if there is any evidence for the action of such a cocktail of factors in promoting endothelial dysfunction in PE subjects, and further if there is evidence this is mediated through changes in Cx43 gap junction function.

2.4 The Evidence That Multiple Cytokines and Growth Factors Abnormally Elevated in PE Subjects Are Also Associated with Endothelial Dysfunction in Humans and Animal Models

As reviewed previously, PE is characterized within weeks by a failure to show pregnancy-adapted vasodilation [1, 2]. Further data suggests that an exaggerated inflammatory response and abnormal vascularization during placentation each have specific roles in the development of PE symptoms [32, 33]. Abnormal placentation during the first stages of pregnancy may lead to a reduction in utero placental perfusion and this may in turn further contribute to production of PE associated factors from the hypoxic/ischemic placenta. The decidua and fetal membranes may also further contribute to increased levels of multiple cytokines (particularly TNF-alpha, IL-6, IL-8 and interferon (IFN)-gamma) and growth factors (particularly VEGF but also bFGF and EGF) in the maternal circulation, and these are associated with the systemic symptoms of PE [34, 35]. We now survey the link between each of these factors in PE subjects, associated endothelial dysfunction, and the possible mediation of such effects through ERK or Src kinase signaling acting to inhibit Cx43 function. For clarity we will first consider the cytokines, and then discuss the growth factors found at chronic wound sites and abnormally elevated in PE subjects.

2.4.1 Evidence for Increased Cytokine Production in PE Subjects

Cytokines normally circulate at higher levels even during normal pregnancy, but in 2003 Redman and Sargent [36] published the theory that excessive maternal intravascular immune/inflammatory response is also a possible cause of PE. Since then, further studies have supported the notion PE is associated with elevation of several cytokines [33] known to be actively involved in controlling endothelial, cardiovascular, and renal function under physiological and pathological conditions [37]. Although the results of studies comparing normal vs. PE pregnancies have been mixed [38, 39], there seems to be growing evidence that in PE subjects there are increased levels of several different cytokines including TNF-alpha [40–45], IL-1beta [46], IL-6 [40, 42, 44, 45, 47, 48], IL-8 [44, 49], and IFN-gamma [33, 50–52]. These same factors all have direct or indirect relevance to Cx43 functional inhibition (see above).

Given PE is a condition of abnormal placentation and associated hypoxia, it is relevant that hypoxia alone can increase production of TNF-alpha, IL-1alpha and IL-1beta even by the normal placenta [53, 54]. Nonetheless, the placenta is not the only source of elevated cytokines in PE subjects. IL-6 is also produced by decidua as well as placenta, and Lockwood [48] suggests the decidua is a greater source of IL-6 in PE pregnancies. In normal pregnancy, IFN-gamma is produced by uterine natural killer cells and has been shown to play a critical part in normal implantation of placenta and spiral artery remodeling. In PE subjects, however, IFN-gamma is further elevated in plasma, circulating leukocytes, and decidual tissue [51]. While the source of elevated IL-8 is more elusive, Kauma [49] showed human umbilical vein endothelial cells (HUVEC) grown in plasma from PE women exhibit a 1.5-fold increase in production of IL-8 [49]. Further evidence for stimulation of cytokine production was provided by Kocyigit et al. [55], who measured serum levels of different cytokines in the third trimester of pregnancy in women with and without PE. They found a significant increase in TNF-alpha during the third trimester, and serum levels of IL-6 and IL-8 were significantly correlated in women with PE. Both TNF-alpha and IL-1beta have since been shown to induce a clear dose-dependent effect on IL-6 release by decidua [48].

2.4.2 Effects of Cytokines in Animals and Isolated Vascular Tissue Studies

The reduced uterine perfusion pressure (RUPP) model [56] is a widely used model of PE because it mimics hypertension and endothelial dysfunction. The RUPP procedure places silver clips

around the right and left ovarian artery and infrarenal aorta to reduce uterine perfusion pressure. Serum taken from RUPP rats has elevated TNF-alpha and IL-6, as well as soluble Flt (sFlt)-1 levels [37, 57–59]. In non-surgical models, infusion of TNF-alpha into pregnant rats resulted in a significant increase in blood pressure and a decrease in renal NO production [60], while similar studies by LaMarca [37] also showed an increase in mean arterial pressure, renal vascular resistance and a decrease in renal glomerular filtration rate. In pregnant baboons, infusing TNF-alpha for 2 weeks resulted in a significant increase in blood pressure compared to control groups [61]. Plasma sFlt-1 also significantly increased over time in the TNF-alpha-treated pregnant baboons but not the non-pregnant group, suggesting TNF-alpha may play a part in elevating sFlt-1 levels in PE patients.

Isolated vascular tissue studies have also been informative. TNF-alpha increased vascular tone in intact endothelial aortic vascular strips from pregnant rats and inhibited pregnancy-enhanced endothelial vasodilation in response to acetylcholine (ACh) and BK, as mediated via the NO/cGMP pathway [62]. These findings were consistent with the results of Chia et al. [63] in which infusion of TNF-alpha into human brachial forearm arteries resulted in a loss of ACh- or BK-induced vasodilation. Similar observations were made in bovine coronary arteries, where BK-induced NO production was impaired following TNF-alpha exposure [64]. In rat mesenteric vessels, exposure to TNF-alpha but not IL-1beta, leads to a loss of vasodilation and NO production in response to ACh and BK [65]. LaMarca BB [66] showed that chronic infusion of IL-6 into pregnant rats resulted in an increase of mean arterial pressure that did not occur when an angiotensin antagonist (Losartan) was given, suggesting that the angiotensin pathway is involved in the long-term response. Nonetheless, a direct action on endothelium was also suggested by Orshal [67], who tested the effects of 1-h treatment with IL-6 on aortic strips from pregnant and virgin rats. They reported that IL-6 substantially reversed the ACh- and BK-mediated pregnancy-enhanced vasodilation, and did so by impairing activation of the NO/cGMP pathway in a manner comparable to the acute actions of TNF-alpha [62].

2.4.3 Evidence for Altered VEGF, bFGF and EGF Production in PE Subjects

While many growth factors may play important roles in control of the uteroplacental vasculature in pregnancy, the one factor investigated most thoroughly in women with PE is VEGF. Elevated levels of VEGF have been reported in some studies of PE subjects (reviewed in [68]), which may be due in part to increased production by trophoblasts in response to hypoxia [69]. Despite complications by method of sample collection in measuring circulating VEGF [70], and the confounding effect of circulating sFlt (a soluble form of VEGF receptor (VEGFR)1 [71]), elevated VEGF in amniotic fluid is a predictor of preterm delivery [72]. VEGF expression has also been shown to be upregulated in both hypoxic and PE placentae [73], consistent with the proposal that VEGF itself is a key factor in the pathogenesis of PE [68].

While the literature on relative VEGF levels in PE subjects is abundant, that on circulating bFGF or EGF levels is comparatively rare. Nonetheless, human studies comparing serum bFGF levels between normal and PE pregnancies have shown the median values were significantly elevated in women with mild or moderate, but not severe PE [74]. EGF is also detectable in amniotic fluid and rises late in pregnancy, with levels being significantly *lower* in subjects giving birth to intrauterine growth restricted infants [75].

2.4.4 Effects of VEGF, bFGF and EGF in Animal and Isolated Vascular Tissue Studies

In mice, VEGF administration can produce a PE-like condition, including placental hypercoagulation and hypertension [76]. VEGF treatment of intact human arteries ex vivo has been shown to impair endothelium-mediated relaxation in response to BK [77, 78]. VEGF treatment of pregnancy-adapted UA Endo preparations have been shown to inhibit subsequent NO production in response to ATP [5] to an extent similar to that observed using GAP27 [6].

In HUVEC, Cx43 inhibition by VEGF is mediated by altered Cx43 phosphorylation [79]. In contrast, studies in microvascular endothelium suggest bFGF actually increases Cx43 expression and cell–cell coupling [80]. Thus, it is of particular relevance that bFGF may act in concert with other circulating factors, because bFGF also mediates TNF-alpha-stimulated endothelial IL-6 production in human microvascular endothelial cells [81]. Although studies of EGF action are rare, EGF has been shown to behave like VEGF, impairing Cx43 gap junction function in isolated HUVEC [82].

2.4.5 Cytokine and Growth Factors Signaling to Endothelium Cx43 via Src and ERK Kinases

Studies in UAEC have already shown expression of multiple cytokine receptors, including those for TNF-alpha, IL-1 and IL-6 [83]. Studies in other cells have further shown that TNF-alpha is capable of signaling to Src kinases [84]. Van Rijen et al. [85] demonstrated that even when TNF-alpha did not appear to affect total cellular Cx43 levels in HUVEC, the cellular localization was still altered, with more perinuclear staining implicating a loss of cell surface function. TNF-alpha has been shown to inhibit Cx43 function via the Src pathway in Human lung endothelial cells [86]. IL-6 also appears to couple to Src and ERK signaling pathways in lymphatic endothelial cells [87]. While there is little evidence the other cytokines (IL-1beta, IL-8 or IFN gamma) directly signal through Src or ERK pathways, it is likely they can mediate negative effects on Cx43 function through altering production of other cytokines that do (above), or transactivation of growth factor receptors (below).

VEGFR1, VEGFR2, multiple FGF receptor isoforms and EGF receptor (EGFR) are all expressed in both P-UAEC [14, 83, 88], and HUVEC-CS [89]. In P-UAEC, VEGFR2 is coupled to ERK [14]. Src activation has not yet been investigated in P-UAEC, but VEGFR2 is already known to couple to both ERK and Src pathways to mediate inhibition of Cx43 in HUVEC [79]. Although the actions of bFGF and EGF have not yet been similarly reported on Cx43 itself in endothelium, bFGF is known to activate both Src and ERK signaling pathways in HUVEC [90], and activation of the ERK pathway was observed in response to mitogenic doses of bFGF, EGF and VEGF in P-UAEC [14, 83, 88] and HUVEC-CS cells [89]. EGFR is not only capable of coupling to the Src signaling pathway in natural killer cells, it may indeed do so in response to IL-8-induced transactivation [91]. Further observation of similar EGFR transactivation by IL-8 in microvascular endothelium [92] is therefore highly relevant to our consideration of the cause of endothelial dysfunction in PE subjects as it suggests elevation of EGF may not be needed for EGFR to mediate negative effects on Cx43 in vivo.

3 The Translational Barrier

Given the endocrine profile of PE is very similar to that of wounding, it is possible the associated loss of pregnancy-adapted endothelial function may well result from Cx43 function being blocked by inappropriate signaling from these PE-associated endocrine factors. More recently we have established that inhibitory regulation of Cx43 is indeed possible in UAEC, and Ca^{2+} bursting is particularly sensitive to factors that increase ERK and Src signaling [10]. Treatment of P-UAEC with TPA resulted in phosphorylation of Cx43 at position s279/282, s368 and y265, indicating that ERK, PKC, and Src regulatory kinases are active and phosphorylating their expected Cx43 sites [10]. The TPA-induced phosphorylation of s279/282 Cx43 was in turn blocked by U0126, confirming the involvement of the MEK/ERK pathway, while PP2 blocked phosphorylation of y265, demonstrating a role for Src family kinases. Of note, reversal of Cx43 phosphorylation at these specific ERK and Src target sites alone were also associated with recovery of pregnancy-adapted Ca^{2+} signaling function [10]. We now need to establish if this is the case in endothelial cells from pregnant *human* subjects, and further expand our studies to include the many 'wound associated' endocrine factors found in PE subjects. Only then can we begin to screen for possible therapeutic compounds that block their combined effects at the level of the ERK

and/or Src pathways. Once a short list of such candidate molecules is identified, we can then examine if these same compounds could help restore normal pregnancy-adapted endothelial function in intact vessels from PE subjects. In order to do all of this, we must first consider just which human vessels are both accessible and have the potential to provide an accurate model of pregnancy-adapted endothelial cell function and PE-associated dysfunction.

3.1 Finding a Suitable Model for Translation

While whole animal and the derived cell culture models have immense research value, PE or equivalent disorders of pregnancy are exceedingly rare in the animal kingdom. As a result many animal models are derived from irreversible surgeries or infusion of endocrine factors to try to reproduce the symptoms of PE (see above) and are at best a close approximation of disease both from a causal and symptomatic point of view. The limitations of studying the molecular aspects of the disease in the human, however, are largely ethical due to the invasive nature of tissue removal from an already compromised system and the especially sensitive nature of fetal drug exposure. Thus, mechanistic human studies have mostly been limited to tissue collected from small uterine myometrial arteries collected by uterine biopsy during caesarian section [93–96]. While such studies allow evaluation of vessel contractility in PE vs. normal pregnancies, they are insufficient to provide a source of high enough quantity or purity of early passage human uterine artery endothelial cells to perform automated tier one drug screening as we propose is necessary to advance the field. There would clearly be considerable value in a cell culture model from an alternate human vessel that shares functional properties with that of the pregnancy adapted uterine artery, and is also easily obtained in large amounts through minimally invasive procedures.

Another question to consider is whether that vessel has to be maternal or even arterial? Some state that PE is a maternal disease, but we would point out the easy measure of maternal parameters and access to maternal tissues may have unconsciously biased our view in this regard. Indeed, when Doppler ultrasound is performed on the fetal circulation in fetal growth restricted pregnancies (often complicated with maternal PE), there routinely are abnormal flow patterns in the fetoplacental circulation, including umbilical artery and vein [97–100]. The studies of Mahdy et al. [101] on maternal human hand vein endothelium show clearly that there are defects in systemic endothelial function in PE subjects that includes *venous* endothelial cell dysfunction. So, despite the attention paid to the uterine arteries, there is a precedent for observed dysfunction in other endothelial cell types of both arterial and venous vascular beds. This makes sense given all endothelial cells may be exposed to the same 'toxic' cocktail of circulating cytokines and growth factors associated with PE.

What these combined observations and the inherent observational bias of the mother over the child suggest to us is that an appropriate cell model may not necessarily have to come from the uterine artery, or even necessarily the maternal circulation. Dysfunction of umbilical endothelium has already been reported [102], and we have recently made observations of parallel reductions in both Ca^{2+} bursting and NO output in umbilical vein endothelium [103]. We propose herein that the HUVEC model may indeed be a completely valid cell model for both mechanistic studies of endothelial dysfunction in PE, as well as tier one screening of drugs to restore endothelial function. From the standpoint of ethical collection of large amounts of tissue, umbilical cord vessels, being waste tissue upon delivery, are without rival. The vessel size and tissue quantity allows for relatively easy endothelial cell isolation and sorting for purity, satisfying the requirements needed for large-scale drug screening and extensive mechanistic profiling. Umbilical cords can also be collected from normal and diseased pregnancies, allowing for direct comparison in culture of any programmed changes in cell behavior (programmed defined as any aspect of cell function reflecting the normal or abnormal condition in vivo and retained even after expansion in primary culture). Most importantly, HUVEC show similar capacity to produce sustained Ca^{2+} responses to multiple agonists, and correspondingly produce NO, as UAEC have

been shown to do. The following sections will now further address the extent of these similarities in more detail.

3.2 Evidence That UV Endo and HUVEC Signal eNOS Activation in a Cx43-Dependent Manner Similar to P-UA Endo and P-UAEC

The essential considerations with regard to the use of alternative cell models of PE for translational research are not simply whether there is ample access to tissue, but also if the relevant properties of that tissue are comparable to the best animal cell models available. In this case, sustained Ca^{2+} signaling and eNOS activity are of the utmost relevance. Fidelity of this signaling pathway in a human cell model is essential in order to test the hypothesis that growth factor and cytokine signaling can inhibit coordinated endothelial cell function through Cx43 phosphorylation. This section presents the case that human umbilical vein (in UV Endo and HUVEC forms) function to produce sustained Ca^{2+} responses and associated NO output through similar signaling components to those already identified in ovine UA Endo and UAEC.

Figure 4.1 compares representative individual cell tracings from ovine UA Endo and human UV Endo preparations using identical methodologies to those previously published [5, 103]. Such analysis

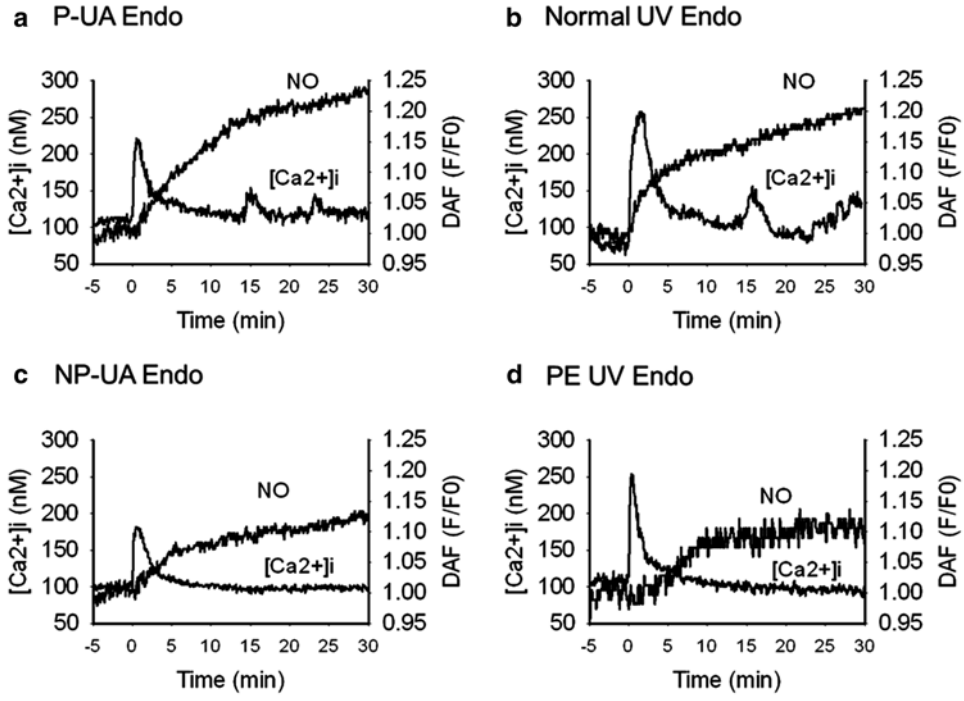

Fig. 4.1 Representative Ca^{2+} and NO tracings from individual endothelial cells on intact vessel segments. Real-time measurements for $[Ca^{2+}]i$ (Fura-2) and NO (DAF-2) in ovine uterine artery (UA Endo, panels **a** and **c**) and human umbilical vein (UV Endo, panels **b** and **d**) segments from select physiological conditions are shown. Methods were exactly as in [111, 114] for UA-Endo and [48] for UV-Endo. The Ca^{2+} stimulating agonist, ATP (100 μM), was added at 0 min and $[Ca^{2+}]i$ and NO production are monitored for 30 min. Panels **a** and **c** show tracings from vessel segments from late gestational pregnant (P-) and luteal phase non-pregnant (NP-) ewes. P-UA Endo show sustained Ca^{2+} burst responses to ATP which correspond to sustained NO production. NP-UA Endo lack such sustained Ca^{2+} responses, and NO production in NP-UA Endo is reduced in comparison to P-UA Endo. Panels **b** and **d** show tracings from human umbilical veins isolated from Normal and PE pregnancies. Normal UV Endo show sustained Ca^{2+} burst responses to ATP and produce NO throughout the 30 min recording. PE UV Endo show blunted Ca^{2+} and NO responses to ATP. Thus, P-UA Endo and Normal UV Endo Ca^{2+} and NO responses are analogous; while NP-UA Endo and PE UV Endo show analogous blunted Ca^{2+} and NO responses

reveals similar broad periodic Ca^{2+} bursts are observed in response to ATP challenge in the endothelium of both vessels. Production of NO is also similarly maintained for the duration of the Ca^{2+} burst response in each case. Gap junctions are also present in the endothelium of both ovine uterine arteries and human umbilical vein. In freshly isolated UA Endo, both Cx37 and Cx43 are detectable by western blot [6]; Cx43 dominates gap junction protein expression in vessels from late-term pregnant ewes, while levels of Cx37 are comparatively low in both the non-pregnant and pregnant state. In P-UA Endo, in spite of expression of multiple gap junctional proteins, the use of GAP inhibitory peptides has confirmed only Cx43 is functionally linked to the agonist-stimulated pregnancy-adapted Ca^{2+} burst response and pregnancy-adapted NO production [6]. Connexin expression in umbilical vein also comprises varying levels of Cx37, Cx40, and Cx43 depending on the preparation. Immunostaining of umbilical veins in situ showed clear homogeneous endothelial expression of Cx40 and Cx43, while Cx37 was expressed in a less uniform manner [104]. More recent western blot for Cx43 expression in freshly isolated UV Endo also gave a strong signal [103], but other Cx isoforms were not investigated.

For both ovine uterine artery and human umbilical vein, isolated endothelial cells in early passage primary culture continue to show repeated Ca^{2+} bursting, albeit with the peaks being narrowed and occurring more frequently (Fig. 4.2). Direct NO measurement is not possible at this time in cultured UAEC or HUVEC

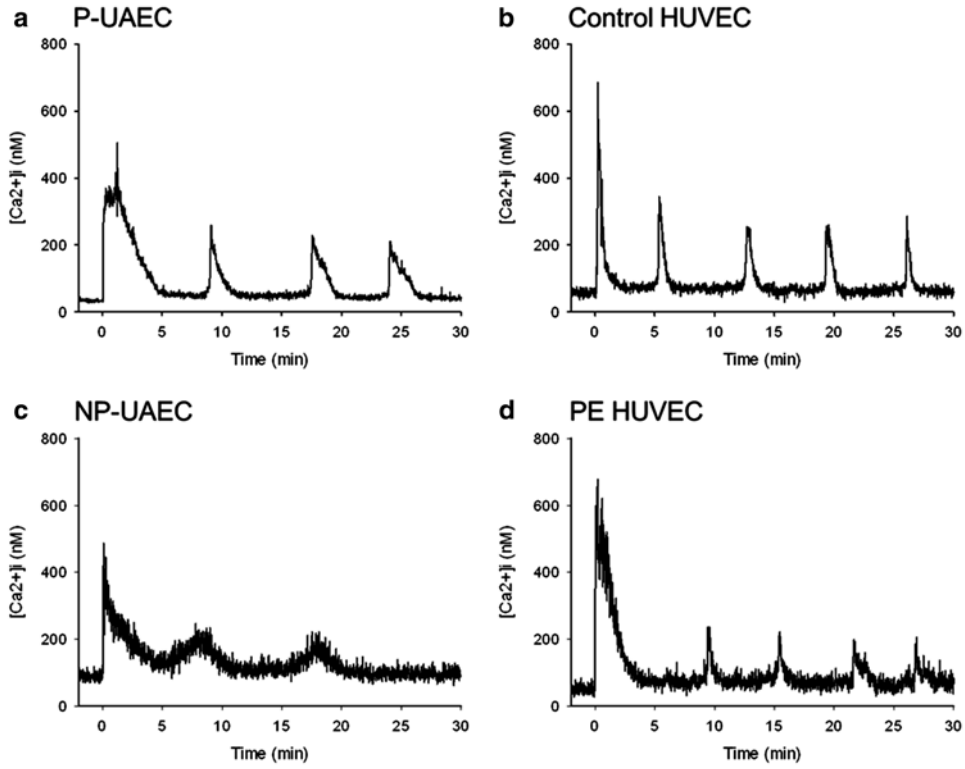

Fig. 4.2 Representative Ca^{2+} tracings from individual endothelial cells in primary culture. Real-time measurements for $[Ca^{2+}]i$ (Fura-2) in passage 3–4 primary endothelial cells from ovine uterine arteries (**a** and **c**) or human umbilical vein (**b** and **d**) from select physiological conditions are shown. The Ca^{2+} stimulating agonist, ATP (100 μM), was added at 0 min and $[Ca^{2+}]i$ monitored for 30 min, exactly as reported in [113] for UAEC and [48] for HUVEC. Panels **a** and **c** show tracings from cells derived from late gestational pregnant (P-UAEC) and luteal non-pregnant (NP-UAEC) ewes. P-UAEC show sustained Ca^{2+} burst responses to ATP, while NP-UA Endo lack such sustained Ca^{2+} responses. Panels **b** and **d** show tracings from human umbilical veins endothelial cells (HUVEC) from Normal (Control) and PE pregnancies. Control HUVEC show sustained Ca^{2+} burst responses to ATP throughout the 30 min recording. PE HUVEC do *not* show reduced burst numbers to Control HUVEC, but do show blunted Ca^{2+} burst amplitude in responses to ATP

using DAF-based imaging dyes, due in part to a drop in eNOS expression in primary culture. Nonetheless, sustained Ca^{2+} responses in the form of transient periodic bursts continue throughout the 30-min recording period in response to ATP in both cell types. As discussed earlier, the initial ATP-stimulated Ca^{2+} signaling events in UAEC are regulated through a classical PLCbeta/IP3R mechanism. While studies in UAEC have identified IP3R2 and TRPC3 isoforms as key players in pregnancy-adapted Ca^{2+} burst responses to ATP (above), other isoforms such as IP3R1, IP3R3, and TRPC6 are also present and possibly have a supporting role [18, 105]. At passage 4, UAEC Cx37 expression drops below the level detectable by western blot, but expression of Cx43 remains high through multiple passages and it is also the only connexin isoform functionally linked to agonist-stimulated and pregnancy-enhanced Ca^{2+} burst responses [7]. Inhibition of Cx43 has also been shown to block pregnancy-adapted eNOS activation in parallel with a loss of sustained Ca^{2+} bursting in P-UAEC [7].

In HUVEC, IP3R1, IP3R2, and IP3R3 are also detectable, albeit with relative levels changing over multiple passages compared to freshly isolated cells [106]. Likewise, in HUVEC multiple TRPC isoforms are detected by western blot, with strong bands observed for TRPC1, TRPC3, TRPC4, and TRPC6 [107, 108]. In UAEC, both TRPC3 and TRPC6 have been easily detected, and TRPC3 is implicated in agonist-stimulated Ca^{2+} responses to ATP stimulation [18]. In Chinese hamster ovary (CHO) cells, VEGFR2 was capable of coupling to recombinant TRPC3 and TRPC6 [109] and in HUVEC, a partial fragment of TRPC3 was capable of inhibiting CCE Ca^{2+} responses [108]. TRPC6 has also been functionally linked with VEGF-stimulated Ca^{2+} responses [107]. In HUVEC maintained in culture, Cx mRNA expression has been analyzed by immunofluorescence and rt-PCR, with Cx37, Cx40, and Cx43 all being readily detectable [104, 110]. Although their distribution and localization was not consistent between isoforms, Cx43 was primarily found at the plasma membrane, while Cx37 and Cx40 were observed primarily within cytosolic compartments [104]. Furthermore, the majority of electrical connectivity in HUVEC was due to Cx43 coupling and this was closely associated with highly efficient Lucifer Yellow dye transfer [104].

Herein we show two additional experiments that illustrate that the same fundamental mechanism regulates sustained Ca^{2+} burst signaling in UAEC and HUVEC (Figs. 4.2 and 4.3). Sustained Ca^{2+} responses in UAEC are dependent on TRPC/IP3R channel interaction, and pretreatment with the IP3R antagonist 2-APB interferes with this interaction, causing profound reductions in sustained phase Ca^{2+} responses in UAEC [13, 18] and indeed UA Endo [4]. When HUVEC are exposed to 2-APB, the initial $[Ca^{2+}]i$ peak is also reduced in size or obliterated, and the sustained phase Ca^{2+} responses to ATP is also almost completely inhibited (Fig. 4.3, middle). The second experiment uses the PKC agonist TPA to reduce Cx43 gap junction function through induction of multiple inhibitory phosphorylations on the c-terminus of Cx43, as we have already reported occurs in P-UAEC [10]. Just as observed with UAEC, sustained phase Ca^{2+} bursting in HUVEC treated with ATP is very strongly inhibited by TPA pretreatment (Fig. 4.3, bottom). Thus HUVEC responses to 2-APB and TPA are consistent with IP3R-sensitivity of Ca^{2+} bursting being under the control of Cx43 that is in turn sensitive to kinase-mediated inhibition of function. As such, even though HUVEC may be both venous and fetal in origin, they may be suitable for preliminary drug screens.

3.3 The Case that Ca^{2+} Signaling in UV Endo of PE Subjects Is Actively Suppressed by Circulating Factors to a Level Analogous to NP Ovine Uterine Artery Model

The underlying assumption that the reduced functionality of the PE uterine artery endothelium is because it functions in a similar manner to non-pregnant uterine artery endothelium is difficult to prove at a mechanistic level given ethical

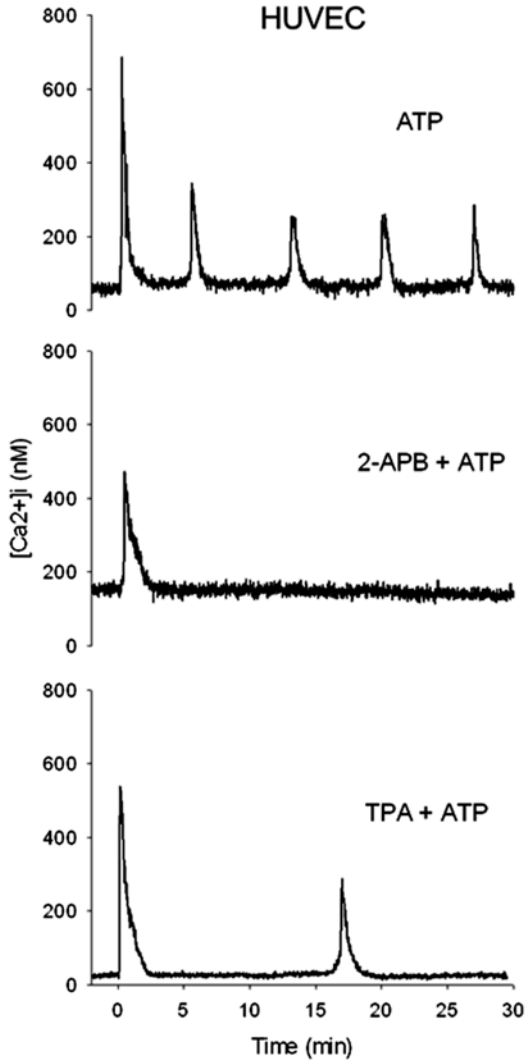

Fig. 4.3 Representative Ca^{2+} tracings in HUVEC with inhibitors to Ca^{2+} signaling machinery. Fura-2 loaded HUVEC were treated with 100 μM ATP at 0 min and changes in $[Ca^{2+}]i$ recorded for 30 min. Control tracing (*top*) is from Fig. 4.2. Pretreatment for 5 min with the IP3R inhibitor, 2-APB (*middle*, 50 μM) reduced the initial peak amplitude and eliminated subsequent sustained burst responses. Pretreatment with the PKC agonist and Cx43 phosphorylating agent, TPA (*bottom*, 1 nM) left the initial peak largely intact while reducing the occurrence of sustained Ca^{2+} bursts in response to ATP

constraints on study in humans. However, the studies of Mahdy et al. [101] were first to show that there is a clear drop in sustained Ca^{2+} responses in *systemic* endothelium in PE subjects that appears strikingly similar to that of non-pregnant subjects. The question then follows, is there an equivalent drop in functionality in HUVEC from PE pregnancies to match the maternal systemic drop? Is this also equivalent to the reduced functionality observed in non-pregnant-derived ovine endothelium when compared to the high level of functionality in pregnant-derived endothelium? A key study by Steinert et al. [102] showed a PE-related drop in histamine-stimulated Ca^{2+} signaling is also observed in HUVEC. In that study, there was clearly a more rapid return of agonist driven $[Ca^{2+}]i$ levels to baseline in cells derived from PE cords than in cells derived from normal cords. While the recordings from this study were relatively short in length, they were long enough to capture the shift from endoplasmic reticulum store release alone to the CCE phase of the early Ca^{2+} response. Of note, the divergence point between the PE and control HUVEC corresponds closely with the similar onset of CCE in UAEC that is otherwise most enhanced in P-UAEC compared to NP-UAEC. This suggested to us that if pregnancy-associated CCE mechanisms had failed in PE subjects, UV Endo from intact cords of PE subjects should lack more sustained Ca^{2+} bursts and show correspondingly blunted NO output. Our recent studies [103] show indeed this is also the case (See also Fig. 4.1). The extended recording time allows us to see that PE-UV Endo have reduced Ca^{2+} burst numbers compared to normal control and this is paralleled by a reduced NO output by the cells down to a level more reminiscent of that observed in nonadapted NP-UA Endo [5].

So the remaining question is whether the lack of a prolonged bursting response in PE subjects is because the enhanced Cx43 function failed to develop in the first place, or was being actively suppressed by circulating factors. If in PE the cells were never programmed for multiple burst function, then they should act like NP-UAEC in culture. Alternatively if they were capable of bursting, but endocrinologically suppressed by circulating factors, then PE-derived HUVEC in primary culture should show recovery of enhanced bursting. The studies of Krupp et al. [103] show clearly that PE-derived HUVEC at

passage 3 are indeed able to recover burst numbers to levels even above that of normal HUVEC. It is therefore likely that the endothelial dysfunction associated with PE in vivo is due to an active suppression of function of the otherwise normal cord. Of note, the 'toxic environment' of a PE cord is what we consider to be the 'chronic wounding' cocktail of growth factors and cytokines. Further studies are necessary now to establish if this is the case, and we propose the UV Endo preparations and HUVEC cell cultures are valid models to use for such studies.

4 Conclusion and Futures Studies

It is now clear that pregnancy-adapted vascular endothelial function in the mother is achieved through an increase in Cx43 functional coupling that is most apparent in uterine artery endothelial cells, and likely also observed throughout the maternal vasculature. Indeed, such maternal adaptive mechanisms to enhance endothelial function during normal pregnancy may also be shared with the fetus. This certainly makes sense given both transport of oxygenated and nutrient rich blood to the uterus would need to be matched by fetoplacental transport from the placenta to the baby. Very similar Ca^{2+} burst profiles are identifiable in the cells of the intact pregnant uterine artery and umbilical vein, and repeat Ca^{2+} bursts in each case are accompanied by prolonged NO output (Fig. 4.1). Of note, the level of dysfunction of Ca^{2+} bursts in UV Endo from PE subjects is similar to that of unadapted NP-UA Endo and equally associated with blunted NO output. While all these points are now clearly established, we need to complete the validation of our proposed hypothesis, namely that PE results from a prolonged wounding-like endocrine profile whereby the cocktail of cytokines and growth factors promotes over activity of ERK and Src signaling pathways. This in turn causes Cx43 phosphorylation to occur, promoting a return of Cx43 function to 'nonpregnancy' levels, which contributes to hypertension. It is noteworthy that over activity of Src may also promote the breakdown of cell–cell contact points between endothelial cells [111–113] and so have a causal role in the onset of proteinuria and even breakdown of the blood brain barrier, leading to seizures. The observation that PP2 is particularly good at rescue of bursting in TPA-treated UAEC is encouraging and we are now in the process of verifying that PP2 can also reverse the inhibitory effects of the endocrine factors found elevated in PE subjects on other aspects of endothelial cell function. Even given successful rescue of endothelial cell function, however, PP2 cannot be used for therapy in vivo. To that end we now propose to establish a high throughput screening methodology based in HUVEC to confirm the wide range of possible inflammatory factors that may inhibit endothelial adaptation at the level of sustained Ca^{2+} bursting, and then identify possible drugs to rescue cells from them. Only when both the long list of factors inducing endothelial damage are verified, and the short list of therapeutic compounds is newly established that can block them can we further extend such studies to establish clinical utility in round two (as follows) on our path to human therapy.

While our focus so far has been on Ca^{2+} bursting, there is also a need to broaden the studies to examine PE-associated defects at the level of the plasma membrane as a whole, i.e., beyond those regions containing Cx43. It is clear Cx43 resides in the membrane in association with endothelial junctional structures [114], and serum from PE subjects is known to promote breakdown of HUVEC monolayer integrity in a manner associated with altered junctional protein distribution [115, 116]. We need to examine this process further using more direct methods to identify the pathways responsible for more permanent breakdown of the host plasma membrane domains in which Cx43 resides. Such studies would not only be informative in themselves but also aid in identifying points of signaling convergence to or divergence from direct Cx43 phosphorylation to further refine therapeutic targeting.

Round two may also require further dissection of cell signaling pathways down to individual kinase isoforms through molecular alteration or mutagenesis techniques. More permanent cell

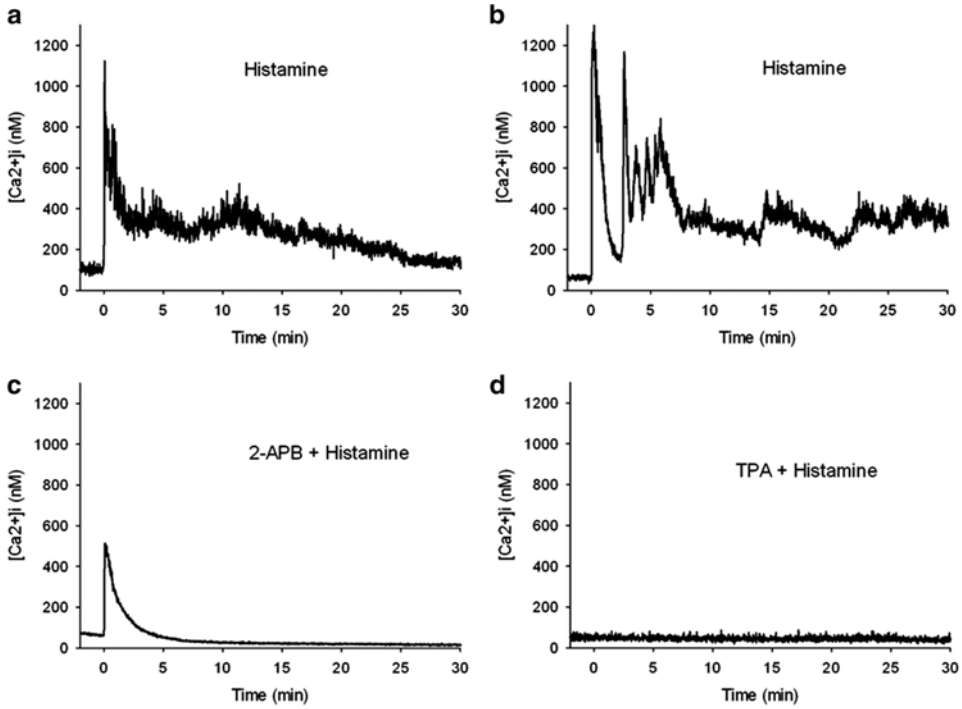

Fig. 4.4 Representative Ca^{2+} tracings in HUVEC-CS cell line. Fura-2 loaded HUVEC-CS were treated with 10 μM Histamine at 0 min and recorded for changes in $[Ca^{2+}]i$ for 30 min. HUVEC-CS methods were exactly as in [48] for HUVEC, except high cell density was not achieved. Panel **a** shows a typical Histamine-stimulated cell. Panel **b** shows a multiple burst pattern in response to Histamine which suggests that the more typical sustained Ca^{2+} response observed in Panel **a** may be a series of fused Ca^{2+} bursts. Panel **c** shows the inhibitory effect of 5 min pretreatment with the IP3R inhibitor, 2-APB (50 μM) on sustained Ca^{2+} bursts upon Histamine application. Panel **d** shows that 30 min pretreatment with the Cx43 phosphorylating agent, TPA (10 nM) completely inhibits Histamine stimulation of Ca^{2+} bursts in HUVEC-CS. Of note, TPA did not completely abolish the entirety of the Ca^{2+} response in all cells, but did so in the majority of the cells monitored

lines and sublines would be of considerable value in this regard. We have previously reported the HUVEC-CS cell line also responds to ATP, but its response is not as clearly delineated into bursts as UAEC or primary HUVEC. However, Steinert et al. [102] previously reported that HUVEC also respond well to histamine, and our prior observation in UAEC that the CCE response to thapsigargin is also enhanced by pregnancy predicts that more than one agonist response may be affected by enhanced Cx43 coupling. It is thus reasonable to assume that other agonists coupled to Ca^{2+} signaling in a manner dependent on IP3R and Cx43 mediated cell communication may be equally useful as a screen stimulus. To that end we report here that HUVEC-CS response to histamine is also sensitive to 2-APB and pre-exposure to TPA (Fig. 4.4) and this is also true for a number of other PLCbeta stimuli, albeit with a lower percentage of cells responding (Table 4.1). As such it may also be possible now to alter signaling in these cells to further examine subpools and even subtypes of ERK and Src that mediate these convergent inhibitory signals on Cx43 function.

5 Closing Remarks

There are clearly difficulties facing us in moving from the realization that PE is essentially a wounding state where the body is misled into inappropriately shutting down the pregnancy adaptation of Cx43 function in vascular endothelium, to the future goal of having identified

Table 4.1 Qualitative assessment of agonist stimulated Ca^{2+} responses in HUVEC-CS

Agonist	Inhibitor	Detectable response	Peak height	Area under the curve
ATP		+	++	+
	2-APB	–	–	–
	TPA	+	+	+
Bradykinin		+	+++++	+++
	2-APB	+	+	–
	TPA	+	+	+
Carbachol		+	+++	+
	2-ABP	+	++	+
	TPA	–	–	–
Seratonin		+	+++++	+++++
	2-ABP	–	–	–
	TPA	–	–	–
Histamine		+++++	+++++	+++++
	2-ABP	+++++	+++	–
	TPA	+	++	+
Adenosine		+	++++	+++
	2-ABP	+	+	–
	TPA	–	–	–

All agonists tested caused a detectable Ca^{2+} response in at least a small proportion of cells observed. Doses of agonist used were: ATP (100 μM), Bradykinin (1 μM), Carbachol (1 μM), Seratonin (10 μM), Histamine (10 μM), Adenosine (100 μM). Histamine strongly stimulated both initial and sustained Ca^{2+} responses as depicted by peak height and area under the curve. Other agonists, such as Bradykinin, Seratonin, and Adenosine recruited relatively few cells, but those that did respond did so in a robust, sustained manner. The results for 2-APB (50 μM) pretreatment indicate that initial peak height is affected by the IP3R2 inhibitor on an individual agonist basis, but sustained phase responses are highly dependent on IP3R activity in all cases. Pretreatment with TPA (10 nM) strongly inhibited both initial peak height and sustained phase Ca^{2+} (AUC) for all agonists, suggesting Cx43 function is critical to agonist stimulated Ca^{2+} responses in HUVEC-CS

+: <20 %; ++: 21–40 %; +++: 41–60 %; ++++: 61–80 %; +++++: 81–100 %; –: 0 %

a new therapy to restore vascular endothelial (and even renal and blood brain barrier) function. Nonetheless, breaking through this barrier is necessary if we are to treat the actual cause of this devastating disease for the first time. For all its imperfections, the proposed use of HUVEC as a *first* round screen has considerable merit, and may be the way to initially screen the many potential cytokines as well as growth factors for their inhibitory effects via each kinase signaling pathway, and then go on to screen libraries of pharmaceutical or even neutraceutical kinase inhibitors to find agents that can be effective and safe for use in pregnant subjects. Even then we will need to undertake testing in intermediate animal models such as the RUPP [56] or renin angiotensinogen transgene cross [117] before proceeding to clinical trials. Nonetheless, the use of HUVEC as a *first* round screen is the only rational strategy that could allow the initial dissection of the endocrine mechanisms behind PE-associated endothelial dysfunction, and lead to a shortlist of possible compounds that could be therapeutically targeted to block the actual cause of dysfunction in PE subjects.

Acknowledgements Many of the authors studies covered in this review were supported by ongoing funding by NICHD and NHLBI, including awards HL56702, HD050578, HL64601, HL079020, HD069181 and P01 HD38843. Predoctoral Trainees were also supported by T32HD41921 awarded to the Endocrinology and Reproductive Physiology Graduate Training Program at Univ Wisconsin Madison SMPH.

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Regulation of Amniotic Fluid Volume: Evolving Concepts

5

Robert A. Brace and Cecilia Y. Cheung

Abstract

Studies in late gestation fetal sheep have provided several new insights into the regulation of amniotic fluid (AF) volume (AFV): There are four quantitatively important amniotic inflows and outflows that include fetal urine production, lung liquid secretion, swallowing, and intramembranous absorption. Of these, AFV is regulated primarily by modulating the rate of intramembranous absorption of AF water and solutes across the amniotic epithelial cells into the underlying fetal vasculature. Modulation of the rate of intramembranous absorption depends on the presence of stimulators and inhibitors present in the AF. A stimulator of intramembranous absorption is present in fetal urine. In addition, AF contains a non-renal, non-pulmonary inhibitor of intramembranous absorption presumably secreted by the fetal membranes. Although passive bidirectional movements of water and solutes occur across the intramembranous pathway, intramembranous absorption is primarily a unidirectional, vesicular, bulk transport process mediated through VEGF activation of transcytotic transport via caveolae. Further, the stimulators and inhibitors of intramembranous absorption alter only the active, unidirectional component of intramembranous absorption while the passive components are not altered under experimental conditions studied thus far. Future progress depends on identifying the cellular and molecular mechanisms that regulate active and passive intramembranous absorption as well as their regulatory components.

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L. Zhang and C.A. Ducsay (eds.), *Advances in Fetal and Neonatal Physiology*,
Advances in Experimental Medicine and Biology 814, DOI 10.1007/978-1-4939-1031-1_5,

Keywords
Amniotic fluid volume regulation • Intramembranous absorption • Sheep fetus • Fetal swallowing • Fetal urine production • Fetal lung liquid secretion

1 Introduction

Although an understanding of the physiological regulation of amniotic fluid volume has long been sought, much remains to be discovered. Over the past 30 years, the pathways of fluid transport into and out of the amniotic cavity have begun to be characterized under diverse experimental conditions. However, the underlying cellular events and the mediators that regulate amniotic inflows and outflows are less known. The current lack of knowledge is due primarily to a combination of two factors: first, there are eight potential pathways (Table 5.1) through which water and solutes such as sodium and chloride can move into and/or out of the amniotic space [1–3]; second, the transfer rates of water and solutes through most of these pathways have rarely been measured simultaneously. Without knowledge of normal flows into and out of the amniotic cavity, an integrated understanding of the complex mechanisms that regulate amniotic fluid volume has been difficult to attain. In this review, we attempt to interpret the experimental data that forms the basis for our current understanding of the amniotic fluid volume regulatory mechanisms in late gestation. A key objective is to present new ideas based on the latest findings that would promote investigations to further advance our knowledge of amniotic fluid dynamics. Further, this review focuses on amniotic dynamics in late gestation as there are little data on amniotic inflows and outflows in early to mid-gestation in contrast with extensive data from late gestation.

1.1 Terminology

Understanding the regulation of amniotic fluid volume requires a distinction between "*intramembranous absorption*" and "*transmembranous absorption*". Intramembranous absorption refers to the transfer of amniotic water and solutes across the amnion and into the underlying fetal vasculature. This occurs across the fetal surface of the placenta in primates and into the vascularized chorion in species such as sheep [4]. As discussed below, the intramembranous pathway is the primary site of regulation of amniotic fluid volume. Transmembranous absorption refers to absorption of amniotic fluid across the amniochorion into maternal blood perfusing the uterine wall [3].

Table 5.1 Amniotic inflow and outflow pathways and average daily amniotic volume flows in late gestation fetal sheep measured under control conditions[a]

Major flows		Minor flows	
Pathway	Daily flow rate	Pathway	Daily flow rate
Renal excretion	+1,010	Oral/nasal secretion	+28
Intramembranous absorption	−810	Transmembranous	−10?
Swallowing	−580	Transcutaneous	–
Lung secretion	+380	Cord surface	–

[a]Values for major daily flow rates are mean values measured simultaneously in 17 fetuses weighing 3.9 kg. Positive values are flows into and negative values out of the amniotic sac. Values are described in text and have units of ml/day. Fetuses were chronically catheterized with ligated urachus and catheterized trachea. All tracheal outflow entered the amniotic fluid

2 Experimental Animal Model

The literature provides data on amniotic fluid volume under normal conditions in a variety of mammalian species including humans. However, simply knowing the volume does not provide insight into the regulatory processes. What is needed is an experimental animal model in which amniotic fluid volume as well as volume flow rates into and out of the amniotic cavity can be measured simultaneously and repeatedly over time. The chronically catheterized late gestation ovine fetus is the only experimental model in which the primary regulated flows have been measured simultaneously. Consequently, new insights into the amniotic fluid volume regulatory mechanisms have been derived largely from studies in this animal model. There are disadvantages of the ovine model including placentation differences from humans and the presence of a second extra-fetal fluid compartment, i.e., the allantoic cavity (vestigial in humans). Most recent studies of amniotic dynamics in the fetal sheep model attempt to minimize the potential effects of the allantoic compartment by ligating the urachus. This eliminates urine entry into the allantoic cavity and reduces allantoic fluid volume to essentially zero [5].

3 Amniotic Inflow and Outflow Pathways

In order to fully understand the complex amniotic fluid volume regulatory mechanism, all amniotic inflows and outflows must be measured simultaneously under normal and experimental conditions. With limited access and eight pathways, such measurements are not possible. However, in late gestation after the skin keratinizes, flows across the fetal skin and surface of the umbilical cord are so small that they can be ignored [3]. This reduces complexity in that six rather than eight pathways must be considered.

3.1 Secretions from the Fetal Head

Early studies in late gestation sheep showed that the rate of fluid secretion from the fetal oral/nasal cavities averaged 28 ml/day [6]. With urine, swallowing and intramembranous flows as much as 1,000 ml/day or more in late gestation (Table 5.1), the fetal oral/nasal cavities do not appear to be a major contributor to amniotic fluid and thus are not considered as a volume regulatory pathway. This further reduces complexity in that only five pathways need to be considered in late gestation.

3.2 Transmembranous Absorption

Transmembranous transfer of amniotic fluid has been implicated to contribute significantly to the regulation of amniotic fluid volume. This view was based on observations that the isolated amniochorion of humans and sheep is relatively permeable to water and solutes [7–10]. However, this assumption does not consider the volume absorptive capacity of the uterine wall. Rather than absorption, the post implantation uterine wall secretes fluid that is the source of first trimester amniotic and coelomic fluids. This is consistent with fluid accumulation within the gestational sac before fetal urine production/lung liquid secretion begins and even before the embryonic heart begins to beat. Further, with a blighted ovum, uterine wall secretions are responsible for fluid accumulation within the fetal membranes in the absence of an embryo [1, 3].

In late gestation sheep, transmembranous transfer of water independent of transplacental transfer could not be demonstrated following injection or infusion of water into the ovine amniotic cavity [11], or when amniotic and allantoic osmolalities were increased [12]. Under hyperosmotic experimental conditions, the calculated maximal transmembranous transfer of water was 10 ml/day for a 3 kg fetus even though the hypertonicity disrupted the fetal membranes. Thus, in late gestation, transmembranous water movement

appears to be negligibly low, at least in the pregnant sheep model. This conclusion is consistent with statements by Seeds in his classic review [3] that in humans "…*only a very small net transfer of water could occur by*… {the transmembranous pathway}… *since the vascularity of maternal tissue in proximity to the chorion laeve and amnion is sparse*."

Collectively, the above supports the concept that the ovine and perhaps human transmembranous fluid transfer rate in late gestation is so low relative to other daily flows that its effect need not be considered. If transmembranous transfer does occur normally, it currently is unclear whether this occurs into or out of the amniotic fluid.

4 Major Contributors of Amniotic Fluid Volume Regulation

With transcutaneous, transmembranous, umbilical cord, and fetal head flow rates negligibly low, four pathways remain as potentially important regulatory pathways for amniotic fluid volume in late gestation. These include two inflow paths: fetal lung liquid secretion and urine excretion; and two outflow paths: fetal swallowing and intramembranous absorption.

4.1 Secretions from the Fetal Lungs

There have been two misinterpretations regarding fetal lung fluid movements that have impacted knowledge of amniotic fluid dynamics. First, observations in stillborn fetuses suggested, based on autopsy results, that the fetal lungs normally absorb amniotic fluid. Prior to the recognition of high intramembranous absorption rates, absorption of amniotic fluid by the fetal lungs was logical [3] in that studies had suggested that the volume of fluid removed from the amniotic cavity by fetal swallowing was significantly less than the volume of urine produced [13, 14]. However, this notion contradicts early reports that the fetal lungs normally secrete rather than absorb amniotic fluid [15–17]. In a classic review, Liley [1] noted that "*Normally there is no net inward flow of fluid to the tracheobronchial tree*…." Subsequently, it was shown in unanesthetized late gestation ovine fetuses that the lungs secrete a volume of liquid averaging 10 % of body weight per day, with roughly 99 % of the secreted volume flowing out through the trachea [18–20]. Fetal lung liquid secretion rates can be reduced by fetal hypoxia [20, 21], prior to birth [19] and by the effects of stress hormones [22–29]. During fetal stress, the fetal lungs occasionally absorb amniotic fluid as illustrated by meconium staining below the vocal cords in 1/3 of the cases of meconium stained amniotic fluid [30]. However, in the normal, non-stressed 3 kg fetus, the lungs secrete an average of 300 ml/day of fluid.

A second misinterpretation, based on early studies in anesthetized animals, was that fetal lung liquid was swallowed as it exited the trachea [15]. However, in the unanesthetized ovine fetus, analysis of the composition of amniotic fluid, lung liquid and swallowed fluid revealed that an average of 18 % of the swallowed fluid was lung liquid with the remainder being amniotic fluid [31]. Only 47–54 % of the lung liquid that exited the fetal trachea was swallowed and the rest mixed with the amniotic fluid under both normoxic and hypoxic conditions. This is consistent with the presence of pulmonary surfactants in normal late gestation amniotic fluid. Fetal oral/nasal secretions also may be swallowed in that swallowed fluid sometimes contains higher potassium concentrations and more mucoid material than present in either lung liquid or amniotic fluid [32].

Even though half of the late gestation lung secretions may enter the ovine amniotic fluid, the quantitative contribution of fetal lung liquid to amniotic fluid volume regulation has yet to be established. There have been no studies of the effect of lung liquid drainage on amniotic fluid volume. Further, continuously replacing lung liquid as it flowed out of the trachea with lactated Ringer's solution altered neither amniotic fluid volume nor intramembranous absorption rate [33]. Lung liquid secretion rate has been reported

to decrease during short-term vascular volume loading [26] but little change occurs during long-term amniotic fluid volume loading [33, 34] even when vascular volume is elevated [35]. Further, no correlations between lung liquid secretion rate and changes in amniotic fluid volume have been reported. Thus, although contributing volume, fetal lungs do not appear to have an active role in regulating amniotic fluid volume. This is consistent with the concept that fetal lung liquid secretion is regulated independently of amniotic fluid volume.

4.2 Role of Fetal Swallowing in Regulating Amniotic Fluid Volume

It has long been recognized that the late gestation fetus swallows large volumes of amniotic fluid daily [13, 36–42]. However, the contribution of fetal swallowing to the regulation of amniotic fluid volume has been unclear because swallowing is regulated by central nervous system activity [42, 43] presumably to meet fetal body needs rather than regulated to maintain a normal amniotic fluid volume. Thus, fetal swallowing passively affects amniotic fluid volume but is not actively regulated in order to maintain amniotic fluid volume near normal [44]. However, in experimental studies, daily swallowed volume increased when amniotic fluid volume was elevated [35, 45] and decreased when amniotic fluid volume was below normal [46], suggesting that the contribution of fetal swallowing varies as a function of amniotic fluid volume.

By combining data from fetuses under different experimental conditions, a strong relationship between amniotic fluid volume and daily swallowed volume was found, with swallowing decreasing to near zero when amniotic fluid volume was far below normal and increasing to a maximum of approximately twice normal as amniotic fluid volume reached polyhydramniotic values of 1,500–2,000 ml [47]. At normal amniotic volumes of 600–800 ml, swallowed volume increased by 0.3 ml/day for each 1 ml increase in amniotic fluid volume. Additionally, during intra-amniotic fluid infusion, swallowing increased by 0.5 ml/day for each 1 ml increase in amniotic fluid volume while intramembranous absorption increased by 3.9 ml/day (unpublished data from [33]). In a more recent intra-amniotic fluid infusion study, swallowing increased by 1.1 ml/day for each 1 ml increment in amniotic fluid volume above normal [48]. Combined, these studies suggest that, within the range of normal amniotic fluid volumes, swallowing in the late gestation ovine fetus may vary by an average of 0.7 ml/day for each 1 ml alteration in amniotic fluid volume. If so, even though a passive response, fetal swallowing would make a more powerful contribution to the regulation of amniotic fluid volume than previously recognized, especially over periods of days to weeks.

4.3 Role of Fetal Urine in Regulating Amniotic Fluid Volume

Fetal urine is the largest volume contributor to amniotic fluid in late gestation and therefore is a determinant of amniotic fluid volume. In renal agenesis, typically but not universally, anhydramnios is present [49]. With normal flows close to 1,000 ml/day in late gestation, drainage of fetal urine results in large decreases in amniotic fluid volume [5, 50–52] as expected and increases in amniotic osmolality [53]. Further, indomethacin administration reduces fetal urine production [54] and amniotic fluid volume [55, 56] while administration of the diuretic furosemide to the fetus increases both urine flow rate and amniotic fluid volume [57].

However, within the physiologic range, the contribution of fetal urine to the regulation of amniotic fluid volume is less clear. One supporting observation is that spontaneous day-to-day changes in ovine amniotic fluid volume correlated positively with urine flow rates [14]. Further, with large data sets, absolute amniotic fluid volume and urine flow rate are positively related [58] although the positive relationship often is not seen with smaller data sets (unpublished). The lack of consistency may be attributed to several factors. First, each fetus

appears to regulate its amniotic fluid volume at a unique set point that is independent of urine flow rate. Second, fetal urine may have multiple effects besides being a major volume contributor. Upon entry into the amniotic sac, it could dilute the effects of regulatory factors present in the amniotic fluid. In addition, our recent findings demonstrated that urine contains yet to be identified factors that stimulate intramembranous absorption [34]. Because of the competing effects of urine, the net effects of fetal urine on amniotic fluid volume in the physiologic range of volume are not easily discerned. However, during conditions of intravascular and intra-amniotic volume loading, urine flow rate and amniotic fluid volume increase in parallel. Importantly, though, the increase in amniotic fluid volume is much less than the excess volume of urine [33, 35, 59] due to an increase in swallowed volume and a large increase in intramembranous absorption [33].

4.4 Intramembranous Absorption

Until the late 1980s, the significant role of intramembranous absorption of amniotic fluid across the amnion and into the underlying fetal vascular had not been recognized other than as a theoretical possibility [8]. Our study in 1989 made two important observations that highlighted the role of intramembranous absorption in regulating amniotic fluid volume: first, injections of water into the amniotic cavity resulted in a decrease in fetal osmolality more rapidly than could be explained by fetal swallowing; second, the decrease in fetal osmolality occurred even more rapidly if the fetus could not swallow due to fetal esophageal ligation [11]. These observations were a breakthrough in advancing the knowledge of amniotic fluid volume regulatory mechanisms in that not only was intramembranous absorption of amniotic fluid quite rapid but also the absorptive capacity of the intramembranous pathway could be upregulated [60].

Several studies showed that sodium, chloride and other solutes were absorbed rapidly by the intramembranous pathway against concentration gradients, against hydrostatic gradients and in the absence of osmotic gradients [61–63]. Elimination of the normal fetal-to-amniotic fluid osmotic difference only slightly diminished the rate of intramembranous absorption [61]. Further, during intra-amniotic fluid infusions, not only do large increases in intramembranous volume absorption occur but the intramembranous clearances of multiple amniotic solutes increase and become equal to the intramembranous volume absorption rate [63]. Finally, the intramembranous transport of albumin occurs rapidly in the amniotic fluid-to-fetal direction but does not occur in the fetal-to-amniotic fluid direction [61]. Collectively, these observations confirmed that intramembranous transport is primarily a unidirectional, bulk transport of amniotic fluid with dissolved solutes, consistent with the characteristic of vesicular transport. Further, under experimental conditions of reduced or increased intramembranous absorption rates, only the active unidirectional component of intramembranous absorption changes while the passive diffusional components remain unchanged [34, 63]. This series of experiments forms the basis for the current concept that amniotic fluid volume is regulated by intramembranous absorption of amniotic fluid primarily by the transport mechanism of vesicular transcytosis [34, 59, 62–64]. This conclusion represents a second major breakthrough in understanding of the amniotic fluid volume regulatory mechanisms. Although recent reviews suggest that passive forces are solely responsible for intramembranous fluid transport [65–67], passive intramembranous fluxes of sodium and chloride are small and in the opposite direction to large net outward fluxes [62, 63].

5 Intramembranous Absorption in Primates

The rate of volume absorption through the intramembranous pathway has been determined only in fetal sheep. However, supportive experimental data suggest that intramembranous absorption likely is rapid in both humans and non-human primates. An early study by Renaud et al. [68] found that, following injection of amino acids

into human amniotic fluid just prior to delivery, amino acid concentration in cord blood was found to be higher than could be expected from fetal swallowing alone. Subsequent study by Gilbert et al. [69] reported rapid intramembranous of technetium in monkeys and Schröder et al. [70] reported that glucose is transferred from the amniotic compartment across the human chorionic plate into the fetal circulation. With such limited data, the contribution of intramembranous absorption to amniotic fluid volume regulation in humans remains speculative and likely would differ quantitatively and qualitatively from that in sheep. However, computer models suggest that intramembranous absorption rates across the human amnion may be quite high [71, 72].

6 Current Estimates of Intramembranous Absorption Rate

The current experimental model used for amniotic fluid studies utilized the chronically catheterized fetal sheep preparation in which fetal urine flow, swallowing, and lung liquid secretion rates are simultaneously and continuously measured over a period of days. The time integrated flow rates combined with amniotic fluid volume measurements at the beginning and end of the flow measurements allow calculation of intramembranous absorption rate while all four of the primary amniotic flows are intact and functional. Under control conditions, intramembranous absorption rate averaged 810 ml/day in late gestation fetuses weighting 3.9 kg (Table 5.1). This value is much greater than the initial indirect estimates of 200–240 ml/day because the early estimates were derived from osmotically induced changes during intra-amniotic water administration [11, 73] or changes in amniotic osmolality during urine drainage plus trachea-esophageal occlusion [74]. Those early estimates, which are a small fraction of the normal daily intramembranous volume flow, represent primarily free water movement through water channels and are overestimates because of passive intramembranous diffusion of solutes into amniotic fluid from fetal blood.

In addition to high basal rates, intramembranous absorption rate can increase to several liters per day in response to intra-amniotic fluid infusion [33]. The latter observation of large increases in absorption rate is consistent with previous indirect estimates during intravascular or intra-amniotic fluid infusion or fetal hypoxia [59, 75, 76].

7 Characteristics of the Intramembranous Absorption Mechanisms

Several pivotal studies form the basis for the concept that modulation of intramembranous absorption rate is the primary mechanism for amniotic fluid volume regulation. First, when ovine fetuses were rendered hypoxic by lowering maternal inspired oxygen content for 4 days, fetal urine flow rate increased gradually, reaching four times normal with 4 l of excess urine excreted over the 4 days of hypoxia [75, 76]. However, amniotic fluid volume increased only by 10 % of the excess urine volume. Because fetal swallowing was unchanged over the 4 days of hypoxia [76], the 4 l of fluid must have been absorbed from the amniotic compartment through the intramembranous pathway [75, 76]. The fetal lungs may have contributed to the clearance of the 4 l of excess fluid because fetal lung liquid secretion was shown to be reduced during hypoxia induced by reductions in uterine blood flow [20, 27]. However, when fetal swallowing of amniotic fluid is prevented by esophageal ligation, the changes in amniotic fluid volume over 9 days were independent of whether lung liquid entered the amniotic cavity or was shunted into the fetal stomach [77]. This observation suggests that the contribution of altered lung secretion to the clearance of 4 l of fluid during hypoxia may have been small.

In a second series of studies, ovine fetuses were volume loaded by intravascular infusion of physiologic saline at successive rates of 1, 2, and 4 l/day over 3 days [45, 59]. Fetal urine production increased in parallel with the infusion rate, with an excess urine volume of 7 l over the 3 days. In fetuses with a ligated urachus, amniotic fluid volume increased by less than 1 l while swallowing

increased by less than 1.5 l. Since lung liquid secretion changes little if at all during fluid infusions [33], the 4.5 l lost from the amniotic sac during the infusion were absorbed through the intramembranous pathway. In these studies, passive intramembranous permeability was not altered as shown by no change in permeability of technetium 99m, consistent with the concept that "*non-passive mechanisms appear to mediate the increase in intramembranous absorption*" [59].

7.1 Passive and Non-passive Intramembranous Solute Transport

In order to isolate the intramembranous absorption pathway, ovine fetal swallowing and lung liquid flow were blocked while fetal urine was drained to the exterior for 8 h to eliminate all major amniotic inflows and outflows except intramembranous absorption [62]. Intramembranous solute fluxes were calculated from mass balances using initial and final amniotic fluid volumes, time integrated amniotic flow rates and solute concentrations. Intramembranous solute fluxes of sodium, potassium, chloride, bicarbonate, glucose, lactate, and calcium were all positively correlated with intramembranous volume absorption rate, consistent with bulk unidirectional transport. In addition, fluxes of sodium, chloride, calcium and bicarbonate were correlated with their respective fetal-to-amniotic concentration differences. On average, there were four times as many sodium and chloride ions transported from amniotic fluid to fetal blood against their concentration differences as was transported passively from fetal blood to amniotic fluid down the concentration gradients. Since net transport of these solutes occurs against concentration gradients, hydrostatic gradient [62, 63] and in the absence of osmotic gradients [61], it is clear that a majority of intramembranous solute absorption was occurring by unidirectional bulk transport consistent with transcellular vesicular transport of amniotic fluid across the amnion and into fetal blood [34, 59, 62, 63].

In a separate study, additional evidence in support of the active unidirectional bulk transport characteristics of intramembranous absorption was the demonstration that sodium and chloride were actively transported outward from the amniotic fluid into fetal blood on average six times more rapidly as transferred passively in the opposite direction under basal conditions [63]. In response to intra-amniotic fluid infusion, intramembranous volume absorption rates were elevated while the active outwardly directed intramembranous sodium and chloride fluxes increased to 38 times the passive inwardly directed fluxes. Because the regression relationships between intramembranous solute flux and volume flux were not altered when intramembranous absorption rate was elevated [63], it was concluded that the increased volume and solute fluxes were mediated solely by an increase in the active, bulk component of intramembranous absorption while the passive diffusional components remained unchanged. Further, the intramembranous sodium, potassium, chloride, calcium, and glucose clearances all increased during the infusion and became equal to the intramembranous volume absorption rate, indicating that water and solutes were transported together by a single mechanism, i.e., unidirectional bulk vesicular transport.

To test the validity of the hypothesis that intramembranous transport is primarily unidirectional bulk flow, we compared the amniotic fluid-to-fetal and fetal-to-amniotic fluid unidirectional transport rates of technetium 99m and ^{14}C-inulin in fetal sheep [78]. The amniotic fluid-to-fetal blood transport rate of technetium (which has high lipid solubility) was 2.1 ± 0.3 times that in the opposite direction. The transport rate of ^{14}C-inulin (which has a higher molecular weight than technetium) from amniotic-to-fetal blood was 4.7 ± 1.2 times that in the fetal-to-amniotic fluid direction. Similarly, Faber and Anderson [61] showed that radio-labeled albumin is rapidly transported in the amniotic-to-fetal but not in the opposite direction. The observations demonstrate that, in addition to a large unidirectional transport, bidirectional transport occurs through the intramembranous pathway for smaller but not large molecules.

In order to explore the role of the amnion in intramembranous transport, permeabilities of technetium and ^{14}C-inulin were measured in isolated ovine amnion mounted in an Ussing chamber

and amnion permeability-surface area products were calculated from total surface areas of the amnion [78]. Unlike *in vivo* conditions, the ratio of the unidirectional permeabilities in the two directions across the isolated amnion was not different from unity, showing that passive diffusion was occurring in the absence of unidirectional transport, consistent with previous studies [9, 79]. Further, the permeability-surface area products for the two tracers were similar in magnitude to their *in vivo* permeabilities. The latter observation shows that the amnion is the rate limiting barrier of intramembranous transport. This concept is supported by our recent amnion resection study in which exposure of large portions of the chorion to amniotic fluid resulted in dramatic reductions in amniotic fluid volume [80]. The studies also suggest that unidirectional transport does not occur across the isolated amnion when bathed in artificial fluids.

7.2 Stimulators and Inhibitors of Intramembranous Absorption

It has been speculated that substances in the amniotic fluid could alter intramembranous absorption. This was based on amniotic fluid washout studies in fetal sheep [81, 82]. Amniotic fluid volume increased during isovolumic washout of amniotic fluid with lactated Ringer's solution, suggesting removal of an intramembranous stimulator by the washout. The increase in amniotic fluid volume during the washout was greater in fetuses with a tracheal-esophageal fistula (abolishing swallowing and the entry of lung liquid into the amniotic fluid) than in fetuses with an intact trachea and esophagus [81, 82]. This difference could be due to either the presence of a stimulator in the lung liquid that was diverted into the fetal stomach by the tracheal-esophageal shunt or to removal of an inhibitor of intramembranous absorption by fetal swallowing. However, subsequent studies [33, 63, 77] showed that fetal lung liquid does not contain a substance that stimulators or inhibits intramembranous absorption. Thus secretions of the fetal lungs do not participate in the active regulation of amniotic fluid volume.

Unlike lung liquid, fetal urine may contain substances that alter intramembranous absorption rates. To test this hypothesis, fetal urine was continuously drained to the exterior and isovolumically replaced with lactated Ringer's solution [34]. Compared to a 2 day control period, urine replacement over 2 days resulted in a doubling of amniotic fluid volume while intramembranous absorption rate decreased by an average of 40 %. Because the volume of fluid entering the amniotic compartment was not altered by urine replacement, it is clear that constituent(s) in fetal urine function as stimulator(s) of intramembranous absorption. Further, because urine replacement with lactated Ringer's solution diluted 50 % with water resulted in the same responses, the decrease in intramembranous absorption and increase in amniotic fluid volume during urine replacement were not due to osmolality or compositional differences between lactated Ringer's solution and fetal urine [34]. These results are crucial evidence that fetal urine is a source of one or more intramembranous regulatory factors. The observation represents a breakthrough in our understanding of amniotic fluid volume regulatory mechanisms because it is the first demonstration of factors mediating regulation of intramembranous absorption rate and thus amniotic fluid volume.

In order to further explore regulators of intramembranous absorption, lactated Ringer's solution was infused at 2 l/day into ovine amniotic fluid for 2 days in order to test the existence of an inhibitory factor in amniotic fluid. During the infusion, fetal urine either entered the amniotic sac or was isovolumically replaced. Intramembranous absorption rate increased above control levels during the 2-day infusion independent of urine entry into the amniotic fluid (unpublished observations). Thus, because fetal membrane stretch does not alter ovine amniotic fluid volume [83], it appears that a powerful inhibitor of intramembranous absorption is present in amniotic fluid and was diluted by the intra-amniotic infusion. The observation that amniotic fluid volume increased over time to very high levels while intramembranous absorption rate remained low following esophageal ligation or placement of a tracheo-esophageal shunt in ovine fetuses [77] further suggests that the intramembranous inhibitor is removed by fetal swallowing.

8 Cellular and Molecular Pathways for Intramembranous Amniotic Fluid Transport Mechanisms

Intramembranous transport of amniotic fluid is mediated by a primary component of unidirectional vesicular transcytosis and a secondary component of passive bidirectional permeation. The observation that intramembranous volume transport rate and amniotic fluid volume are negatively correlated under basal, urine replacement and volume loading conditions [84, 85] demonstrates the importance of this regulatory pathway as a key determinant of amniotic fluid volume homeostasis. The transport of amniotic fluid from the amniotic compartment into the fetal-placental circulation occurs across tissue layers that include the amniotic epithelial cells, chorionic tissues and vascular endothelium of the microvessels that perfuse the chorion and the surface of the placenta. The unique characteristic of the vesicular transport pathway is the outward unidirectionality from the amniotic cavity into fetal blood. In human and non-human primates, intramembranous absorption occurs across the placental amnion whereas, in species such as sheep, absorption occurs across the entire amnion as fetal vessels perfuse the connective tissue between amnion and chorion as well as the entire chorion [4]. Although the chorionic membrane may also participate, we and others have shown that the amnion is the rate-limiting layer for transport as permeability of the chorion is several times higher relative to that of the amnion and does not demonstrate unidirectionality [10, 86]. The role of the vascular endothelium in intramembranous absorption remains unclear as fluid normally filters out of the blood in most capillary beds whereas fluid is absorbed into the intramembranous vasculature.

8.1 Regulators of Intramembranous Transport

Based on our previous studies, the primary mechanism of intramembranous transport is consistent with the process of vesicular transcytosis [34, 59, 62, 63, 87]. The secondary component of passive transfer of water and solutes occurs through water channels and through other diffusion limited processes (Fig. 5.1).

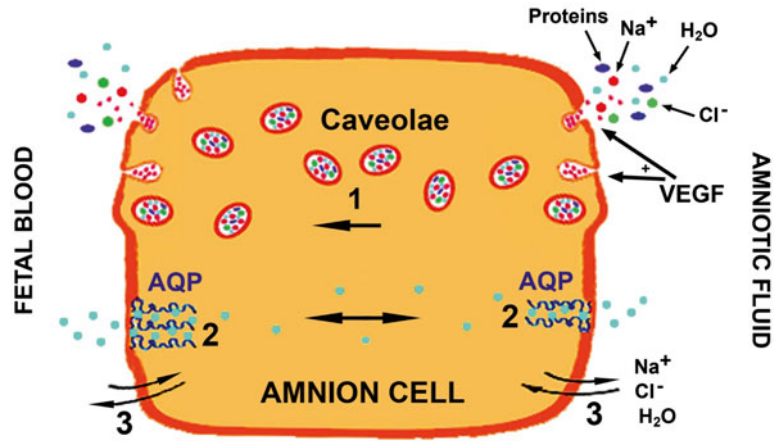

Fig. 5.1 Schematic representation of amniotic fluid transport across amnion cells. (*1*) Unidirectional caveolar transcytosis activated by VEGF. (*2*) Water transport facilitated by aquaporin channels. (*3*) Bidirectional diffusion of water and solutes

Our approach toward understanding the regulation of the intramembranous pathway is to identify individual components of the pathway and explore the cellular regulatory mechanisms, characterize the source and function of the regulatory factors, and determine conditions under which individual components are altered potentially leading to aberrant fluid transport with resulting abnormal amniotic fluid volumes. The sensor(s) that activates these pathways could be mechanical, such as membrane stretch, or biochemical, such as a substance(s) present in the amniotic fluid. The mechanisms mediated by the sensor would in turn activate specific pathways to affect transcellular fluid transport across the amnion. Our current hypothesis, supported by experimental data, indicates that active vesicular transport across amnion cells is stimulated by vascular endothelial growth factor (VEGF) to initiate vesicular transcytosis mediated via mobilization and transcellular shuttling of caveolar vesicles across the amnion cell, while the passive transcellular transfer of water is facilitated by aquaporin water channels localized on amnion cell membrane.

9 Membrane Stretch

The amniotic membrane has a high degree of distensability in order to accommodate the growing fetus with advancing gestation. The gain in fetal size is paralleled by expansion of amniotic fluid volume through much of gestation [88]. Any expansions or reductions in volume would induce changes in the stretch of the amniotic membrane. An extended or excessive stretch could lead to membrane rupture so that a feedback mechanism would need to be in place in order to regulate the amount of fluid within a normal range. In chronically catheterized fetal sheep, alternate inflation and deflation of intra-amniotic balloons (650 and 1,300 ml) at 3 day intervals had no effect on amniotic fluid volume in spite of changes in intrauterine volume [83]. Thus, in sheep, membrane stretch would not serve as a sensor for initiating intramembranous transport.

In an *in vitro* model of human amniotic epithelial cells, chronic static stretch applied for 36 h induced the expression of PBEF (Pre-beta cell colony enhancing factor) and its responsive effectors resulting in increased amniotic cell survival [89]. The anti-apoptotic effect of PBEF would maintain membrane integrity allowing amniotic fluid volume to increase, as in cases of amniotic fluid volume excess. More importantly, the significance of PBEF upregulation is due to its ability to increase VEGF action through enhanced expression of VEGF and its receptor VEGFR-2 in human amniotic epithelial cells to initiate transcytotic amniotic fluid transport [90]. This PBEF mediated pathway may represent an important cellular mechanism for augmenting intramembranous absorption thereby returning amniotic fluid volume to normal particularly in clinical conditions of polyhydramnios.

Because the ovine and human studies led to contrasting conclusions regarding the role of membrane stretch as a regulator of amniotic fluid volume, further studies will be required to re-evaluate the mechanism of membrane stretch and its effect on intramembranous absorption.

10 Biological Factors and Sources

Our recent studies suggest the presence of stimulatory and inhibitory factors in the amniotic fluid that modulate the rate of intramembranous absorption [34]. The potential sources of these regulatory factors include fetal urine, lung liquid and the fetal membranes.

10.1 Prostaglandin E_2

Amniotic fluid contains various proteins, endocrine factors, and cytokines in addition to the major solutes. Early studies have shown the presence of prostaglandin E_2 (PGE_2) in amniotic fluid and that it is primarily derived from fetal urine [91, 92]. Indeed it has been speculated that PGE_2 entry into the amniotic fluid may be important

particularly in the regulation of amniotic fluid volume homeostasis [92]. In addition, PGE_2 is known to stimulate VEGF expression in rat osteoblasts [93] and rat retinal endothelial cells [94]. Thus, it is possible that PGE_2 in the amniotic fluid would similarly activate VEGF production in the fetal membranes to initiate intramembranous transport. To test the role of PGE_2 in amniotic fluid volume regulation, we determined the relationships between intramembranous absorption rate and amniotic fluid PGE_2 levels during conditions of normal, high or low absorption rates in pregnant sheep [85]. We found that urinary PGE_2 concentrations are twice amniotic PGE_2 levels and that amniotic PGE_2 concentrations correlated positively with urinary PGE_2 levels. Further, the studies showed that urinary PGE_2 is a primary contributor to the amniotic PGE_2 pool. However, no relationships were observed between intramembranous absorption rate and amniotic PGE_2 or urinary PGE_2 concentrations under the experimental conditions studied. These findings preclude a role for amniotic and/or urinary PGE_2 as a regulatory factor of amniotic fluid volume homeostasis.

10.2 Vascular Endothelial Growth Factor

The growth factor VEGF is expressed in the amnion, released into the amniotic fluid, and has been implicated as a regulatory factor of intramembranous absorption [64]. In addition to its angiogenic properties, VEGF is a potent vascular permeability factor known to increase transport across capillary endothelial cells primarily by activating transcellular transport through the process of vesicular transcytosis [87, 95, 96]. We and others have shown that $VEGF_{165}$ and its receptors are expressed in fetal membranes and placentas of humans [97, 98] and sheep [99–103] and that VEGFR-1 and R-2 are present in ovine amniotic epithelial cells [104]. Further, vesicles and vacuolar-like organelles are localized in ovine amniotic membrane as shown by electron microscopy [105] with aggregates along the periphery of the amnion cells. These vesicles are enriched in caveolin-1, the structural protein of caveolae [104]. Caveolae are plasma membrane microdomains enriched in sphingolipids and cholesterol. Upon activation, the caveolar domains are internalized, released from the apical plasma membrane as caveolae and mobilized across the cell to the basolateral membrane for exocytosis. Thus, in the amnion, components are in place for a VEGF mediated caveolar transcytotic pathway for unidirectional bulk transport of amniotic fluid. In support of this hypothesis, our studies in ovine fetuses showed that $VEGF_{165}$ expression is up-regulated in the amnion during conditions of increased intramembranous absorption induced by blockade of fetal swallowing [106], intravascular volume loading [59] or hypoxia [107, 108]. In human amniotic epithelial cells *in vitro*, we found that permeability to inulin was significantly reduced by treatment with NEM (*N*-ethylmaleimide), an inhibitor of vesicular transport in lipid rich vesicles.

VEGF binds to two transmembrane receptors, VEGFR-1 (flt-1) and VEGFR-2 (flk/KDR), to initiate its biological effects. VEGFR-2 activation has been shown to initiate the majority of the $VEGF_{165}$ effects [109], including vascular permeability and vesicular transcytosis [96]. Our observation that $VEGF_{165}$ increased caveolin-1 mRNA levels and activated caveolin-1 phosphorylation in primary cultures of ovine amniotic epithelial cells is consistent with the existence of this caveolar transport pathway in the amnion. The VEGF signaling cascade appears to be mediated through c-Src protein kinase since PP2 (pyrazolopyrimidine 2), a protein kinase inhibitor, abrogated the $VEGF_{165}$ induced caveolin-1 phosphorylation [104]. Activation of caveolin-1 is an obligatory step in the formation and internalization of caveolae.

Soluble VEGFR-1 (sFlt-1) is expressed in placental tissues [97, 110] and is released into amniotic fluid [111]. sFlt-1 binds to $VEGF_{165}$ with high affinity and PlGF (placental growth factor) competes for the binding to sFlt-1. As such, sFlt-1 can modulate VEGF activity by reducing the bioavailability of VEGF for interaction with VEGFR-2, thus playing an inhibitory role in intramembranous transport. The role of sFlt-1 as an inhibitory factor awaits future studies.

The VEGF-A gene consists of eight exons and differential splicing generates multiple families of isoforms. A variant family of isoforms, referred to as VEGF-b, is formed by an alternate selection of exon 8 splice sites [112]. $VEGF_{165}b$ is a peptide of the same length as $VEGF_{165}$ but with a C-terminal sequence that differs by six amino acids. $VEGF_{165}b$ binds to VEGFR-2 with similar affinity as $VEGF_{165}$ but does not activate the post receptor signaling sequence [113]. It therefore acts as a negative regulator of $VEGF_{165}$ bioactivity. In most mammalian tissues, both forms are expressed at differing proportions. In normal tissues, the major isoform is $VEGF_{165}b$. An exception is the human placenta where $VEGF_{165}b$ levels are low relative to $VEGF_{165}$. However, under abnormal pregnancy conditions such as preeclampsia, the ratio of $VEGF_{165}$ to $VEGF_{165}b$ may be altered due to upregulation of $VEGF_{165}b$ and down-regulation of $VEGF_{165}$ [114]. This suggests that pathological conditions could lead to changes in the proportion of $VEGF_{165}$ relative to $VEGF_{165}b$ by preferential selection of splice sites.

Based on evidence that $VEGF_{165}$ is a stimulatory factor for vesicular transcellular transport through the intramembranous pathway and that $VEGF_{165}b$ can antagonize the effect of $VEGF_{165}$ thus acting as an inhibitory factor of transport, a potential mechanism for amniotic fluid volume regulation can be formulated. The rate of intramembranous transport and thus amniotic fluid volume may be regulated by the ratio of $VEGF_{165}$ to $VEGF_{165}b$ in normal pregnancy. Modifications in the ratio of $VEGF_{165}$ to $VEGF_{165}b$ would alter transport rate resulting in abnormal amniotic fluid volume. To test this hypothesis, we determined the relative abundance of $VEGF_{165}$ and $VEGF_{165}b$ mRNA in the fetal membranes and placentas of sheep and humans. In the normal ovine fetus near-term, the mRNA levels for both isoforms are lowest in the amnion and highest in the placenta. In the amnion and chorion, the relative quantities of $VEGF_{165}$ and $VEGF_{165}b$ mRNA were similar yielding a ratio of unity. This implies that normal amniotic fluid volume in pregnant sheep may be maintained by a balance of the $VEGF_{165}$ to $VEGF_{165}b$ level of expression. In contrast, in humans at normal term pregnancy, the mRNA level of $VEGF_{165}$ is significantly higher than $VEGF_{165}b$ in the placental amnion. This results in a $VEGF_{165}$ to $VEGF_{165}b$ ratio of 4:1 [115]. These studies revealed significant species differences and that the ratio of $VEGF_{165}$ to $VEGF_{165}b$ may be of greater importance in maintaining normal amniotic fluid in humans than in other species such as the sheep. Further studies on the ratio of $VEGF_{165}$ to $VEGF_{165}b$ in pregnancies with abnormal amniotic fluid volumes would provide key information necessary to define the role of VEGF isoforms in the regulation of amniotic fluid volume.

10.3 Fetal Solutes as Determinants of Amniotic Fluid Transport

A component of the intramembranous pathway is passive bidirectional movement of solutes across the amnion that is dependent on concentration differences between amniotic fluid and fetal blood. In the near-term ovine fetus under normal conditions, fetal blood is hypo-osmotic relative to maternal blood while amniotic fluid osmolality is lower than that of fetal blood. In response to fetal intravascular volume infusion, the fetus disposes of the excess fluids through its kidneys resulting in a small increase in amniotic fluid volume while most of the infused fluid is transferred across the placenta into the maternal circulation [45, 59]. High solute concentrations in the fetal circulation could similarly increase fetal blood volume due to osmotically induced increases in transfer of fluid from the maternal compartment into the fetal circulation [116]. Our previous studies showed that infusions of physiologic saline into fetal sheep resulted in transfer of sodium and chloride along with water to the ewe [35, 45, 117].

To directly test whether sodium and chloride availability would determine fetal fluid balance, a 5 M sodium chloride solution was infused intravascularly into the fetus for 3 days [118]. The infusion reduced but did not reverse the normal maternal-to-fetal sodium and chloride concentration differences. The fetuses diuresed extensively

but were not edematous with normal amniotic and allantoic fluid volume. These results indicate that large amounts of sodium and chloride were transferred to the maternal circulation. To further test the effects of solutes on fetal fluid transfer, 5 M sodium lactate was infused into the fetal circulation for 3 days [119]. The fetuses similarly diuresed, however, the excess fluid excreted was accumulated in the amniotic compartment resulting in an amnio-allantoic fluid volume of over 5 l compared to 1 l in the normal fetus. These findings rule out an osmotic role for sodium and chloride in effecting fluid transfer across the amnion and placenta, while larger molecules such as lactate are osmotically important determinants of fluid transfer between mother and fetus and hence amniotic fluid volume. Conversely, infusion of concentrated sodium lactate into amniotic fluid, while increasing amniotic lactate concentration to several times normal, did not alter amniotic fluid volume [120]. Thus, unlike the placenta, concentration differences may have only small effects on intramembranous transport. This is not meant to imply that concentration differences do not affect intramembranous transport because infusion or injection of large volumes of warm distilled water into the amniotic compartment cause rapid intramembranous water fluxes [11, 73].

10.4 Aquaporins and Intramembranous Transport of Water

Passive water permeation across the amnion has been estimated to constitute as much as 30 % per day of overall intramembranous transport under normal conditions [61, 62]. However, aberrations in this pathway over the course of pregnancy could have significant consequences on amniotic fluid volume homeostasis that could lead to polyhydramnios or oligohydramnios if no compensatory mechanisms develop.

Passive intramembranous transfer of water is facilitated by aquaporin (AQP) water channels. Aquaporins are a family of transmembrane proteins that function as specialized water channels permeable to water but not organic and inorganic molecules [121], except for a subfamily of aquaporins with high structural homology to AQP1 that allows passage of certain solutes such as urea and glycerol in addition to water [122]. Aquaporin 3 and 9 are members of this subfamily termed aquaglyceroporins. Currently five aquaporins, AQP1, AQP3, AQP8, AQP9 and AQP11, are known to be expressed in the amnion and chorion of humans [123–130] and mice [131]. In AQP1 knockout mice, polyhydramnios develops while amniotic fluid osmolality decreases [132]. However, in idiopathic polyhydramnios, AQP1, AQP8 and AQP9 mRNA levels in the fetal membranes are elevated [124, 133], while in oligohydramnios the relative quantities of AQP1 and AQP3 are reduced [134]. The latter findings contrast with the expected changes in aquaporin expression if aquaporins were to mediate water transfer outward from the amniotic fluid compartment. Thus, although these studies support the concept that aquaporin water channels play a role in amniotic fluid volume regulation [65, 135], they are more likely to be involved in mediating compensatory mechanisms. As such, even though changes in aquaporin activity may cause abnormal amniotic fluid volumes, the changes appear more likely a consequence of the changes in amniotic fluid volume.

An important regulatory factor for water movement across aquaporin channels is osmolality. Expressions of AQP1, AQP3, AQP8 or AQP9 in various cell types have been shown to be modified by osmotic stress; however, the responses were variable. Hypertonicity was shown to increase AQP1 expression in kidney cells [136] while hypotonicity enhanced AQP1 expression in choroid plexus [137]. In human amnion cells, AQP8 expression was increased by hypoosmotic stimulus and decreased by hyperosmolality [138]. Because an osmotic difference normally exists between amniotic fluid and fetal blood, amniotic fluid osmolality could be a primary driving force for water movement out of the amniotic cavity into fetal blood. This would be particularly important when amniotic fluid volume is reduced and amniotic fluid osmolality augmented due to conditions such as placental insufficiency [139] or post-term pregnancies [140].

To gain insights into the relative importance of the five aquaporins as water transport facilitators in the amnion, we determined their expression patterns in the amnion. In the mouse at gestational age from E14 to E19, all five aquaporins are present with a differential expression pattern. The most abundant aquaporin expressed is AQP8, nearly tenfold higher than AQP1 while AQP3 and 11 are expressed at levels similar to that of AQP1. The least expressed is AQP9 at levels less than 1 % of AQP1, 3, or 11. In human term gestation, the placental amnion similarly expresses AQP1, 3, 8, 9 and 11. There were large and highly significant differences in the relative quantities of mRNA amongst individual aquaporins. AQP1, 3, 9, and 11 mRNA levels were 230, 227, 119, and 3.7 times that of the placental AQP8 mRNA level. AQP8 is least expressed with AQP11 the second least while AQP1 and 3 are the most abundant forms. AQP9 expression is 50 % of AQP1 and AQP3. The species difference in the pattern of aquaporin expression supports the notion that utilization of the various aquaporins in the amnion is different among species.

The importance of the simultaneous presence of five aquaporins in the amnion at various abundances may have functional significance. Either a high degree of redundancy exists to ensure adequate water transport capability or individual aquaporins may have different functional roles. AQP1 is a water selective channel that primarily functions to facilitate water transport. AQP3 and AQP9 are members of the aquaglyceroporin subfamily that permit transport of urea and glycerol in addition to water, whereas AQP8 is distinct with low structural homology. AQP11 is a member of the "superaquaporin" subfamily with a low degree of structural homology with conventional water selective aquaporins [141]. Due to a specialized N-terminal modification of the signature motif, the water permeability of AQP11 is low compared to AQP1. With an intracellular localization, AQP11 may have unique functions different from water transporting aquaporins. Given such structural diversity, the five aquaporins in the amnion may mediate separate transport functions for water and small molecules at varying capacity. Until further discovery of the structure–function relationships amongst the different aquaporins, the identification of effectors for intramembranous water transport would focus on those aquaporins with established water transport function.

11 Summary

Over the 30 years since we began exploring the regulation of amniotic fluid volume, our understanding continually has evolved from not knowing if volume regulation occurs to knowing not only that modulation of intramembranous absorption is the primary site of regulation but also that it is the active, unidirectional component of intramembranous transport that is regulated while the passive, bidirectional components change little. Concepts evolved from little known to multiple pathways involving complex transport mechanisms. Future progress depends on identifying the cellular and molecular mechanisms that regulate active and passive intramembranous absorption as well as their regulatory components.

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6 Gestational Diabetes, Preeclampsia and Cytokine Release: Similarities and Differences in Endothelial Cell Function

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Abstract

Gestational diabetes, pre-eclampsia as well as intra-uterine infection during pregnancy affects the function of the endothelium both in the mother and the fetus leading to endothelial dysfunction. Gestational diabetes is also associated with an increased incidence of pre-eclampsia and it is likely that both the hyperglycemia as well as the release of cytokines especially TNFα during hyperglycemia may play an important role in the pathogenesis of endothelial dysfunction leading to preeclampsia. Similarly, some but not all studies have suggested that infection of the mother under certain circumstances can also lead to preeclampsia as women with either a bacterial or viral infection were at a higher risk of developing preeclampsia, compared to women without infection and infection also leads to a release in TNFα. Endothelial cells exposed to either high glucose or TNFα leads to an increase in the production of H_2O_2 and to a decrease in endothelial cell proliferation. The cellular and molecular mechanisms involved in this phenomenon are discussed.

Gestational diabetes, pre-eclampsia as well as intra-uterine infection during pregnancy has profound effects on the fetus and long term effects on the neonate. All three conditions affect the function of the endothelium both in the mother and the fetus leading to endothelial dysfunction. Gestational diabetes is also associated with an increased incidence of pre-eclampsia and it is likely that both the hyperglycemia as well as the release of cytokines especially

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L. Zhang and C.A. Ducsay (eds.), *Advances in Fetal and Neonatal Physiology*, Advances in Experimental Medicine and Biology 814, DOI 10.1007/978-1-4939-1031-1_6,

TNFα during hyperglycemia may play an important role in the pathogenesis of endothelial dysfunction leading to preeclampsia. It has also been suggested although not universally accepted that under certain circumstances maternal infection may also predispose to pre-eclampsia. Pre-eclampsia is also associated with the release of TNFα and endothelial dysfunction. However, the cellular and molecular mechanism(s) leading to the endothelial dysfunction by either hyperglycemia or by the cytokine TNFα appear to be different. In this chapter, we explore some of the similarities and differences leading to endothelial dysfunction by both hyperglycemia and by the inflammatory cytokine TNFα and the cellular and molecular mechanism(s) involved.

Keywords
Gestational diabetes • Pre-eclampsia • Endothelial dysfunction • Hyperglycemia

Gestational diabetes, pre-eclampsia as well as intra-uterine infection during pregnancy has profound effects on the fetus and long term effects on the neonate. All three conditions affect the function of the endothelium both in the mother and the fetus leading to endothelial dysfunction. Gestational diabetes is also associated with an increased incidence of pre-eclampsia and it is likely that both the hyperglycemia as well as the release of cytokines especially TNFα during hyperglycemia may play an important role in the pathogenesis of endothelial dysfunction leading to preeclampsia. It has also been suggested although not universally accepted that under certain circumstances maternal infection may also predispose to pre-eclampsia. Pre-eclampsia is also associated with the release of TNFα and endothelial dysfunction. However, the cellular and molecular mechanism(s) leading to the endothelial dysfunction by either hyperglycemia or by the cytokine TNFα appear to be different. In this chapter, we explore some of the similarities and differences leading to endothelial dysfunction by both hyperglycemia and by the inflammatory cytokine TNFα and the cellular and molecular mechanism(s) involved.

1 Effects of Gestational Diabetes During Pregnancy

Increasing levels of plasma glucose leads to increased insulin level in the fetus to counteract the hyperglycemia. This increase in insulin levels in the fetus in turn stimulates growth of the fetus thereby leading to increased fetal birth weights above the 90th percentile. The increase in fetal growth and insulin levels are mainly responsible for an increase in primary cesarean deliveries and neonatal hypoglycemia. Exposure to maternal hyperglycemia during pregnancy is also associated with birth defects and effects on childhood growth and glucose regulation [1]. Most of the increased prevalence of childhood type 2 diabetes during the last 30 years is also attributable to increasing exposure to maternal diabetes during pregnancy [2].

2 Gestational Diabetes, Hyperglycemia and Endothelial Dysfunction

The cord blood serum c-peptide level is usually above the 90th percentile in gestational diabetes suggesting inflammation of the endothelial cells [3]. Prevalence of atherosclerotic disease is also markedly increased among individuals with diabetes mellitus [4, 5]. Endothelial dysfunction comprises a number of functional alterations in the vascular endothelium, such as impaired regulation of vasodilation and vasoconstriction, impaired or excessive angiogenesis, decreased barrier function, and increased inflammatory activation, all of which are associated with cardiovascular disease [6]. In type 2 diabetes, the relationship between endothelial dysfunction and diabetes is complex, as endothelial dysfunction is

initiated well before the onset of overt diabetes [6]. The mechanisms underlying endothelial dysfunction in diabetes differ between type 1 (T1D) and type 2 diabetes (T2D): hyperglycemia contributes to endothelial dysfunction in all individuals with diabetes, whereas the causative mechanisms in T2D also include impaired insulin signaling in endothelial cells, dyslipidemia and altered secretion of bioactive substances (adipokines) by adipose tissue. The close association of so-called perivascular adipose tissue with arteries and arterioles facilitates the exposure of vascular endothelium to adipokines, particularly if inflammation activates the adipose tissue [6]. The timing of endothelial dysfunction is equally unclear in gestational diabetes as well [6]. Hyperglycemia-induced endothelial dysfunction is also characterized by enhanced inflammatory cytokine and adhesion molecule expression including intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1, leading to endothelial-monocyte adhesion. The expression and secretion of various adhesion molecules, which can be activated by cytokines, are implicated in leukocyte migration and the development of atherosclerosis [7]. There is also an impairment of the release of vasodilator substances such as nitric oxide (NO) [8], in addition to the impairment of endothelium induced vasodilation [9]. Chronic levels of inflammation, marked by elevated levels of plasma C-reactive protein (CRP), as well as cytokines such as TNFα, have been noted in patients with longstanding diabetes [10]. In addition, the expression of VCAM-1, ICAM-1, and E-selectin in endothelial cells indicates the existence of chronic inflammation in diabetes [10]. Furthermore, inflammatory cytokines such as TNFα can act as mediators of insulin resistance by impairing the tyrosine kinase activity of both the insulin receptor (IR) and insulin receptor substrate (IRS-1), thus inhibiting insulin signaling [11].

3 Insulin and Endothelial Function

There appears to be a bi-directional relationship that exists between hyperinsulinemia and low grade chronic inflammation in diabetes. Hyperinsulinemia can lead to vascular inflammation which can cause insulin resistance, thereby leading to hyperinsulinemia [12]. At physiological concentrations, insulin exerts mainly anti-inflammatory effects whereas hyperinsulinemia increases levels of oxidative stress and inflammation [12]. It has been demonstrated that insulin at pathophysiological concentrations alone or in combination with low concentrations of TNFα has the ability to promote VCAM-1 expression through increasing the steady-state levels of mRNA via the activation of transcription factors, such as NF-kβ, which have been linked to VCAM-1 activation [13]. In this manner, hyperinsulinemia leads to increased monocyte adhesion to endothelial cells [13, 14]. An interesting feature of insulin resistance is the fact that the normal route for insulin to activate the PI-3 kinase and Akt dependent signaling pathway is impaired, whereas hyperinsulinemia over activates the mitogen activated protein kinases (MAPK) pathways thereby creating an imbalance between PI-3 kinase and MAP-kinase dependent functions of insulin [14]. This leads to a decrease in the production of nitric oxide (NO) which is a vasodilator and an increase in endothelin (ET)-1 which is a vasoconstrictor. These findings may explain the endothelial dysfunction and hypertension observed in gestational diabetes, and may also account for the increased incidence of preeclampsia [14].

4 Hyperglycemia, Endothelial Cells and Reactive Oxygen Species

In addition to the increased expression of adhesion molecules observed, hyperglycemia also stimulates reactive oxygen species (ROS) production by four major sources which include glucose auto oxidation [15], mitochondrial superoxide production [16], e-NOS uncoupling [17] and advanced glycation end products (AGE) dependent NADPH oxidase activation [18]. Glucose auto oxidation and mitochondrial superoxide are likely to be the initial contributors to ROS mediated dysfunction caused by hyperglycemia [18].

5 Similarities in the Effects of Gestational Diabetes and Maternal Infection on Endothelial Function

There are some similarities between the effects of gestational diabetes and maternal infection on endothelial cell function. Some but not all studies have suggested that infection of the mother under certain circumstances can also lead to preeclampsia [19, 20]. Women with either a bacterial or viral infection were at a higher risk of developing preeclampsia, compared to women without infection [19]. More recently, an association between maternal periodontal disease and preeclampsia has been reported [20]. In this study, the risk of preeclampsia was increased in pregnant women with urinary tract infection (pooled odds ratio, 1.57; 95 % CI, 1.45–1.70) and periodontal disease (pooled odds ratio, 1.76; 95 % CI, 1.43–2.18). More studies are required to verify this as well as to explore whether or not such relationships are causal and, if so, the mechanisms involved.

During maternal infection there is also an increase in the release of pro-inflammatory cytokines like TNFα as well as an increase in ROS [20] similar to that observed in GDM [15–18]. Through an increase in ROS, both hyperglycemia as well as inflammatory cytokines can lead to endothelial dysfunction along with functional alteration in the endothelial cells of both the mother and the fetus. Similarly, TNFα release by leukocytes in pre-eclampsia indicates activation of TNFα producing leukocytes by the disease process thereby leading to endothelial dysfunction [21]. For example, endothelial cells from umbilical cords obtained from pre-eclamptic women appeared oval, round elongated, flattened, triangular or of polygonal shapes [22]. The nuclei displayed shallow and deep invaginations of nuclear envelope [22]. The endoplasmic reticulum appeared highly dilated and vacuolated [22]. The mitochondria appeared abnormal and showed cristae fragmentation [22]. Areas of focal necrosis were appreciated throughout the cytoplasm [22]. In addition, a marked enlargement of sub-endothelial space and the presence of an electron dense granular material were also found [22]. On this basis the authors concluded that their findings revealed both activation and injury of endothelial cells and disruption of the endothelial cell layer in pre-eclampsia [22].

6 Energy Production and Utilization by Endothelial Cells in Hyperglycemia and Infection: Similarities and Differences at the Cellular Level

Physiologically, endothelial cells function as a barrier between the circulating blood and the vascular smooth muscle. Unlike most cells, it is currently accepted that the energy utilized by endothelial cells is characterized by glucose fermentation to lactate [23–26] even under normoxic conditions (“aerobic glycolysis”), similar to the utilization of energy by malignant cells.

Most data on endothelial cells have been obtained following their isolation and culture in vitro, an important distinction from the environment and situation of endothelial cells in vivo [23]. All enzyme activities were found to be increased in isolated HUVEC within 2 weeks in culture [23]. It is therefore important that any study utilizing endothelial cells in vitro must be performed with cells in early passages. We therefore utilized endothelial cells up to passage 3 for all our studies. In normal HUVEC, we observed an increase in H_2O_2 production over time, following exposure to TNF-α, which was accompanied by a decrease in the viable cell count, most likely reflecting a decrease in proliferation of HUVEC (unpublished observations). This decrease in viable cell count was not due to apoptosis as we [27], and others [28] have demonstrated, that TNFα alone does not cause apoptosis of HUVEC, but does so only in the presence of cycloheximide or actinomycin D [28].

We also observed that the decrease in viable cell count of HUVEC mediated by H_2O_2 following exposure of HUVEC to TNFα was through down regulation of the TAZ (Transcriptional co-Activator

with PDZ-binding motif) protein. TAZ is a biologically potent transcriptional co-activator and functions by binding to the PPXY motif present in several transcription factors. Notably, TAZ behaves as a transducer linking cytoplasmic signaling events to transcriptional regulation in the nucleus. This TNFα induced down regulation of TAZ protein was mediated by NFkβ (unpublished observation). Similarly, exposure of HUVEC to high glucose (25 mm) also increased H_2O_2 production over time and this was also accompanied by a decrease in viable cell count as well as decreased expression of the TAZ protein (unpublished observation). However, unlike the decrease in TAZ observed following exposure of HUVEC to TNFα, the decrease in TAZ following exposure to high glucose was not mediated through the NFkβ pathway. This indicated that while some of the effects of TNFα and hyperglycemia on HUVEC may be similar the cellular mechanism(s) may be different.

We also observed that the oxygen consumption rate (OCR) increased in HUVEC exposed to TNFα, but the extra cellular acidification rate (ECAR) (indicating glycolysis) showed no change and thereby the OCAR/ECAR ratio was increased in HUVEC exposed to TNFα (unpublished observation). This indicated that HUVEC exposed to TNFα increased energy formation by oxidative phosphorylation and not by glycolysis as it did not further increase the glycolytic process in HUVEC. By contrast, we observed that in the presence of oligomycin which inhibits mitochondrial ATPase, addition of TNFα to HUVEC increased the ECAR (indicating increase in glycolysis) demonstrating that HUVEC exposed to TNFα has the capacity to compensate for the loss of ATP synthesis by the mitochondria by its ability to redirect the synthesis of ATP through glycolysis.

By contrast, when HUVEC were exposed to high glucose (25 mm) simulating that observed during hyperglycemia, there was only a moderate decrease in OCAR but an increase in ECAR. This was an effect that was not observed in control cells which were not exposed to high levels of glucose (unpublished observation). It therefore appears that HUVEC exposed to high glucose decreases mitochondrial ATP synthesis which is compensated by ATP production by glycolysis. This highlights another difference as indicated earlier that HUVEC exposed to TNFα increases ATP synthesis by the mitochondria and only when mitochondrial function is affected, it increases glycolysis. This indicates that the cellular mechanism of energy generation by HUVEC when exposed to either TNFα or to high glucose is different.

Following exposure of HUVEC to either TNFα or to high concentration of glucose showed a decrease in some of the intermediates of the tricarboxylic acid cycle (TCA cycle). The TCA cycle (also known as the Krebs cycle) is a series of enzyme-catalyzed chemical reactions that form a key part of aerobic respiration in cells. Here again, the mechanism (s) of the decrease in some of the intermediates of the TCA cycle appeared to be different with respect to HUVEC exposed to high levels of TNF α, when compared with HUVEC exposed to high levels of glucose. In HUVEC exposed to TNFα, the CO_2 elimination rate (CDER) appeared to be increased (unpublished observations). As CO_2 is formed mainly as a byproduct of the TCA cycle, it would suggest that the decrease in the intermediates of the TCA cycle was most likely due to increased activity of the TCA cycle in HUVEC exposed to TNFα. The potential of NADH and $FADH_2$ which are the end products of the TCA cycle is converted to more ATP through an electron transport chain with oxygen as the "terminal electron acceptor". This further confirms that most of the ATP produced by HUVEC following exposure to TNFα as indicated previously is by aerobic cellular respiration and which is primarily by oxidative phosphorylation. Conversely, in HUVEC exposed to high glucose, the CDER was decreased supporting the concept that there was decreased activity of the TCA cycle (unpublished observations). This would indicate that in HUVEC exposed to high levels of glucose, the ATP synthesis was geared more towards glycolysis when compared to HUVEC exposed to TNFα which were more likely to utilize ATP synthesis through oxidative phosphorylation.

Despite these differences, there were some common features associated with endothelial

dysfunction caused by TNFα and that produced by hyperglycemia. Increased levels of TNF α, as well as high levels of glucose, led to an increase in the production of ROS, eventually leading to decreased endothelial cell proliferation (unpublished observations). Most [29, 30], but not all [31] studies indicate that TNFα decreased nitric oxide synthase (NOS) function and thereby NO production by various mechanisms. Similarly high levels of glucose also inhibited NO production in HUVEC by various mechanisms [32, 33]. This may be possible through the formation of advanced glycation end products (AGEs) on gene expression and synthesis of TNFα and endothelial NO synthase by endothelial cells. AGEs promote mRNA expression and secretion of TNFα and reduce the endothelial NOS (eNOS) mRNA and protein expression in HUVEC [34]. Therefore, the effect of elevated TNFα and decreased NO activity on endothelial functions and intercellular interaction could play an important role in pre-eclampsia.

7 Conclusions

Endothelial dysfunction is characteristics of pre-eclampsia. Both hyperglycemia and release of inflammatory cytokines in the diabetic state as well as the release of inflammatory cytokines during maternal and fetal infection lead to endothelial cell activation. Some studies indicate that under certain circumstances, both the diabetic state [35] as well as maternal infection [19, 20] may be associated with preeclampsia. Since endothelial dysfunction is a key feature of pre-eclampsia, there may be much to gain from assessing the similarities in cellular and molecular changes observed with HUVEC when they are exposed to either TNFα or to high levels of glucose, and compare these changes with those obtained from pregnancies affected by pre-eclampsia.

Acknowledgement This work was partially presented at a symposium to honor Lawrence D. Longo M.D. for his successfully forming and leading the Center for Perinatal Biology at Loma Linda University, California.

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Heart Disease Link to Fetal Hypoxia and Oxidative Stress

7

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Abstract

The quality of the intrauterine environment interacts with our genetic makeup to shape the risk of developing disease in later life. Fetal chronic hypoxia is a common complication of pregnancy. This chapter reviews how fetal chronic hypoxia programmes cardiac and endothelial dysfunction in the offspring in adult life and discusses the mechanisms via which this may occur. Using an integrative approach in large and small animal models at the *in vivo*, isolated organ, cellular and molecular levels, our programmes of work have raised the hypothesis that oxidative stress in the fetal heart and vasculature underlies the mechanism via which prenatal hypoxia programmes cardiovascular dysfunction in later life. Developmental hypoxia independent of changes in maternal nutrition promotes fetal growth restriction and induces changes in the cardiovascular, metabolic and endocrine systems of the adult offspring, which are normally associated with disease states during ageing. Treatment with antioxidants of animal pregnancies complicated with reduced oxygen delivery to the fetus prevents the alterations in fetal growth, and the cardiovascular, metabolic and endocrine dysfunction in the fetal and adult offspring. The work reviewed offers both insight into mechanisms and possible therapeutic targets for clinical intervention against the early origin of cardiometabolic disease in pregnancy complicated by fetal chronic hypoxia.

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L. Zhang and C.A. Ducsay (eds.), *Advances in Fetal and Neonatal Physiology*, Advances in Experimental Medicine and Biology 814, DOI 10.1007/978-1-4939-1031-1_7,

Keywords
Hypoxia • Oxidative stress • Antioxidants • IUGR • Programming

1 Fetal Hypoxia

Fetal hypoxia is one of the most common consequences of complicated pregnancy and it may occur by insufficiency of either uterine blood flow, increased placental vascular resistance with a consequent decrease in umbilical blood flow, or by a decrease in the maternal arterial oxygen content [1–3]. Other mechanisms such as fetal anaemia or increased fetal oxygen consumption (e.g. in pyrexia) are relatively rare in clinical practice [4]. Risk factors which predispose to the development of fetal hypoxia can be classified into maternal, intra-partum and iatrogenic. Maternal risk factors include diabetes, pregnancy induced or chronic hypertension, Rh sensitization, maternal infection, sickle cell anaemia, chronic substance abuse, asthma, seizure disorders or smoking [5–13]. Intra-partum risk factors for fetal hypoxia encompass multiple pregnancy, pre or post-term birth, prolonged labour, placental abruption, placenta praevia, prolapsed umbilical cord or abnormal presentation of the fetus [14–21]. Maternal hypotension, which may accompany epidural anaesthesia is an example of an iatrogenic risk factor for the development of fetal hypoxia [22].

2 The Fetal Defence to Hypoxia

In contrast to glucose or protein, which can be stored as reserves in our body for prolonged periods of time, there is no similar mechanism for the long-term storage of oxygen in our bodies either before or after birth. Consequently, before and after birth, our physiology is largely dependent on a constant supply of oxygenated blood. Not surprisingly, the source of this constant delivery of oxygen is drastically different between the intra- and extra-uterine environments. Therefore, it should also be unsurprising that the strategy of our cardiovascular system to defend tissues against periods of reduced oxygenation is also dramatically different during the fetal and postnatal periods. Outside the womb, there is a vast supply of oxygen from the atmosphere. In the adult period, a reduction in oxygen supply to the tissues is met with an increase in ventilation to increase the level of oxygenation in our pulmonary blood. This luxurious supply of atmospheric oxygen allows the adult cardiovascular system to increase perfusion not only to essential vascular beds but also to peripheral tissues, maintaining their oxygenation during periods of systemic hypoxia [23]. Within the womb, the supply of oxygenated fetal blood is dependent on the placenta. In contrast to pulmonary ventilator processes, intrauterine mechanisms to increase the input and output of oxygenated blood are limited. However, a number of adaptations, unique to life in the womb, permit the supply of oxygen to the fetus to exceed its metabolic needs, equipping the unborn child with a considerable margin of safety for oxygenation under basal conditions during development. For example, relative to the adult, these adaptations allow the fetus to bind greater concentrations of oxygen in its haemoglobin, to have an increased basal blood flow to most tissues, and to relinquish the bound oxygen to the fetal tissues at lower oxygen tensions [24]. In addition, shunts in the fetal circulation and preferential streaming ensure an adequate supply of oxygenated blood to tissues most at risk of damage during reductions in oxygenation [24, 25]. Finally, the fetus has a greater capacity than the adult to hinder oxygen-consuming processes [24]. Consequently, the fetal defence strategies during episodes of hypoxia capitalise on increasing the efficiency of these fetal adaptations,

thereby either consuming even less oxygen, extracting even more oxygen from haemoglobin and ultimately making better use of this finite supply of oxygenated blood [25]. The defence responses to episodes of hypoxia of the fetal cardiovascular system exemplify some of these strategies. In the late gestation fetus, an episode of acute hypoxia triggers fetal bradycardia [26]. Deceleration of the fetal heart has several advantages as it permits maintenance of normal levels of myocardial oxygen consumption despite hypoxic conditions [27] and prolongs the beat-to-beat interval, thereby increasing end diastolic filling volume, which helps maintain fetal cardiac output [28]. Reducing the velocity of blood flow through the fetal coronary circulation will also prolong diffusion time, an effect likely to increase the efficiency of myocardial blood gas exchange. During episodes of acute hypoxia, the fetal cardiac output is also redistributed due to differential vasomotion. For instance, circulations perfusing the fetal brain undergo active vasodilatation and those perfusing peripheral circulations undergo active vasoconstriction [25, 26]. Therefore, the fetal cardiac output follows the path of least resistance, becoming diverted from less essential vascular beds towards those perfusing the brain—the so called brain sparing effect. The fetal bradycardia and brain-sparing circulatory responses to acute hypoxia have been conserved across all species studied to date, from the chick embryo to the sheep fetus, the non-human primate and the human fetus (see [29]).

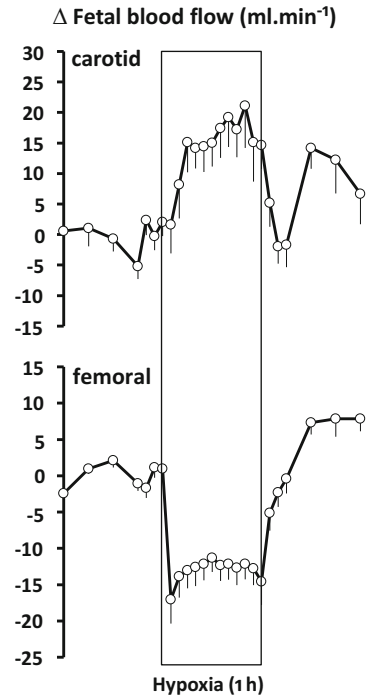

Fig. 7.1 Fetal brain sparing effect during acute hypoxia in real time *in vivo*. Values are mean ± S.E.M. of change from baseline in carotid blood flow and femoral blood flow measured simultaneously in 14 fetal sheep between 118 and 125 days gestation (term ~145 days) during basal conditions and in response to a 1 h period of acute hypoxia (*box*). Fetal descending aortic PO_2 during acute hypoxia was reduced from 23.4 ± 0.7 to 13.2 ± 0.3 mmHg. Redrawn from [30]

3 The Physiology Underlying the Fetal Cardiovascular Defence to Hypoxia

More than two decades ago, for the first time, Transonic flow probes were surgically implanted around the carotid and femoral arteries of late gestation fetal sheep in long-term preparations to be able to visualise the fetal brain sparing response to acute hypoxia in real time ([30]; Fig. 7.1). The data revealed that the fetal peripheral circulatory response occurs within seconds of the onset of hypoxia, suggesting a neurally-triggered response. It is now established that while the increase in cerebral blood flow during acute hypoxia results from an increase in nitric oxide (NO; [31]), both the bradycardia and the peripheral vasoconstriction in the fetus are initially triggered by the same carotid body chemoreflex, as bilateral section of the carotid sinus nerves prevents them from occurring [30]. Once triggered by the carotid chemoreflex, fetal peripheral vasoconstriction is maintained by the release of constrictor agents into the fetal circulation, such as catecholamines, vasopressin and neuropeptide Y [32–34].

We now know that this neuro-endocrine peripheral constrictor drive is fine-tuned by opposing dilator influences, such as an increase in NO production in fetal peripheral vasculature during acute hypoxia [35]. Data to support this comes from studies using fetal *in vivo* treatment with a NO clamp. The latter is a technique that permits blockade of *de novo* synthesis of NO during acute hypoxia while maintaining basal cardiovascular function [36, 37]. Application of the NO clamp to the late gestation sheep fetus markedly enhanced the magnitude of the femoral vasoconstrictor response, revealing the full strength of the unopposed chemoreflex and endocrine constrictor responses [35]. Most recently, we have discovered that the bioavailability of NO in the fetal peripheral vascular beds is itself limited by the generation of reactive oxygen species, such as the superoxide anion ($^{\bullet}O_2^-$), during acute hypoxia. Therefore, it is the actual interaction between $^{\bullet}O_2^-$ and NO that provides a local oxidant tone to the fetal vasculature, whereby a fall in the ratio favours dilatation and an increase promotes vasoconstriction. We have shown that this vascular oxidant tone is operational in fetal life and that it can be easily manipulated [29, 38–40]. For instance, fetal treatment with antioxidants or with statins markedly diminished the magnitude of the femoral vascular resistance response to acute hypoxia. This effect was by increasing the bioavailability of NO, as fetal treatment with antioxidants or with statins during NO blockade with the NO clamp restored the full magnitude of the femoral vasoconstrictor response [29, 39]. Combined, therefore, data show that the peripheral vasoconstrictor response to acute hypoxia in the fetus is an aggregate of mechanisms, resulting from carotid chemoreflex stimulation, enhanced endocrine vasoconstrictors and a local vascular oxidant tone determined by the interaction between $^{\bullet}O_2^-$ and NO.

4 Fetal Chronic Hypoxia

In fetal physiology, it is a well accepted view that should the duration of the period of fetal oxygen deprivation become prolonged, then the fetal circulatory defence response to acute hypoxia persists. Therefore, in response to chronic fetal hypoxia, such as may occur during preeclampsia or placental insufficiency, persisting redistribution of blood flow away from peripheral circulations is believed to maintain oxygen and nutrient delivery to the fetal brain. The biological trade-off is asymmetric fetal growth restriction, whereby babies are not only small but they are thin for their length with a low ponderal index [41]. These infants also show a greater impact of hypoxia on body growth relative to brain growth, usually represented in neonatology by an increase in the bi-parietal diameter to the body length ratio of the infant [41]. Persisting redistribution of blood flow away from vascular beds considered less essential in fetal life, such as those perfusing the fetal kidneys or the fetal pancreas, may also explain the reduced endowment of kidney nephrons and of beta cells in the islet of Langerhans in intrauterine growth-restricted (IUGR) offspring [42, 43]. Data to support persisting redistribution of blood flow away from less essential vascular beds towards the fetal brain in pregnancy complicated by chronic fetal hypoxia is difficult to obtain, but the group of Professor Longo at Loma Linda University has been able to just do that. Exploiting the natural hypobaric hypoxia of pregnancy at high altitude and combining it with expertise to measure regional blood flow changes with radioactively labelled microspheres, they reported that during long-term high altitude hypoxia in sheep, blood flow to the fetal brain is maintained and there is a sustained decrease in blood flow to the fetal carcass ([44]; Fig. 7.2). In comparison to asymmetric IUGR, the less well described side effect of persisting redistribution of blood flow away from peripheral circulations may be endothelial dysfunction leading to an increase in fetal peripheral vascular resistance, which will increase fetal cardiac afterload if cardiac output is maintained. Persisting increases in fetal cardiac afterload may overwhelm the Frank-Starling mechanism and trigger changes in the morphology and function of the fetal heart and aorta [28]. Accordingly, several studies in chick embryos and fetuses of mammalian species have now reported that developmental hypoxia promotes cardiac and aortic wall hypertrophic growth, altered cardiac function, pulmonary

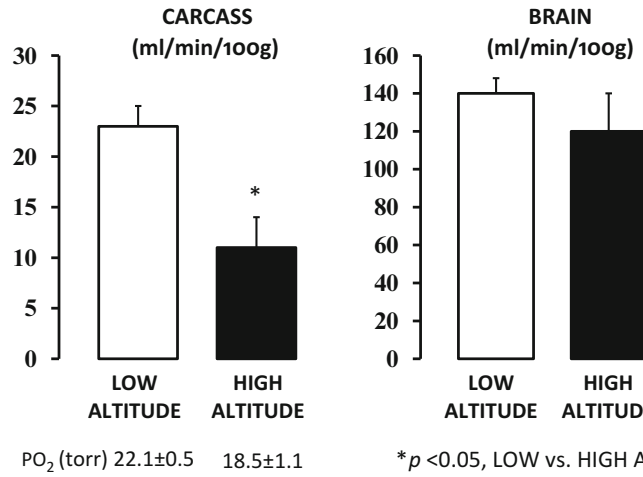

Fig. 7.2 Fetal brain sparing effect during chronic hypoxia. Values are mean ± S.E.M. of brain and carcass basal blood flow in late gestation fetal sheep from control low altitude pregnancy or pregnancy exposed to high altitude from 30 to 135 days of gestation. Blood flow was measured at 135 ± 1 days of gestation (term ~146 days). *P < 0.05, low altitude *vs.* high altitude hypoxia. Redrawn from reference [44] with permission

hypertension, sympathetic hyper-innervation of peripheral resistance arteries and NO-dependent peripheral vascular endothelial dysfunction (see [45] for review). The cardiac and aortic wall remodelling and asymmetric growth restriction that occurs in sea level chick embryos incubated at high altitude is no longer observed in sea level embryos incubated at high altitude with oxygen supplementation [46, 47], underlying the direct effects of chronic hypoxia on fetal growth and on cardiovascular development. In the clinical setting, fetal aortic wall thickening is particularly relevant as, in humans, an increase in large artery stiffness independently predicts cardiovascular risk [48], being a key component in the aetiology of cardiovascular diseases including hypertension, atherosclerosis and coronary heart disease [49]. An increase in wall thickness, in particular, in the aorta has also been proposed as the first physical sign of atherosclerosis [50]. In human perinatal clinical studies, there have been four additional reports linking babies born from pregnancies complicated by placental insufficiency with aortic wall thickening, increased aortic stiffness and reduced distensibility [51–54]. In addition, developmental hypoxia in animals and humans induces neonatal pulmonary hypertension with a marked cardiopulmonary remodelling [55–57]. A remarkable number of unique studies by the groups of Gilbert, Pearce and Longo have reported that in fetal sheep subjected to high altitude from day 30 of gestation to term (*ca.* 145 days), there is evidence of a fetal origin of cardiac dysfunction. They reported an inability of the chronically hypoxic ovine fetus to maintain cardiac output, secondary to a decrease in the contractile function of myocardial cells (see [45, 58] for review). Similarly, Keller and colleagues using the chick embryo reported that sustained hypoxia during incubation decreased ventricular pressures and ventricular ejection fraction, consistent with depressed systolic function [59]. Therefore, sustained increases in fetal cardiac afterload may not only overwhelm the Frank-Starling mechanism but also the compensatory ventricular hypertrophic growth, eventually leading to hallmarks of heart failure. Therefore, chronic fetal hypoxia is not only an immediate threat to fetal survival, but it is also an important environmental influence triggering asymmetric IUGR and a fetal origin of cardiac as well as pulmonary and peripheral vascular disease.

5 Programming of Cardiovascular Dysfunction in Adulthood by Fetal Chronic Hypoxia

Over the last 5 years, there has been a surge of studies reporting programming of cardiovascular dysfunction in adult offspring of hypoxic pregnancy. The laboratory of Zhang, also at Loma Linda University, in an exceptional series of

investigations has consistently reported that hypoxic pregnancy in rats increases the susceptibility of cardiac ischaemic-reperfusion (I/R) injury at adulthood (see [45, 60] for review). The programming of this cardiac defect at adulthood is associated with the decreased expression of the cardio-protective gene protein kinase C epsilon (PKCε). Further, repression of cardiac PKCε gene expression in fetal rat pup hearts of hypoxic pregnancy is epigenetically regulated [60]. They reported that the increase in the promoter methylation and the reduced expression of the PKCε gene in fetal rat pup hearts of hypoxic pregnancy could be prevented by treatment with a DNA methylation inhibitor. Conversely, treatment of hearts from adult offspring of normoxic pregnancy with a PKCε translocation inhibitor could mimic the defects in hearts of offspring from hypoxic pregnancy. The laboratory of Davidge reported that hypoxia-induced IUGR is associated with the development of chronic cardiopulmonary dysfunction during ageing, identifying a mismatch in glucose metabolism, leading to proton accumulation in the post-ischaemic myocardium of offspring born IUGR as a potential mechanism involved (see [45, 61] for review). Further, they reported that a postnatal high fat diet accelerated cardiac dysfunction in IUGR offspring of hypoxic pregnancy. In addition, Niu and colleagues have reported that hearts of adult offspring of hypoxic pregnancy have in addition a sympathetic dominant phenotype, being more responsive to β_1 adrenergic agonists and less responsive to muscarinic agonists [62]. The latter is of further clinical relevance, as heightened sympathetic excitation with diminished parasympathetic reactivity is an unsustainable situation to maintain cardiac output, and such a cardiac phenotype has been strongly associated with eventual heart failure in humans [63, 64]. Accordingly, exposure of chick embryos, mice and rat pups to prolonged developmental hypoxia promotes dilated cardiomyopathy, with evidence of pump dysfunction that is demonstrable in the offspring and maintained into adulthood (see [45] for review). Other studies in chickens and rodents have consistently reported that hypoxic development can also programme peripheral vascular dysfunction in adulthood, showing defects in the capacity of peripheral vascular resistance vessels to relax during stimulation with NO-dependent dilator agonists, such as with acetylcholine (see [45] for review). Data are also beginning to surface to suggest the programming of an insulin resistant phenotype in adult offspring of hypoxic pregnancy [65–67].

6 Intervention Against Programming of Cardiovascular Dysfunction in Hypoxic Pregnancy

With accumulating evidence pointing towards the programming of a metabolic syndrome by development complicated by fetal chronic hypoxia, which encompasses cardiac sympathetic dominance with increased susceptibility to I/R injury, peripheral endothelial dysfunction and insulin resistance, there is intensifying interest in identifying potential clinical therapy. In recent publications, using an integrative approach at the *in vivo*, isolated organ, cellular and molecular levels, our group has proposed the hypothesis that the molecular basis underlying the programming of cardiovascular dysfunction in the adult offspring of hypoxic pregnancy is through the generation of oxidative stress *in utero* [62]. This offers the exciting prospect that maternal supplementation with antioxidants in pregnancy complicated by fetal chronic hypoxia may protect against the development of cardiovascular disease in the offspring, even before they are born. Accordingly, in a longitudinal study in rats, we reported on the effects of maternal treatment of hypoxic pregnancy with the antioxidant vitamin C on the cardiovascular system of the offspring at the end of gestation and at adulthood. In the fetus, pregnancy under hypoxic conditions promoted aortic thickening with enhanced nitrotyrosine staining and an increase in cardiac HSP70 expression, both robust measures of vascular and cardiac oxidative stress, respectively [62]. By adulthood, offspring of hypoxic pregnancy had *in vivo* evidence of altered baroreflex function,

markedly impaired NO-dependent vasorelaxation in peripheral resistance arteries and a cardiac sympathetic dominant phenotype. Maternal treatment with vitamin C prevented these cardiovascular defects in fetal and adult offspring of hypoxic pregnancy [62, 68]. In addition to antioxidant effects on the fetal cardiovascular system, additional protective effects of maternal treatment with vitamin C on the chronically hypoxic fetus may be due to alterations in the vascular oxidant tone at the level of the placental circulation. We have previously reported that vitamin C enhances umbilical blood flow via NO-dependent mechanisms [38]. Maternal treatment with vitamin C may therefore quench free radicals and increase NO bioavailability, shifting the placental vascular oxidant tone towards dilatation. Antioxidants may thus further protect fetal oxygen delivery by increasing in umbilical blood flow in complicated pregnancy [69, 70].

7 Antioxidant Protection in Hypoxic Pregnancy of Large Mammalian Species

With the perspective of translating the findings in rodent species to the human situation, the British Heart Foundation requested evidence that maternal treatment with antioxidants was protective in fetal and adult offspring of hypoxic pregnancy in larger mammalian species, such as in sheep. In contrast to rodent species, sheep permit surgical instrumentation of the mother and fetus for long-term recording, allowing *in vivo* real time evaluation of the safety of antioxidant therapy on the maternal and fetal physiology as the chronic hypoxic pregnancy is occurring. At the Barcroft building of the University of Cambridge, we have now created four isobaric hypoxic chambers able to maintain chronically-instrumented maternofetal ovine preparations for the duration of pregnancy under controlled hypoxic conditions. The chambers are supplied with nitrogen and air provided by nitrogen generators and air compressors from a bespoke nitrogen generating system (Domnick Hunter Gas Generation, Gateshead, Tyne & Wear, UK). We have also created a wireless data acquisition system (CamDAS, Maastricht Instruments, The Netherlands), which is able to record maternal and fetal arterial blood pressure and blood flow in four circulations, such as the uterine, umbilical, fetal carotid and fetal femoral vascular beds. Exteriorised maternal and fetal catheters and flow probe leads terminate in miniaturised pressure and flow boxes, which are housed in a bespoke jacket worn by the ewe. Electronic signals are then transmitted via bluetooth technology to a laptop sitting outside the chambers, thereby recording continuous maternal and fetal cardiovascular function without interruption of the hypoxic exposure. In this set up, we have taken great care to minimise maternal stress. An additional advantage of this system is that the degree of fetal chronic hypoxia induced is not restricted to the level achieved by ovine pregnancy at 3,500–4,000 m above sea level, which results in fetal arterial PO_2 values of *ca.* 18 mmHg [44]. Consequently, one is able to determine the effects of significant chronic fetal hypoxia, resulting in fetal arterial PO_2 of *ca.* 11–13 mmHg, akin to values reported in human pregnancy complicated by severe IUGR [71]. Using these hypoxic chambers for the first time, our investigations revealed that hypoxic pregnancy, reducing the maternal arterial PO_2 from 107.0 ± 1.5 to 46.8 ± 0.4 and the fetal arterial PO_2 from 22.1 ± 1.1 to 12.0 ± 0.2 mmHg during the last third of gestation in sheep, promoted asymmetric fetal growth restriction, systolic and diastolic cardiac dysfunction in the fetus and significant hypertension at adulthood. Intravenous treatment of pregnant ewes with vitamin C for the last third of gestation prevented these defects in fetal and adult offspring of hypoxic pregnancy (unpublished data).

8 Future Perspectives

While our studies in rodent and ovine pregnancy offer insight to mechanism and thereby closer targets for human clinical intervention against developmental origins of cardiac and peripheral vascular dysfunction in offspring of risky pregnancy, there is a caveat. In both our rodent and

ovine studies, only maternal treatment with vitamin C in very high doses, incompatible with human treatment, are effective. The reason is because the kinetics of the reaction between $^{\bullet}O_2^-$ and NO is so fiercely fast, that for vitamin C to be able to compete *in vivo* with NO for any given concentration of $^{\bullet}O_2^-$, its concentrations must exceed that of NO by a factor of 100,000. Therefore, for vitamin C to be able to work as an antioxidant *in vivo*, its concentrations would need to be *ca.* 10,000 μmol l^{-1} or 10 mmol l^{-1} to prevent the interaction between $^{\bullet}O_2^-$ and NO [29, 62]. This may explain the inability of maternal treatment with vitamin C to protect against placental vascular oxidative stress in preeclamptic pregnancy in all reported clinical trials to date [72, 73]. Interestingly, in all trials, the dose of maternal vitamin C administration was 1 g per pregnant woman per day, yielding circulating maternal concentrations of vitamin C of ~120 μmol l^{-1}, far short of the 10,000 μmol l^{-1} range required for the antioxidant vitamin to be effective *in vivo*. Since excessive doses of vitamin C promote oxaluria leading to the formation of kidney stones and several studies have suggested pro-oxidant activity of ascorbic acid with pharmacological doses even in healthy pregnancy [74, 75], there is increasing interest in alternative antioxidant therapies, compatible with human treatment. One interesting candidate is a mitochondrial-targeted antioxidant. Mitochondria are a major site of reactive oxygen species (ROS) production, therefore protecting them from oxidative damage should be one of the most effective antioxidant therapeutic strategies. However, conventional antioxidants are ineffective because they cannot penetrate the mitochondria [76]. Part of the problem relates to the difficulty of delivering antioxidants to mitochondria *in situ*. Dr Mike Murphy from the Mitochondrial Biology Unit, Addenbrookes Hospital, Cambridge and colleagues have developed the mitochondrial targeted antioxidant MitoQ that overcomes the problem. MitoQ consists of a quinone moiety covalently attached to a triphenylphosphonium (TPP) cation. The TPP cation is lipophilic, and its positive charge results in the 100–1,000-fold accumulation of MitoQ in the mitochondrial matrix, driven by the negative trans-membrane potential. Once inside the matrix, MitoQ is reduced by complex II in the respiratory chain to the active quinol form of the antioxidant. This will react with any ROS present, recycling MitoQ back into the quinone form and removing the ROS. Initial experiments with MitoQ demonstrated its ability to prevent lipid peroxidation in cell culture [77]. The benefits of MitoQ have now been revealed in a range of *in vivo* studies in rats and mice and in two phase II human trials [76, 78]. For instance, Dominiczak and colleagues showed elegantly that in vivo treatment with MitoQ of stroke-prone spontaneously hypertensive rats reduced arterial blood pressure, cardiac hypertrophy and improved NO-dependent endothelial function [78]. In contrast to vitamin C and other conventional antioxidants, MitoQ demonstrates no pro-oxidant activity and long-term administration to mice for 28 weeks revealed no toxic effects [76, 79]. However, the antioxidant benefits of MitoQ in risky pregnancy of any species await investigation. Future studies in our laboratory will determine the protective effects of MitoQ against the programming of cardiovascular disease in pregnancy complicated by fetal chronic hypoxia in small and large animal models. This offers exciting potential for human clinical intervention.

Acknowledgements Dino Giussani is Professor of Cardiovascular Physiology & Medicine at the Department of Physiology Development & Neuroscience at the University of Cambridge, Professorial Fellow and Director of Studies in Medicine at Gonville & Caius College, a Lister Institute Fellow and a Royal Society Wolfson Research Merit Award Holder. He is supported by the British Heart Foundation, The Biotechnology and Biological Sciences Research Council and the Isaac Newton Trust.

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Fetal Breathing Movements and Changes at Birth

8

Brian J. Koos and Arezoo Rajaee

Abstract

The fetus, which develops within a fluid-filled amniotic sac, relies on the placenta for respiratory gas exchange rather than the lungs. While not involved in fetal oxygenation, fetal breathing movements (FBM) nevertheless have an important role in lung growth and in development of respiratory muscles and neural regulation. FBM are regulated differently in many respects than postnatal respiration, which results from the unique intrauterine environment. Prominent distinctions of FBM include its episodic nature and apnea-sensitivity to hypoxia. The latter characteristic is the basis for using FBM in the assessment of fetuses at risk for hypoxic injury. At birth, the transition to continuous postnatal respiration involves a fall in temperature, gaseous distention of the lungs, activation of the Hering-Breuer reflexes, and functional connectivity of afferent O_2 chemoreceptor activity with respiratory motoneurons and arousal centers. Importantly, exposure to drugs or adverse conditions *in utero* not only can change patterns of FBM but also can lead to epigenetic dysregulation in postnatal respiration. Such changes, can blunt respiratory and arousal defenses against hypoxic challenges in sleep. Thus, fetal hypoxia and/or drug exposure may in later life dispose sleeping infants, children, and adults to hypertension, diabetes mellitus, brain injury, and sudden death.

Keywords

Brain • Carotid body • Fetus • Hypoxia • Newborn • Respiration • Sleep

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L. Zhang and C.A. Ducsay (eds.), *Advances in Fetal and Neonatal Physiology*, Advances in Experimental Medicine and Biology 814, DOI 10.1007/978-1-4939-1031-1_8,

> When an animal is born and begins its struggle towards an independent existence, its first efforts are those of breathing. Breathing is living; the onset of respiration is the beginning of life.
>
> —D. H. Barron

The transition from fetal to postnatal life critically depends on a timely transfer of respiratory gas exchange from the placenta to the newborn's lungs. Thus, it is not surprising that fetal respiratory muscles are exercised *in utero*. Besides conditioning respiratory muscles, fetal breathing movements (FBM) contribute to normal lung differentiation and growth and likely to the development of neural circuits involved in respiratory control.

FBM have unique characteristics that suggest they are regulated differently than breathing in the neonate or adult. These distinctive features of FBM are largely an appropriate adaptation to the intrauterine environment. Despite these differences, FBM respond similarly to many modulators of postnatal respiration. A clinically important discovery is that exposure to drugs or adverse conditions *in utero* not only can alter FMB but also can lead to epigenetic changes in postnatal respiratory control. This chapter reviews the progress in our understanding of respiratory regulation in the fetus and its changes at birth.

1 History

That the fetus is able to use its respiratory muscles has been known for some time. In the nineteenth century, breathing movements were observed in fetal guinea pigs within amniotic sacs immersed in warm saline [1]. In 1936, breathing activity reportedly occurred in rabbit fetuses that were released into warm Ringer's solution [2]. FBM have also been visualized *in vivo* through intact uteri of rats, rabbits, and dogs [2, 3].

Spontaneous breathing movements did not occur in mature fetal sheep that were delivered by hysterotomy under spinal anesthesia on a warm table with an intact umbilical circulation [4, 5]. But FBM have been observed through the transilluminated uterus [5, 6]. In 1936, Sir Joseph Barcroft and Donald Barron observed FBM in young ovine fetuses (<0.4 term) delivered under spinal anesthesia in a warm saline bath [4]. Spontaneous bursts of respiratory activity have been reported in older fetuses (~0.47–0.66 term) under similar conditions [7].

Breathing efforts in human fetuses have been observed as early as 12 weeks of gestation upon disruption of placental gas exchange [6]. That FBM normally occur in human fetuses was suggested by German obstetricians who detected rhythmic movements on the gravid abdomen at a rate (~40–60/min) that was distinct from that of maternal respiration (~15/min) and pulse (~80/min) [8, 9].

These observations established that the fetus has the ability to exercise respiratory muscles, but it was not known whether this activity was spontaneous or elicited by asphyxia or stimulation [5, 6]. This gap in knowledge regarding FBM persisted through the late 1960s [5]. The normality of FBM was established in 1970 with the development of the PO_2 electrode and the chronically catheterized fetal sheep preparation [10]. The subsequent introduction of ultrasound techniques in obstetrics allowed the detection of FBM in human fetuses [11–14].

2 Sheep Fetus

2.1 Characteristics

Much of what is known about FBM has come from studies in chronically catheterized fetal sheep [10, 15, 16]. FBM (>0.8 term) primarily involve diaphragmatic excursions, which reduce intrathoracic pressure by ~3–4 mmHg. Each breathing movement is associated with a negligible change (<1.0 ml) in lung volume due to the high viscosity of liquid (relative to air), upper-respiratory-tract resistance, and relatively short inspiratory times. Although lung volume remains virtually unchanged for a single breath, it can be reduced significantly over an episode of FBM because of a net efflux of fluid that results from laryngeal dilatation [17]. Apneic periods are associated with reduced efflux of fluid that is continuously secreted by lungs (~4 ml/kg/h) due to laryngeal constriction and pulmonary elastic recoil.

The result is an increase in lung volume and intrathoracic pressure [17]. Other differences from postnatal respiration include high rates (as high as four breaths/s) and episodic occurrence [10].

2.2 Behavioral States

Breathing episodes of ~10–30 min duration typically occur in cycles alternating with apneic periods. Overall, FBM are present 20–30 min/h. FBM (>0.8 term) characteristically occur in low-voltage electrocortical (ECoG) states associated with rapid-eye movements (REM). The peak incidence and amplitude of FBM occur at 1900–2100 h with a nadir at 0400–0900 h. The circadian variability in fetal breathing incidence coincides with diurnal changes in the incidence of low-voltage ECoG, which is apparently entrained by the maternal melatonin rhythm [18, 19]. Thus, the changes in breathing incidence over time coincide with sleep-state cycles as determined by measurements of ECoG, REM, and nuchal muscle activity.

Fetal behavioral states (>8 term) are similar to REM (low-voltage ECoG, REM, absent nuchal muscle tone) and quiet (high-voltage ECoG, absent REM, nuchal muscle activity) sleep. Wakefulness has not been rigorously established. FBM can be detected as early as 40–50 days of gestation (~0.3 term). Prior to differentiation of electrocortical activity (~0.8 term), FBM occur almost continuously, with apneas generally less than 2 min. FBM become episodic with maturation of the forebrain and the development of high-voltage ECoG. FBM do not occur in high-voltage ECoG even when breathing is stimulated by hypercapnia. The apnea of high-voltage ECoG may involve inhibition of neurons that generate the respiratory rhythm or depression of medullary and/or spinal motoneurons that regulate respiratory muscles.

2.3 Glucose

The incidence of breathing activity is greatly reduced in hypoglycemic fetuses of ewes undergoing prolonged fasting [10]. In contrast, hyperglycemia does not significantly alter the occurrence of FBM in this species [20].

2.4 Respiratory Gases and pH

Normal fluctuations of fetal PaO_2, $PaCO_2$, and pH do not affect the incidence or amplitude of FBM [10]. However, greater changes significantly alter FBM [10, 15, 16].

2.4.1 Carbon Dioxide

A sustained rise in fetal $PaCO_2$ of 5–10 mmHg increases the incidence of FBM by stimulating REM states. Hypercapnia also increases the amplitude and regularity of breathing but fails to elicit FBM in high-voltage ECoG. Hypocapnia diminishes breathing without altering the normal cycling of REM or ECoG.

2.4.2 Oxygen

Isocapnic hypoxia inhibits FBM in a dose-dependent manner once the average end-capillary PO_2 of the fetal brain falls below 13 Torr, as shown in Fig. 8.1. Hypoxia does not alter amplitude or cycle time, which indicates that the depression results from a reduction in the behavioral state coincident with FBM. The inhibitory effects of hypoxia on FBM and REM, relative to

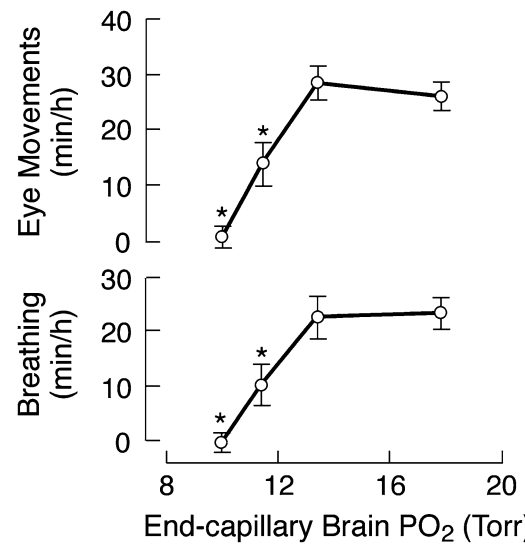

Fig. 8.1 Effects of acute graded isocapnic hypoxia on the incidence of fetal rapid-eye movements (REM) and breathing activity (FBM). Incidence is shown as a function of the calculated effective PO_2 at the end of an average brain capillary. The incidence of low-voltage electrocortical activity (not shown) was not significantly affected under these conditions. *Vertical bars* represent SE. Modified from Koos et al. [21]

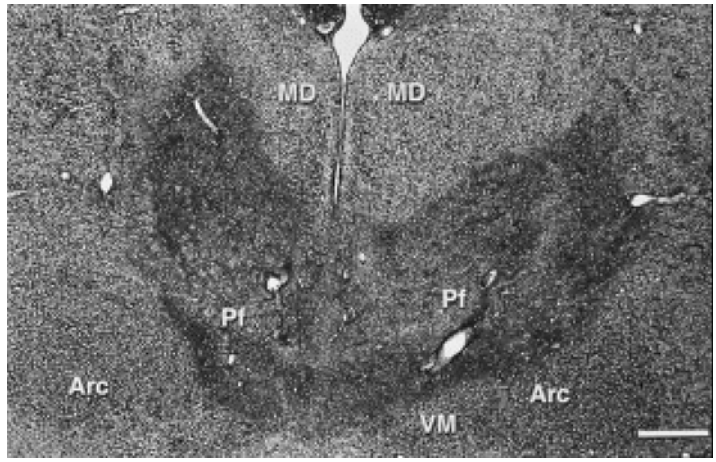

Fig. 8.2 Photomicrograph of Nissl-stained transverse section of the fetal sheep thalamus depicting a lesion that abolished hypoxic inhibition of FBM. The intensely *gray regions* represent neuronal destruction and gliosis induced by ibotenic acid microinjections. *Arc* arcuate n., *MD* mediodorsal n., *Pf* parafascicular n. (includes n. centrum medianum), *VM* ventromedial n. The magnification bar = 1 mm. Reproduced from Koos et al. [22] with permission of the American Physiological Society

fetal brain PO_2, are virtually identical [21]. But this level of fetal O_2 deprivation does not significantly reduce the incidence of low-voltage ECoG [21]. Disruptions of the pons and/or midbrain abolish the depressant effects of hypoxia, which indicates that an O_2-sensitive locus rostral to the medulla triggers the inhibition.

We have identified a thalamic sector as being a crucial rostral component of the neuronal circuitry involved in hypoxic depression of FBM [22, 23]. This critical locus lies within and/or immediately adjacent to the parafascicular nuclear complex (Pf), as shown in Fig. 8.2. Pf is associated with increased cortical discharge rates in desynchronized states of wakefulness and REM. Thus, the involvement of Pf in hypoxic inhibition is consistent with the hypothesis that acute, moderate hypoxia indirectly depresses FBM through affects on behavioral state (e.g., phasic REM).

The fall in breathing incidence elicited by moderate hypoxia is transient, with a return to normal levels within 4–12 h of O_2 deprivation. Such recovery does not occur with greater O_2 deficiency accompanied by a progressive fall in arterial pH [16].

Severe hypoxia can induce low-frequency, deep inspiratory efforts that resemble gasping. These strong respiratory efforts can increase lung liquid volume by 10–30 ml [10, 24].

Raising mean fetal PaO_2 from 26 to 47 Torr through hyperbaric oxygenation promotes arousal and the percentage of REM coincident with breathing activity [25]. But increasing fetal PaO_2 to ~65 Torr for up to 19 h through extracorporeal membrane oxygenation does not alter the incidence of high-voltage ECoG or FBM [26]. Thus, hyperoxia does not change the episodic nature of FBM.

2.5 Chemoreceptors

2.5.1 Brain

2.5.1.1 Hydrogen Ions

Central H^+ chemoreceptors modulate FBM, as illustrated by the augmentation in breath amplitude with acidic cerebroventricular perfusion [27, 28]. FBM remain episodic despite the stimulation. Denervation of the peripheral arterial chemoreceptors has no significant affect on hypercapnia-induced hypernea, which also supports central H^+ chemosensitivity [16].

2.5.1.2 Oxygen

That the depressant effects of hypoxia on FBM and REM are mediated by a central O_2 sensor is supported by several findings: (1) the inhibitory effects of O_2 deficiency are dose-dependent ([21], Fig. 8.1); (2) sinoaortic denervation does not

eliminate the depressant effects of hypoxia [29]; and (3) supramedullary disruptions of the brainstem abolish hypoxic inhibition [10, 28, 30]. In more recent studies, we have identified a locus encompassing Pf in the posteromedial thalamus as the crucial rostral component of the neuronal circuitry that mediates hypoxic inhibition of FBM [22, 23, 31]. The O_2 chemosensory mechanism involves a hypoxia-induced rise in brain adenosine (ADO) concentrations that activate central ADO A_{2A} receptors [31–34]. This central O_2 sensor likely activates a multisynaptic inhibitory pathway that projects ultimately to medullary respiratory neurons.

2.5.2 Peripheral Arterial Chemoreceptors

The fetal carotid bodies are sensitive to both CO_2 and O_2 [35]. That peripheral arterial chemoreceptors facilitate FBM under normal conditions *in utero* is suggested by the reduced incidence and amplitude of FBM with ablation of afferent input from the carotid and aortic chemoreceptors [29, 36]. But sinoaortic denervation does not significantly alter the depressant effects of hypoxia on FBM, although it does increase the lag time from the initiation of O_2 deprivation to the onset of inhibition [29]. Thus, the peripheral arterial O_2 sensors are not primarily involved in eliciting the depressant effects of hypoxia, although they do trigger cardiovascular chemoreflexes in acute O_2 deprivation [31].

Brain lesions can unmask the normal hypoxic hyperpnea that is observed postnatally. For example, hypoxia increases the rate and amplitude of breathing movements in fetuses with pons/midbrain disruptions that eliminate hypoxic inhibition [10, 28, 30]. This respiratory chemoreflex is mediated by carotid body O_2 sensors and involves activation of ADO A_{2A} receptors [31–33, 37, 38], as depicted in Fig. 8.3. Thus, in normal fetuses, hypoxia activates the carotid bodies, but excitatory input to respiratory neurons in the medulla appears to be blocked by supramedullary brainstem sectors

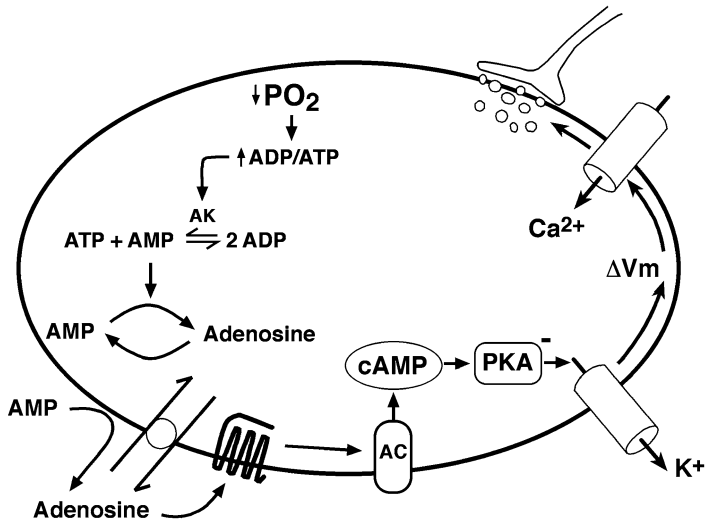

Fig. 8.3 Proposed transduction mechanism involved in O_2 sensing by the fetal carotid body. A hypoxia-induced fall in mitochondrial oxidative phosphorylation activates adenylate kinase, which catalyzes the conversion of two molecules of ADP into ATP and AMP. The resulting rise in the AMP/ATP ratio leads to the intra- and extra-cellular formation of adenosine from AMP. Intracellular adenosine is transported across the cell membrane into the extracellular space. Adenosine subsequently binds to extracellular A_{2A} receptors, which triggers a transduction cascade involving activation of adenylyl cyclase (AC), formation of cAMP, inhibition of potassium currents, decrease in membrane potential (ΔVm), and activation of voltage-gated Ca^{2+} channels. The resulting rise in intracellular Ca^{2+} levels elicits secretion of neurotransmitters that excite the carotid sinus nerve. Reproduced from Koos [31] with permission of the American Physiological Society

[16, 31, 35]. More rostral lesions involving thalamic Pf (Fig. 8.2) abolish the depressant effects of hypoxia on FBM without altering rate or amplitude [22].

In contrast to postnatal effects, hypoxia does not induce arousal in the fetus. This unresponseness is appropriate because an arousal-induced rise in motor activity would not restore oxygenation and in fact would be counter-productive by increasing O_2 consumption. Thus, afferent traffic from the carotid bodies appears to be gated out of central arousal mechanisms as well as respiratory motoneurons.

2.6 Hering-Breuer Reflex

Hering-Breuer inflation reflex in fetal sheep can be induced experimentally, confirming that the involved pulmonary mechanoreceptors and afferents are functional. Under normal conditions *in utero*, the minimal changes in lung volume per breath (<1 ml) are insufficient to activate the stretch receptors in pulmonary smooth muscle and epithelium [10].

2.7 Continuous FBM

The normal episodic pattern of FBM can become continuous through several interventions. For example, fetal administration of cyclo-oxygenase inhibitors, such as indomethacin, evokes prolonged regular, deep breathing efforts that persist through high-voltage ECoG. But REM and ECoG activity continue to cycle normally [39]. The effects on FBM result from inhibition of prostaglandin production in the fetal brainstem [28]. Cooling is a powerful respiratory stimulus. In exteriorized and apneic fetuses, lowering fetal temperature by forcing airflow over wet skin evokes prolonged regular breathing activity [5]. Cutaneous cooling has also been shown to induce FBM in high-voltage ECoG activity in chronically catheterized fetuses *in utero* [40]. Increasing fetal PaO_2 to 40–60 Torr by distending the lungs with 100 % O_2 by continuous positive airway pressure can elicit arousal and FBM in high-voltage ECoG, although breathing generally remains episodic. In older fetuses (>0.9 term), increasing fetal PaO_2 to ~65 Torr by a similar technique evokes arousal and continuous fetal breathing that depends on intact vagal nerves [25, 41, 42]. FBM becomes nearly continuous several days after section of the pons/midbrain [10]. These latter findings are consistent with the involvement of thalamus in sleep-state regulation of FBM.

3 Human Fetus

3.1 Characteristics

In the human fetus, breathing movements can be detected by ultrasound imaging of the paradoxical motion of the sternum and abdominal wall. FBM can also be detected by Doppler velocimetry of umbilical venous blood flow and by color Doppler detection of nasal and tracheal flow [16]. The incidence of FBM is ~2 % of the time at 10 weeks of pregnancy and increases with gestational age, with median values of ~12 % at 20–22 weeks and ~35 % at 30–32 weeks of gestation

3.2 Behavioral States

The human fetus exhibits recurrent, nonrandom behaviors in which the defining coincident behavioral components change at virtually the same time. Four behavioral states have been identified on the basis of eye movements, gross body movements, FBM, and heart rate accelerations, which are more clearly evident after 36 weeks of gestation with the emergence of synchronized patterns of behavior [43]. In the human fetus, quiet-active cycles are present by 24–28 weeks of gestation. REMs develop by ~20 weeks' gestation, while non-REM episodes emerge at 28–31 weeks. At 36–40 weeks' gestation, REMs are present ~60 % of the time. Between 28 and 36 weeks, FBM become increasingly correlated with REM and less associated with non-REM states. By 36 weeks, FBM are more commonly present in active 2F states, which

are characterized by the coincidence of eye movements, gross body movements, irregular FBM, and heart rate accelerations. Less-frequent FBM occur in quiet (non-REM) 1F states, which are defined by absence of eye movements, regular FBM, and incidental gross body movements and heart rate accelerations [43, 44].

Negative intrathoracic pressures generated by fetal inspiratory efforts can induce circulatory changes that significantly alter pressure waveforms in the umbilical vein and inferior vena cava (IVC), reduce flow in the inferior vena cava, and modulate Doppler velocimetry of the umbilical and middle cerebral arteries [45, 46].

3.3 Glucose

In the human fetus, FBM become sensitive to changes in glycemia in the second half of gestation. FBM occur more frequently after maternal meals or glucose ingestion. As a result, the maximum duration of apnea periods decrease to <45 min [47, 48]. Gravidas with gestational diabetes have longer episodes of FBM, which likely result from greater fetal glycemia [49]. The increase in breathing incidence is not associated with alterations in breath interval or amplitude. Thus, the rise in breathing incidence is due to factors other than direct effects on the central chemoreceptors or respiratory neurons. A glucose-independent rise in the incidence of FBM occurs in early-morning hours (0400–0700 h).

3.4 Respiratory Gases

Changes in maternal $PaCO_2$ also modulate the incidence of breathing movements in the human fetus. Hypercapnia increases the incidence, while hypocapnia reduces it. Such changes in $PaCO_2$ do not alter somatic activity. FBM are more sensitive to the inhibitory effects of hypoxia than are limb or body movements [50]. Severe O_2 deprivation induces deep, gasping-type efforts that likely account for the aspiration of meconium and other amniotic-fluid debris in asphyxiated fetuses and stillbirths [6, 10].

3.5 Respiratory Depressants

Compared to postnatal respiration, FBM have greater sensitivity to central-nervous-system depressants, such as alcohol and opiates. Reduced breathing activity can also accompany chorioamnionitis as well as induction of labor with prostaglandins. As in sheep, the incidence of FBM declines prior to the onset of labor [16].

4 Birth Transition

The transition from fetal to postnatal breathing involves circulatory changes, absorption of lung liquid, gaseous expansion of the lungs, activation of Hering-Breuer reflexes, and the onset of continuous respiration. Factors implicated in establishing continuous respiration at birth include asphyxia, vagal afferent traffic related to respiratory/circulatory transitions, umbilical cord occlusion, cutaneous cooling, and the rise in PaO_2 with the first postnatal breath [5]. The onset of continuous respiration at birth depends neither upon afferent input from the carotid sinus and vagus nerves [51] nor interruption of the umbilical circulation [52]. Furthermore, it can occur in noncyanotic (non-asphyxiated) human infants or following a prolonged single inspiratory effort or asphyxial gasping [53]. Thus, it is unlikely that a single factor initiates continuous respiration at birth.

5 Postnatal Respiration

5.1 Characteristics

The high median respiratory rates (~40 breaths/min) of the neonate fall progressively with advancing age. Tidal volume (~6 m/kg) remains virtually the same from birth to adulthood. In early life, higher ventilation requirements are generally met with an increase in respiratory frequency rather than tidal volume. The newborn's respiratory system differs from that of the adult with respect to the upper airways, chest-wall properties, and lower resting functional reserve capacity [54].

5.2 Sleep States

Minute ventilation falls ~10 % in sleep compared to the awake state. The regular respiration found in non-REM sleep is governed primarily by metabolic stimuli, which principally involve CO_2. In contrast, respiration in REM sleep is irregular with variable rate and tidal volume [54]. During postnatal development, respiratory control in sleep changes with respect to ventilatory drive, respiratory mechanics, neuromotor activity, and arousability [54–57]. Compared to term infants, preterm infants respire more irregularly in sleep and are disposed to periodic breathing (5–10 s apneas) and apneas (>20 s apnea) in wakefulness, REM, and quiet sleep. Apneas are more frequent and severe in REM than in non-REM sleep. Both periodic breathing and apnea can significantly impair oxygenation and elicit brain injury [58].

5.3 Glucose

Alterations in glycemia within the normal range do not affect respiration.

5.4 Respiratory Gases

5.4.1 Carbon Dioxide

Inhaled CO_2 increases ventilation in newborn infants. The respiratory response in the newborn is similar to that of the adult on a per-kg bodyweight basis [58].

5.4.2 Oxygen

Normal respiratory response to hypoxia involves an initial stimulation triggered principally by the carotid chemoreflex, which is followed by a decline or roll-off in ventilation despite maintenance of low PaO_2 [15, 31]. In human infants, a decrease in frequency rather than tidal volume largely accounts for the fall in ventilation [58]. In early preterm infants, hypoxia has virtually no stimulatory effect on respiration while eliciting a pronounced respiratory depression [58]. A biphasic respiratory response to hypoxia can also be demonstrated in adults, although the roll-off is less pronounced than in newborns [15, 31].

5.5 Chemoreceptors

5.5.1 Brain

5.5.1.1 Hydrogen Ions

Central H^+ sensors modulate respiration in the newborn. For example, hypercapnia increases ventilation in awake lambs with denervated carotid bodies [59]. *In vitro* studies in newborn rats have demonstrated the CO_2/H^+ sensitivity of pre-inspiratory neurons [60].

5.5.1.2 Oxygen

Although other factors may be involved, the principal mechanism for the second-phase fall in ventilation appears to involve central inhibition elicited by a fall in brain PO_2 [15, 31]. The roll-off response to hypoxia in neonatal and adult mammals is attenuated by non-selective blockade of ADO receptors [15, 31]. Our studies in developing lambs have shown that activation of central ADO A_{2A} receptors triggers hypoxic ventilatory depression [61], as shown in Fig. 8.4. Thus, as in the fetus, ADO A_{2A} receptors are critically involved in the central O_2 sensory mechanism that depresses breathing in O_2-deficient states. These experiments do not exclude the potential involvement of other neurotransmitters or modulators.

Studies in several species have identified sectors in the rostral brainstem that are involved in the roll-off. But evidence is emerging that diencephalon is involved as well. As an extension of our previous work in the fetus, preliminary mapping studies indicate that a thalamic locus involving Pf is a crucial component of the neuronal substrate mediating the roll-off [62]. This novel observation is consistent with the posteromedial thalamus playing a crucial role in respiratory depression in both fetal and postnatal life.

5.5.2 Peripheral Arterial Chemoreceptors

The rise in PaO_2 at birth initially silences afferent traffic in the carotid sinus nerves. After this transition, O_2 responsiveness of carotid O_2 sensors increases with maturation [35, 55]. Although not required for initiating continuous breathing, the peripheral arterial chemoreceptors critically support neonatal respiration. For example, bilateral

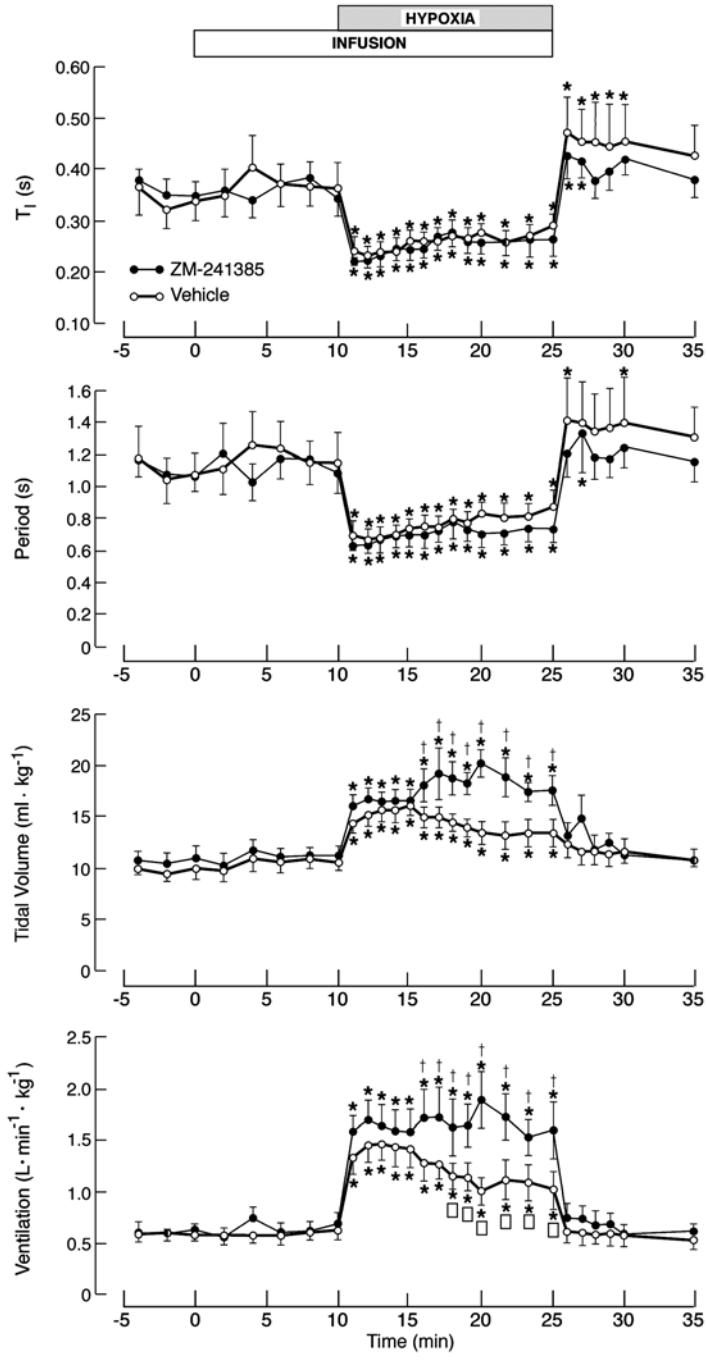

Fig. 8.4 Effects of isocapnic hypoxia on respiration in developing lambs. Inspiratory time (T_I), respiratory period, tidal volume, and minute ventilation are shown during infusion of vehicle (*closed circles*). The ventilatory response is biphasic with initial stimulation followed by a roll-off. Blockade of central adenosine A_{2A} receptors (*open circles*) abolishes the roll-off. *$P<0.05$ compared to 10 min. †$P<0.05$ compared to vehicle. $P<0.05$ compared to peak minute ventilation at 12–13 min. Reproduced from Koos et al. [61] with permission of the American Physiological Society

carotid denervation results in hypoventilation, prolonged apnea, and a high rate of mortality. But respiratory drive in adults is much less dependent on carotid sinus nerve activity [55]. In developing lambs, activation of ADO A_{2A} receptors is not significantly involved in hypoxic excitation of the carotid bodies, although it modulates O_2 sensitivity in other species [61]. With respect to CO_2, carotid sinus nerve discharges in response to hypercapnia increase with age, although the ventilatory response is unaffected [55].

5.6 Hering-Breuer Reflex

The Hering-Breuer inflation reflex is more prominent in the newborn than in later life. In fact, small increments in lung volume can induce apnea [58].

6 Summary

Table 8.1 compares factors involved in breathing movements in fetal sheep with respiration in developing lambs. As in postnatal respiration, FBM are stimulated by hypercapnia and central acidosis, modulated by sleep states, and inhibited by central O_2 sensors that involve ADO A_{2A} receptors. Hypoxia also increases discharges in the fetal carotid sinus nerve in a dose-dependent manner. In contrast, FBM differ with respect to inhibition by exteriorization and restraint, episodic occurrence *in utero*, primary dependence on sleep or behavior-related stimuli, negligible tidal volume, glucose sensitivity, inactive Hering-Breuer reflexes, extent of A_{2A} receptor involvement in carotid O_2 chemoreception, and lack of hypoxic hyperpnea. The latter has been attributed to central gating that prevents integration of afferent O_2 chemosensory activity into respiratory drive as well as arousal. Thus, the transition to postnatal breathing involves a fall in temperature, gaseous distention of the lungs, activation of Hering-Breuer reflexes, a rise in PaO_2 with subsequent blunting of carotid O_2 sensitivity, functional connectivity of afferent O_2 chemoreceptor activity with respiratory motoneurons and arousal centers, and the onset of continuous respiration.

Table 8.1 Comparison of fetal breathing and postnatal respiration in sheep

	Fetus	Developing lamb
Occurrence	Episodic	Continuous
Sleep state	Modulates	Modulates
Glycemia	Modulates	No effect
Lungs		
Distending medium	Fluid	Air
Lung liquid volume	50 ml/kg	
Functional residual capacity		20–30 ml/kg
Respiratory rate	~60/min	~60/min
Tidal volume	0.5 ml/kg	~10 ml/kg
Hering-Breuer reflexes	Inactive	Active
Blood gases and pH		
Normal fluctuations	No effect	Modulate
Hypercapnia	Stimulates	Stimulates
Hypocapnia	Inhibits	Inhibits
Central acidosis	Stimulates	Stimulates
Hypoxia	Inhibits	Stimulates/ inhibits
Carotid body O_2 sensor		
Neuromodulator	Adenosine (ADO)	Not established
Receptor	ADO A_{2A}	Not established
Central O_2 sensor		
Neuromodulator	Adenosine	Adenosine
Receptor	ADO A_{2A}	ADO A_{2A}
Neuronal substrate	Thalamic Pf	Thalamic Pf

7 Clinical Implications

A number of conditions can restrict O_2 supply to the fetus, including decreased maternal arterial O_2 content, reduced uteroplacental perfusion, placental dysfunction, disruption of the umbilical circulation, and various fetal disorders [31]. Commonly recognized causes of fetal O_2 deprivation include high altitude, maternal vascular diseases, uterine contractions, placental separation, and umbilical cord compression.

FBM are substantially reduced or abolished by acute fetal hypoxia. The incidence of FBM is also decreased in chronic O_2 deficiency associated with fetal growth restriction. Thus, ultrasound detection of FBM remains a key component of the biophysical profile that is used clinically to assess the wellbeing of fetuses potentially at risk for hypoxic injury. Biophysical profiles are optimally performed 2 h after maternal meals when FBM are more likely to occur within the 30 min epoch of observation.

Recent reports have implicated maternal sleep-disordered breathing and upper-airway obstruction as etiologic factors in intermittent fetal hypoxia. Such respiratory disruptions

have been linked to snoring, obstructive sleep apnea, chronic hypertension, gestational hypertension, preeclampsia, pulmonary hypertension, nocturnal asthma, and panic attacks [63–67]. Thus, repeated bouts of hypoxia are likely to be more prevalent in the fetus than previously realized.

Sustained or intermittent hypoxia or drug exposure (e.g., nicotine) *in utero* may result in epigenetic dysregulation of postnatal respiration that alters responses to hypoxia and may impair respiratory and arousal defenses to hypoxic challenges in sleep, such as the ability to clear an upper-airway obstruction [31]. The resulting functional disruptions in the peripheral and/or central O_2 sensors and their central integration may blunt arousal and respiratory responses to hypoxia. Thus, fetal hypoxia or drug exposure may in later life dispose sleeping infants, children and adults to hypertension, diabetes mellitus, brain injury, and sudden death [31, 68, 69].

8 Future Studies

Several important gaps remain regarding the regulation of FBM. These include understanding neuronal mechanisms relating to: (1) inhibition in non-REM states, (2) hypoxia-induced gating out of carotid sinus nerve activity from respiratory motoneurons, and (3) central inhibition in O_2 deficiency. Inhibition of FBM in both non-REM states and hypoxia potentially involves rhythm-generating neurons, upper respiratory motoneurons, and phrenic motorneurons. But the integrated role of each in central respiratory depression in both conditions needs to be established. With respect to central O_2 sensors, studies should be performed (1) to identify the locus of ADO A_{2A} receptors that trigger inhibition, and (2) to establish whether thalamic Pf neurons depress FBM through excitatory or inhibitory projections to brainstem respiratory neurons.

Acknowledgment Supported in part by National Institute of Child Health and Human Development Grant HD-18478

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From Fetal Physiology to Gene Therapy: It All Started in Loma Linda

9

Ronald H.W. Lorijn

Abstract

Just having finished medical school in the Netherlands, without basically any serious research experience, Lawrence Longo, Gordon Power and Ray Gilbert received this 25 year young man with open arms at the end of July 1977. The Center for Perinatal Biology of Loma Linda University (CPB) would become for the next 3 years not the just the center of my postdoctoral activities, it would also lay the foundation for the following decades. The next paragraphs will describe three key success factors that can be traced back to these formative years that have contributed so much to my professional career.

Keywords

Fetal physiology • Postdoctoral fellowship • Key success factors

Just having finished medical school in the Netherlands, without basically any serious research experience, Lawrence Longo, Gordon Power and Ray Gilbert received this 25 year young man with open arms at the end of July 1977. The Center for Perinatal Biology of Loma Linda University (CPB) would become for the next 3 years not the just the center of my postdoctoral activities, it would also lay the foundation for the following decades. The next paragraphs will describe three key success factors that can be traced back to these formative years that have contributed so much to my professional career.

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1 Lawrence D. Longo the Teacher

Trying to have this student not to lose heart at the start of his fellowship, Larry told me what his professor of Physiology at Penn State University had told him at the time Larry applied at his department: "You're an obstetrician/gynecologist, Mr. Longo, and you can read and write?" As it turned out, not only could Larry read and write, he proved during my stay that he could educate a somewhat ignorant student from the Low Countries what basic research was all about.

L. Zhang and C.A. Ducsay (eds.), *Advances in Fetal and Neonatal Physiology*,
Advances in Experimental Medicine and Biology 814, DOI 10.1007/978-1-4939-1031-1_9,

Having been assigned a study cubicle in the room just across the lab, my workplace and soon thereafter my activities were clear for the next 3 years. A huge stack of research papers welcomed me on my first day. Larry, Gordon and Ray were there to answer any questions. The focus of the first couple of weeks was to get acquainted with the basic publications on the transport of oxygen across the placenta. For this would become the subject of my research project. To be more specific: fetal oxygen consumption and placental oxygen exchange. Besides recent papers related to this topic, several historical publications had been added too. This allowed me to understand much better the way science had evolved over the years in its apprehension on how the fetal metabolism interacted with its maternal source. Papers by Barcroft [1] and Dawes [2] were key in that sense.

Larry's passion for history is an additional element that adds so much to his qualities as a teacher. An excellent teacher knows the flow of history and how it connects with his field of expertise. On top he has the skills to propagate this to his pupils in a natural way. To have the skills to put current know-how in the context of the past searches for answers on crucial questions in life is of enormous value to students. For, the lessons (in terms of the reasoning, assumptions and study designs) one can draw from investigations done by those who lead us in unraveling the physiologic processes form the basis of today's research. In Larry's own words: "*It is important for physicians to have an appreciation of from whence they have come.*" Larry's publication list contains a substantial number of papers describing important historical contributions and celebrities in the field of medicine. As an example I like to refer to the impressive compilation of historical, innovative papers that contributed to obstetrics and gynecology, which Larry together with my later Dutch tutor, the late Professor Tom Eskes, published in 1994 [3].

Two other key components in Larry's teaching that stood out and became important for me later in life, when I was given the task to teach others, were the freedom and responsibility he trusted me with. First during my years as obstetrician/gynecologist training residents and later as a manager in the biotechnology industry leading teams I learned that the meaning of "leading by example" includes handing over responsibilities and showing one's confidence by allowing people to execute their task.

2 Lawrence D. Longo the Scholar

Inseparably connected with and an integral part of the Teacher is the Scholar Larry. Searching for places of excellence where I could do a postdoctoral fellowship in fetal physiology Tom Eskes suggested to write a letter to Larry. At the time I contacted Larry his department at LLU just existed 4 years, but thanks to its publications had already achieved a reputation as a dynamic and innovative group of researchers in this field. Within a fortnight the answer came back: you are more than welcome, but please bring your own financial support as we have only limited resources at this time. For a young, enthusiastic and somewhat naive graduate in medicine this response was the best he could have hoped for. For, finding the money to finance his fellowship (for him and his young family of 4) shouldn't be that difficult ……. Especially, as Tom Eskes had assured me that once people would understand where I would go, they would open their purses with pleasure! The normal, frustrating battle applying for a scholarship to any and all foundations and grant institutes in the Netherlands resulted in as many rejections. When the last refusal by ZWO (the Dutch NIH) came in, the case seemed to be at a dead end. At the insistence of my wife, I called the responsible person at ZWO for an explanation why the application had not been granted. The man who answered the phone must have been very wise and patient. He understood that the young doctor he was speaking to needed above all a good advice on how to write a grant application. On top of that, luck was with me. NATO's basic research grants in the Netherlands were managed by ZWO and still had one grant had to be given out. The next week a new application went out and 4 weeks later we had obtained the NATO grant covering the stay for me and my family for 1 year. Later this grant was

extended for another year and the last year LLU was willing to pay me a small monthly compensation. An amount I could round up with personal funds making it possible to finish the project.

The basic research project I was assigned to was focused on the transfer of oxygen across the placenta. Over the course of the first months in Loma Linda, and as a result of the Larry's guidance 12 questions were formulated that would guide my research project. Those questions were:

(a) How is the physiologic function of maternal hemoglobin correlated with its chemical structure?
(b) What changes in maternal blood oxygen affinity and capacity occur during pregnancy, and what is their effect on placental oxygen exchange?
(c) What are the biologic and physiologic differences of embryonic and fetal hemoglobin as compared with that of the adult?
(d) How do variations in fetal blood oxygen affinity and capacity affect placental oxygen transfer?
(e) What is the relation of maternal and fetal arterial oxygen tensions to placental oxygen exchange?
(f) How is the exchange process affected by acute and chronic maternal hypoxia as during exposure to high altitude or in the presence of cyanotic congenital heart disease?
(g) How is placental oxygen exchange affected by changes in maternal or fetal blood oxygen affinity, as with fetal intrauterine transfusion or the presence of abnormal hemoglobins?
(h) To what extend do maternal and fetal placental blood flows affect oxygen transfer?
(i) Of what value is the concept of hemoglobin flow and how do alterations in hemoglobin flow affect the oxygen exchange process?
(j) What are the normal relations of maternal and fetal placental blood flows, and how does the distribution of these flows affect respiratory gas exchange?
(k) How do variations in hydrostatic pressure in the maternal vascular bed affect umbilical flow, and vice versa?
(l) To what extent is placental flow and oxygen exchange altered during uterine contractions?

In order to make me understand the continuity of basic research I also needed to formulate important topics in this area that would require further research after I had finished my project.

At the same time, the Dutchman needed to learn how to write a research protocol and last but not least how to perform surgery on fetal sheep.

The team effort that was required to ensure good results of the several ongoing research projects was another important experience during those years. This element was not only present during the surgery or the execution of the sheep experiments. I vividly remember the weekly conferences on the Friday afternoon, where we presented and discussed the ongoing projects. There was no ranking in who could or could not ask or answer questions. It all was a matter of challenging each other's argumentation and rationale. Putting the bar high, demanding only first-class research, competing in a sportsmanlike way with other research groups and at the same time being fair and treating your colleagues with respect that is how I remember doing research in Larry's lab.

These 3 years at CPB brought me much more than publications [4–7], presentations and a PhD thesis [8]. Here I learned the fundamentals of basic research. The basic analytical skills, the design of the appropriate study and the critical review of data generated became crucial elements for me later in life. The challenges, and sometimes even the struggle and disappointments, but also the excitement that comes with executing good research with a team of great collaborators is an experience I wish many young students may have. It certainly has also been character-forming and prepared me for leading my own teams later in life. The exemplary role Larry set turned out to be very important. For Leadership is not about being elected or appointed to an office. The office does not teach someone how to be a leader. Leadership is an attitude cultivated over time. At CPB I found, unknowingly for me at that time, an important part of that attitude.

Returning to my alma mater at the University of Nijmegen in the Netherlands, I started the 6 years residency in obstetrics and gynecology. After a tenure of 4 years as staff member at the

Red Cross teaching hospital in The Hague I came at a cross road in my professional career when the pharmaceutical company Organon International asked me to join them as their head of medical affairs and product surveillance. As (in my view) academic life in those days in the Netherlands for clinicians who wanted combine clinical duties with serious research was losing its prospects, I choose for this new challenge. An unknown path to me and seen by some of my colleagues as crossing the Rubicon.

Taking charge of Organon's clinical support department I had the chance to learn the ins and outs of the pharmaceutical industry. Organon's R&D, a leader in those days in women health care, made a good match with my professional background. To understand better the business aspects I took it upon me to do an MBA.

Four years later a small start-up company, Centocor, founded by fellow countryman Hubert Schoemaker, invited me to join them in their efforts to build up the European organization. An exciting challenge I didn't want to skip. In those days Centocor was at the forefront of developing humanized monoclonal antibodies with its lead product, Centoxin, as a revolutionary medicine against gram negative sepsis [9, 10]. The story of that company [11, 12] could fill an entire business case course, but for this essay I will shorten it to a couple of sentences. After filing the R&D data with the European registration authorities in London (named EMEA in those days) we obtained the marketing approval and the take-off in the market was above anyone's expectations. However, when 1 year later the FDA decided that additional data was required before a possible US approval could be given, the Company entered into an extreme difficult situation. Within 6 months we had to close the European facility while Centocor came close to a bankruptcy. The final rescue came when Johnson & Johnson took the company under its wings. And the rest is history. Centocor today is part of the J&J Corporation flourishing and developing innovative therapies. To me these 3 years, which sometimes felt more like a roller coaster, taught me many important lessons. Not only the huge risks that are part and parcel of the development of innovative medicines, but also how paramount it is to manage a team through difficult times. Larry's maxim "PERSEVERE" remained at the forefront of my thoughts during many of those turbulent days.

At the time Centocor was forced to withdraw from Europe a new and again exciting opportunity crossed my path. After it had laid the groundwork for its future in the USA, the biotech company Amgen wanted to expand its activities in Europe. With two very innovative and promising medicines, the colony-stimulating factors Epogen® (epoetin alfa) and Neupogen® (filigrastim), the Company hoped to grow quickly to become a leader in its field. During the next 8 years I build up and managed Amgen's clinical operations in Europe. Getting the chance to add with the help of the European team (growing from 4 to over 150 people) important building blocks to Amgen's product portfolio and in that way serving patients by transforming the promise of science and biotechnology into therapies that have the power to restore health or even save lives. During the often intense team discussions about clinical studies, registration strategy, data analysis and safety profiles of the products under research, to me the lessons learned in Loma Linda were often a beacon in the sea. The last 4 years I served Amgen as their Vice President of Business Development guiding the company in its expansion strategy into the Middle East, Central and Southern East Europe and North Africa. This exciting time came to an end when a whole new challenge appeared on the horizon: Gene Therapy.

Already during the last 4 years at Amgen I had been much more involved with business management than with overseeing clinical development projects. Before I had been in many different clinical development projects and had seen the implications of the R&D successes and failures on a company. But being the end responsible person for the entire business was a new challenge that presented itself early 2005. The academic hospital of the University of Amsterdam had decided to withdraw its support for a group of gene therapy researchers. When I was consulted about the options that might remain for this team,

I was quickly contaminated with this incredible virus, called gene therapy. What an opportunity if together we could turn this dream into reality and offer patients suffering from serious and often life threatening, mono-genetic diseases a real cure!

After securing a 1 year funding extension from the Academic Hospital, I identified 4 top tier venture capital firms and raised $30 million in capital funding in 9 months. That laid the financial foundation for AMT (Amsterdam Molecular Therapeutics). At the start we had one gene product in development, which required a focused clinical development plan and a registration strategy for both Europe as well as North America. At the same time, we needed to turnaround the production process and last but not least add people to the company with the right experience and know-how. During the next 18 months AMT was revitalized, a unique GMP manufacturing process established and the R&D pipeline filled with 4 additional gene therapy projects. We all realized that the lead product, Glybera® (alipogene tiparvovec), was not "just" a new medicine in development. If it would obtain the blessing of the regulatory authorities it would be the first gene therapy on the western world market and become the door opener for this new therapeutic modality. Quite an exciting challenge.

Glybera® was developed for the treatment of lipoprotein lipase deficiency (LPLD), a very rare inherited condition associated with increased levels of chylomicrons, particles carrying certain fat in the blood. LPLD is caused by errors in the gene that codes for a protein called lipoprotein lipase (LPL). The LPL protein has an important role in dealing with the fats from the food that we eat. When the LPL protein does not work properly, or there is not enough of it, fat levels in the blood increase dramatically causing all kinds of complications with pancreatitis as one of the most dramatic. At that time, no treatment other than a fat-free diet could be advised to these patients. On top of that, even a strictly kept diet does not prevent the rise of intravascular chylomicrons and thus keeping the risk of complications high.

Glybera® introduces a normal, healthy LPL gene into the body so that it can make functional LPL protein. The product is administered via a one-time series of small intramuscular injections in the legs. One of the major hurdles that had been conquered during the research phase, was the selection of the right (read: safe and effective) carrier that could deliver the gene into the nucleus of the cell. In the end, adeno-associated virus (AAV), serotype 1, was chosen as the most reliable and safe delivery vector as it has a natural propensity towards muscle cells. As muscle cells are normally the most important tissue contributing to healthy LPL protein production, this particular AAV is very suitable for correction of LPLD. In other words, we had the right gene and the correct vector that would do the job [13, 14].

The clinical development project for this revolutionary medicine became a real challenge. Not only were we dealing with a very rare disease (the estimates varied between 6,000 and 8,000 people in the world), but we could also not perform a classic phase I study in healthy volunteers. Which healthy person wants to obtain a gene he/she already carries? In consultation with the European registration authority (EMA) a number of clinical studies with a limited number of patients in the Netherlands and Canada were planned that, we hoped, could bring us sufficient and reliable data that would allow the Company to obtain the marketing approval [15, 16].

As so often in academic research as well as in the pharma and biotech industry, the clinical development and registration path of this innovative medicine was characterized by moments of great joy but also many obstacles, and setbacks. The challenges even became so enormous that the continued existence of the company became at risk after the share value had dropped to below two euros. After intervention by the major shareholders (the original venture capital investment firms) and a major reorganization (both the COO and I resigned; reduction of staff by 70 %; delaying several clinical projects), the company was taken private again under a new name (Uniqure). On the 25th of October 2012 Glybera® received European Marketing approval by the EMA, becoming the first approved gene therapy in the Western World [17]. The company was on its feet

and on its way to more successes. Although I had loved to have been present at that special moment on October 25, I felt very pleased to see that the efforts my team and I had made, ultimately resulted in a major milestone for patients suffering from monogenetic diseases. Larry's leitmotiv "*The reward of a thing well done is to have done it*" certainly applied in this case [18].

3 Larry The Friend

Arriving early August 1977 at LAX Larry and his daughter Lisa were waiting for me to drive me to their house. Larry and BJ offered me a warm home, in which I felt welcome and that allowed me to adapt quickly to the new environment. BJ was very helpful to make sure that our small family would find a good place to live. So when 6 weeks later, my wife and our little daughter and the 7 weeks old baby arrived, we were ready to embark on our Californian adventure. During all those years the Longo's were friends in the true meaning of the word sharing their love and support. Later in life when I had the pleasure to guide young people, being it as residents in training or staff reporting to me, I always remembered the friendship I got from Larry and the important example he gave: offer your people a stimulating and pleasant workplace and they will work with their whole heart using all their capabilities for the best of the project and the company.

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30+ Years of Exercise in Pregnancy

10

Frederik K. Lotgering

Abstract

In 1980 I came to Loma Linda to study maternal exercise, with Dr. Longo as my mentor. For millennia strenuous exercise was considered harmful for the fetus. Early studies reinforced that idea, by showing that exercise reduced uterine blood flow and fetal PO_2 by up to 40 and 29 %, respectively. But utero-placental reserve is ~50 %. So why was fetal PO_2 so much reduced during exercise?

Methods proved to be important. It took chronically instrumented animals accustomed to the laboratory environment, experiments standardized to fitness of the individual (%VO_2max), measurement of total uterine blood flow, and blood gas values corrected for body temperature. The results were simple and hold till this day. Uterine blood flow decreases linearly with maternal heart rate increase, which depends on exercise intensity and duration. Maximal reduction in uterine blood flow is ~20 % and uterine O_2-uptake remains unaltered because blood flow reduction is compensated by increases in hematocrit and uterine O_2-extraction. Fetal body temperature increases with that of the mother by ~2 °C at maximal exercise and fetal blood gas values are little affected by exhaustive maternal exercise, if properly corrected for temperature. So I left Loma Linda knowing that pregnant sheep can exercise to exhaustion without harm to the fetus, thanks to effective compensatory mechanisms.

After returning to Erasmus University Rotterdam further studies in humans showed that physical fitness is unaffected by pregnancy, weight-gain affects performance, and strenuous exercise in healthy pregnant women does not harm the fetus. Thus, the millennia-old perspective has changed.

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L. Zhang and C.A. Ducsay (eds.), *Advances in Fetal and Neonatal Physiology*,
Advances in Experimental Medicine and Biology 814, DOI 10.1007/978-1-4939-1031-1_10,

Keywords
Exercise • Maternal • Fetal

This chapter presents a review on exercise in pregnancy, in honor of Prof. Lawrence D. Longo for 40 years of leadership of the Center for Perinatal Biology at Loma Linda University.

When I had finishing my residency training in Obstetrics/Gynecology at Erasmus University Rotterdam, I asked Henk Wallenburg, my chief at Erasmus, where I should go to get the best training in basic research in Perinatology. He recommended Lawrence D. Longo and his Center for Perinatal Biology in Loma Linda for scientific excellence, yet small scale and personal. So, in 1980 I came to Loma Linda on a NATO grant to become a fellow of Larry Longo and Ray Gilbert. When I arrived, Larry wished my wife and me that our years in Loma Linda would be the best of our lives. It turned out they were.

1 Before 1980

In 1980, as it had for millennia, strenuous maternal exercise was considered unhealthful for the fetus. Some 2000 years ago Soranus wrote that even though "… she ought to promenade, … take active exercise …," "one must beware of every excess … for the seed [fetus] is evacuated … through vigorous exercise" [1]. Nonetheless, some women decided differently for themselves. In 1956 Olympic Diving Gold Medalist Patricia McCormick "continued training throughout her pregnancy and swam 800 metres a day until 2 days before childbirth" [2]. At the World Championships in 1958 the gymnastics athlete Larissa Latynina, "won five titles while 4 months pregnant" [3]. Apparently without adverse outcome.

Little was known about the physiology of exercise in pregnancy, yet the limited data available seemed to confirm that strenuous exercise jeopardized fetal health. Several studies in chronically instrumented sheep had shown that during maternal exercise fetal PO_2 decreased by up to 29 % [4–6]. However, the reduction in fetal PO_2 could not be explained solely by a reduction in uterine blood flow, as the observed reduction varied between 0 [7] and 40 % [5, 6] and uteroplacental reserve was known to be approximately 50 % [8]. Why than was it that PO_2 decreased so much during maternal exercise?

2 Loma Linda, 1980–1982

2.1 Preliminary Experiments and Methods

Preliminary experiments soon showed that exercise experiments in chronically-instrumented pregnant sheep aren't easy. Previous studies had used a fixed speed and angle of the treadmill to study exercise responses. Yet, sheep differ in size and fitness. So the same treadmill setting that proves an easy walk for a big fit sheep may well represent an exhausting challenge for a tiny one in lesser shape. Sheep also vary in temperament, some accept their fate and run, while another may freak out and prefer to make a head roll on the treadmill rather than to run. Uterine blood flow was commonly measured with the use of an electromagnetic flow at a distal branch of the uterine vasculature, assuming that the changes measured in that branch would represent total uterine blood flow. Yet the leads of such probes are so rigid that the probe may actually kink the vessel when the animal starts to move, which results in unpredictable reductions in presumed uterine flow. The blood gas analyzer was set at an assumed fetal temperature of 39.5 °C as the standard setting for fetal blood gas analyses, yet has the option to correct for the actual temperature of the blood sample. After an exhaustive treadmill run, the sheep definitely looked hot. So, what were we measuring?

To make the experiments physiologically meaningful, we needed unstressed sheep, exercise them for a given time at a level relative to their individual fitness, measure total uterine blood flow without artifacts, and correct measured fetal blood gases for the temperatures at which they were sampled. This required some adjustments of the protocol.

Knowing that if one sheep leaps over the ditch all the rest will follow, all sheep new to the lab were shown how their predecessors were running. Thereafter they themselves ran on the treadmill apparently unstressed and without hesitation. Individual fitness was measured as maximal oxygen consumption (VO_2max) during an incremental treadmill test after inserting a thermo-dilution catheter. VO_2max was calculated from measured cardiac output and the arterial-venous O_2 difference at each exercise level. VO_2max was defined as the level of exercise at which VO_2 did not increase any further despite a further increase in demand. Experiments were performed at 70 % VO_2max for 10 and 40 min and at 100 % VO_2max for 10 min; exhaustion was reached at 40 min 70 % VO_2max as well as at 10 min 100 % VO_2max. Figure 10.1 shows a pregnant sheep on the treadmill, near exhaustion.

It was difficult to find a way to measure total uterine blood flow in a stable way instead of as a variable fraction of uterine flow and a variable amount of kinking of the probe during exercise. We made plastic casts of the pregnant sheep distal aorta and it branches, which showed that the common internal iliac artery represents total uterine blood flow if one ties off the dorsal sacral and lateral sacral arteries, as shown in Fig. 10.2. That proved doable through a flank incision and allowed stable positioning of the flow probe and its lead without movement during exercise.

It took some time before we realized that we should make temperature corrections in order to obtain real life PO_2 values. If one takes blood at 41.0 °C and measures it in an analyzer set for 39 °C the PO_2 value obtained is falsely low, unless one makes the necessary corrections, as shown by Severinghaus [9]. Such corrections had not been made in previous studies on the subject [4–6]. Thus, we decided to measure maternal and fetal arterial temperatures through implanted thermistors and to correct all blood gas values for the temperatures obtained.

Fig. 10.1 Pregnant sheep on the treadmill, near exhaustion

2.2 Results

The results were actually quite simple, as shown in Figs. 10.3 and 10.4. Average uterine blood flow decreases linearly with maternal heart rate increase (Fig. 10.3), which in turn depends on exercise intensity and duration. The average maximal reduction in uterine blood flow is about 20 % (Fig. 10.4) [10]. Despite the reduction in uterine blood flow uterine O_2-uptake does not decrease during exercise because the reduction in blood flow is compensated by an increase in hematocrit (so that the flow of O_2 is less reduced than that of the blood), and by an increase in uterine O_2 extraction (Fig. 10.4) [11, 12]. When maternal body temperature increases during exercise, from about 39 to 41 °C, the fetal body temperature also increases by 2 °C, but it lags behind that of the mother during warming up and cooling down (Fig. 10.4) [11]—probably because it is surrounded by the amniotic fluid compartment

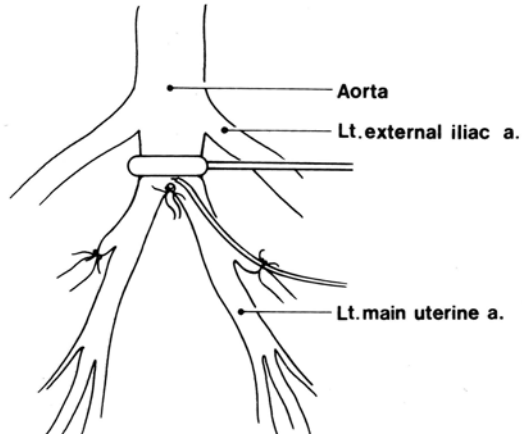

Fig. 10.2 Schematic representation of the distal branches of the aorta in sheep, with electromagnetic flow probe, catheter and ligatures in place. Reproduced from J Appl Physiol 1983 (10), with permission of the American Physiological Society

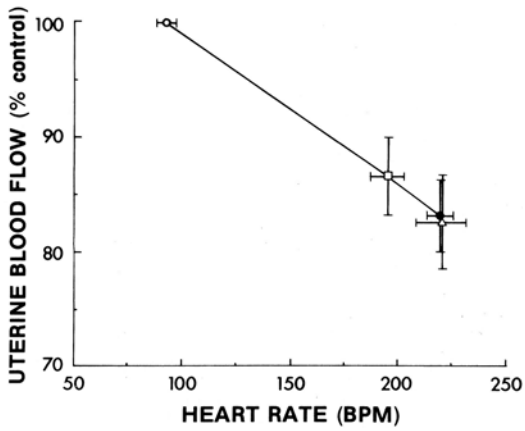

Fig. 10.3 Relation between heart rate and total uterine blood flow in near-term pregnant sheep. Reproduced from J Appl Physiol 1983 (10), with permission of the American Physiological Society

acting as a heat reservoir. Fetal blood gas values corrected for actual body temperature are little affected even by exhaustive maternal exercise, as are fetal stress hormones and cardiac output distribution that are usually altered with fetal distress [11]. Apparently the compensatory physiological mechanisms are so effective that healthy pregnant sheep can exercise to exhaustion without harm to the fetus. The results of the experimental data were published in the Journal of Applied Physiology [10, 11], and an extensive review, in fact the first ever on maternal and fetal responses to exercise in pregnancy, was published in Physiological Reviews [13].

So, when I left Loma Linda and returned to Erasmus in 1982, we knew that in pregnant sheep exhaustive exercise was apparently safe, and why. That message was well received by the field and back home in The Netherlands it earned me my Ph.D., (cum laude) from Erasmus, the Netherlands Award for Sports Medicine, and the Centennial Award for Best Scientific Thesis from the Dutch Society of Obstetrics and Gynecology. The papers have been well cited and the results have remained unchallenged. But the question remained if the overall conclusion, that exhaustive exercise in healthy pregnancy is safe, is true also in humans.

3 Rotterdam

Back at Erasmus, it took some time before I got further human studies going. Meanwhile, a study emerged that suggested that VO_2max was 12 % reduced by pregnancy [14], thereby implicating that pregnant women are in poor shape. Because that was counter-intuitive to me I searched for methodological shortcomings. It showed that the study was cross-sectional in design, with two separate groups of women, pregnant and non-pregnant, and VO_2max was expressed per kilogram body weight to correct for differences in body size. VO_2max depends primarily on muscle mass or lean body mass. One may use VO_2max/kg body weight to normalize differences between small and big people only if they have a similar body composition, otherwise it will distort the picture rather than optimize it [13, 15]. During pregnancy body weight increases by an average of 12 kg, without affecting lean body mass. It makes as little sense to correct VO_2max for pregnancy weight increase as it does for a 12 kg backpack. It seemed likely that preganancy affects just load, not power or fitness.

So we set out to measure absolute VO_2max longitudinally, in the same women during pregnancy and postpartum, for several types of

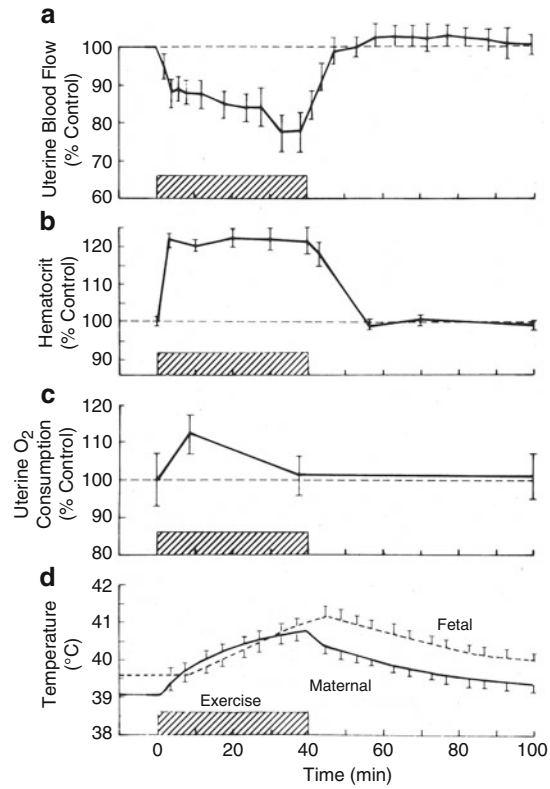

Fig. 10.4 Physiological changes in response to 40-min of exercise at 70 % VO_2max in pregnant sheep: uterine blood flow, hematocrit, uterine VO_2, and temperature. Reproduced from Am J Obstet Gynecol 1984 (12), with permission of the C.V. Mosby Company

exercise. Marieke van Doorn, the Ph.D. student who performed the studies, was the perfect woman to perform these studies. She was Gold Medalist Field Hockey at the 1984 Olympic Games, later hockey coach and sports physician for the 1992 Mount Everest Expedition. During the studies she made all participants do their utmost to achieve true VO_2max, pregnant or not. The studies confirmed that VO_2max is dependent on the type of exercise: the larger the muscle mass used, the higher the VO_2max (running > cycling > swimming). More importantly, it demonstrated that VO_2max in absolute terms, as well as the anaerobic threshold and respiratory compensation point, are unaffected by pregnancy, as is power (Fig. 10.5) [16, 17]. As to be expected with the same power and fitness, during pregnancy maximal speed is inversely related to body weight in weight-bearing exercise (running) and unaffected in non-weightbearing exercise (cycling, swimming). So, physical condition is unaffected by pregnancy, but body weight matters.

Safety for the unborn infant remained a concern, especially after a case report in The Lancet, that showed fetal bradycardia at 60 bpm throughout 20 min of bicycle exercise and thereby suggested great danger to the fetus [18]. Most amazingly, however, fetal heart rate abruptly returned to normal immediately after the prolonged bradycardia—without the gradual recovery or compensatory tachycardia that every physiologist or obstetrician would expect. As we had not observed anything like that in our pregnant sheep, again the search was on for methodological error. Fetal heart rate had been monitored with the use of an external ultrasound transducer, which reacts to anything that moves. With a similar device it proved easy to mimic fetal bradycardia in a man exercising on a bicycle. Apparently, ultrasound reflections from the abdominal muscles exercising at 60 rpm may create a false image of fetal bradycardia. Yet, women experience less fetal movement during exercise. This could be either to save fuel for the

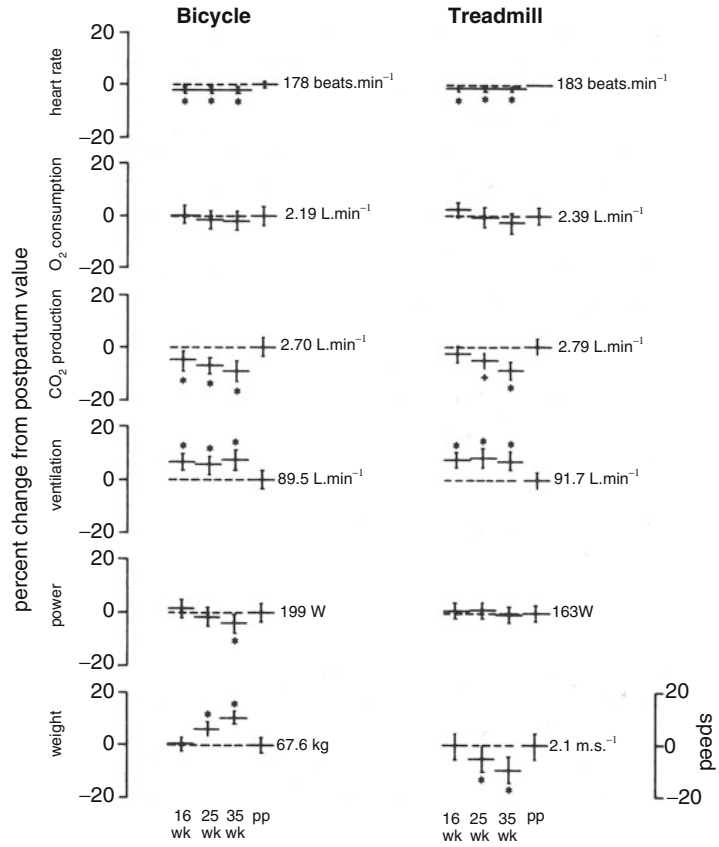

Fig. 10.5 Maternal variables in response to bicycle and treadmill exercise, expressed as percent changes from nonpregnant (postpartum) values of the same women. Reproduced from J Appl Physiol 1983 (16), with permission of the American Physiological Society

exercising muscles or because they pay less attention while being busy exercising.

Wilma Spinnewijn studied the fetal heart rate in women who underwent elective induction of labor, using an electrode attached to the fetal scalp after artificially rupturing the membranes. After a 20 min baseline, exercise was performed for 20 min at a maternal heart rate of 140 bpm. The most prominent change observed was an increase in uterine contractions during exercise that subsided during recovery (Fig. 10.6) [19]. This probably reflects mechanical stimulation of the uterus in women with a cervix ready for delivery. Fetal heart rate showed only a small increase in basal heart rate, probably as a result of temperature increase, but no change in behavioral state [19]. This suggests that the fetus is not quiescent to safe fuel during maternal exercise but rather that women subjectively experience less fetal movements when they are busy exercising.

From our studies we concluded that even strenuous exercise seems safe in healthy women with a normal pregnancy. We did not study the potential benefits of exercise in pregnancy in any detail, but noticed that women participating in the studies maintained their fitness, which seemed good for their self-esteem. Women who like to exercise should feel free to do so as long as they listen to their body, adjust their training schedule for pregnancy weight gain, and prevent dehydration and trauma.

4 Meta-analysis and Guidelines

A Cochrane analysis (2006) confirmed the conclusion that exercise in pregnancy has the advantage that fitness is maintained but found no evidence that it prevents any major obstetric problem, such as gestational diabetes or preeclampsia [20].

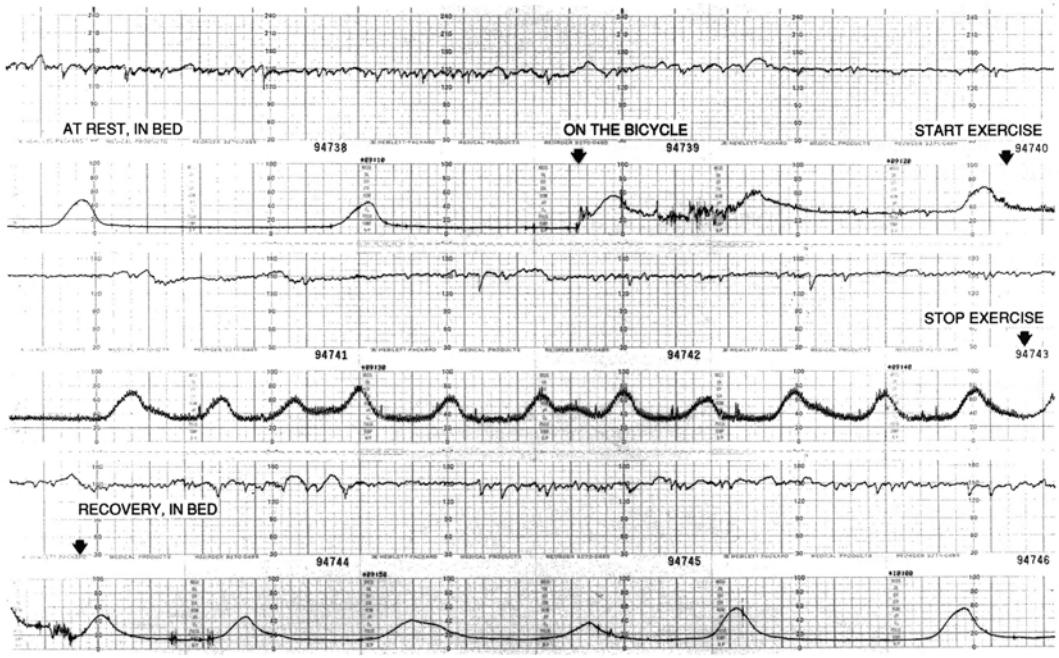

Fig. 10.6 Cardiotocogram before, during, and after maternal exercise, with fetal signal from fetal scalp electrode. Reproduced from Am J Obstet Gynecol 1996 (19), with permission of the C.V. Mosby Company

Several national colleges have accepted the new paradigm on exercise in pregnancy being quite safe and have implemented that in guidelines and recommendations [21, 22]. Most guidelines have remained on the safe side, limiting exercise to moderate duration and intensity, probably for medico-legal reasons. It is beyond the scope of this paper to review the current recommendations in detail.

5 Nijmegen

Formerly preeclamptic women often have signs of the metabolic syndrome and a relatively poor circulation, with low plasma volume, low cardiac output, high systemic vascular resistance and somewhat increased mean blood pressure. Exercise training improves cardiovascular fitness and may improve endothelial function and metabolic factors associated with cardiovascular risk. Therefore, formerly preeclamptic women are likely to benefit from exercise training. Ralph Scholten trained normotensive formerly preeclamptic women as well as matched controls for 1 h at 70–80 % of their heart rate reserve 3 times per week for 12 weeks [23]. The results confirm that formerly preeclamptic women have on average a somewhat higher heart rate and mean arterial pressure, insulin and triglyceride concentration and albuminuria, while BMI and VO_2max are not significantly different from controls. Training improves fitness as well as most markers of vascular risk, including endothelial function measured as flow-mediated vasodilation, and metabolic function. The response to 12 weeks of aerobic training in formerly preeclamptic women is largely similar to that of controls. Albeit it seems beneficial for formerly preeclamptic women to improve their health by exercise training, it remains to be elucidated to what extent such training prevents recurrence of preeclampsia in a next pregnancy and/or cardiometabolic and cardiovascular disease in later life.

6 Scranton and Loma Linda

Meanwhile, in Scranton and Loma Linda, Zavorky and Longo proposed more liberal recommendations for exercise in pregnancy [24].

They asked themselves the question if there are valid concerns for completing a marathon at 39 weeks of pregnancy, and answered that question with a clear No [25].

7 So Where Do We Stand in 2013?

Nowadays, women run or walk the marathon and participate in the Olympic games, and they wonder why some people make a fuss about it. Dr. Longo has contributed greatly to this change of perspective. That may seem remarkable because he (co)authored only 10 papers on the subject, just 3 % of his extensive scientific output expressed as Pubmed articles. Yet, the articles were well cited and sparked further interest in the field.

I will always be thankful for the privilege to have had my Ph.D.-training at the Center for Perinatal Biology, a most wonderfully stimulating research environment, with Larry as my great mentor in Science.

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Gap Junction Regulation of Vascular Tone: Implications of Modulatory Intercellular Communication During Gestation

11

Bryan C. Ampey, Timothy J. Morschauser, Paul D. Lampe, and Ronald R. Magness

Abstract

In the vasculature, gap junctions (GJ) play a multifaceted role by serving as direct conduits for cell–cell intercellular communication via the facilitated diffusion of signaling molecules. GJs are essential for the control of gene expression and coordinated vascular development in addition to vascular function. The coupling of endothelial cells to each other, as well as with vascular smooth muscle cells via GJs, plays a relevant role in the control of vasomotor tone, tissue perfusion and arterial blood pressure. The regulation of cell-signaling is paramount to cardiovascular adaptations of pregnancy. Pregnancy requires highly developed cell-to-cell coupling, which is affected partly through the formation of intercellular GJs by Cx43, a gap junction protein, within adjacent cell membranes to help

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L. Zhang and C.A. Ducsay (eds.), *Advances in Fetal and Neonatal Physiology*, Advances in Experimental Medicine and Biology 814, DOI 10.1007/978-1-4939-1031-1_11,

facilitate the increase of uterine blood flow (UBF) in order to ensure adequate perfusion for nutrient and oxygen delivery to the placenta and thus the fetus. One mode of communication that plays a critical role in regulating Cx43 is the release of endothelial-derived vasodilators such as prostacyclin (PGI_2) and nitric oxide (NO) and their respective signaling mechanisms involving second messengers (cAMP and cGMP, respectively) that are likely to be important in maintaining UBF. Therefore, the assertion we present in this review is that GJs play an integral if not a central role in maintaining UBF by controlling rises in vasodilators (PGI_2 and NO) via cyclic nucleotides. In this review, we discuss: (1) GJ structure and regulation; (2) second messenger regulation of GJ phosphorylation and formation; (3) pregnancy-induced changes in cell-signaling; and (4) the role of uterine arterial endothelial GJs during gestation. These topics integrate the current knowledge of this scientific field with interpretations and hypotheses regarding the vascular effects that are mediated by GJs and their relationship with vasodilatory vascular adaptations required for modulating the dramatic physiological rises in uteroplacental perfusion and blood flow observed during normal pregnancy.

Keywords

Connexins • Nitric oxide • Endothelium • Cyclic nucleotides • Vasodilation • Uterine blood flow

1 Introduction

Cell-to-cell communication is essential for normal multicellular tissue and organ function. Vascular cell responses rely on coordination and synchronization to elicit physiological processes such as changes in vascular tone, cell growth and differentiation, as well as the coordinated contractions of cells. Comparable analogous models have been described in cardiac and myometrial tissue [1–5], however, we will focus this review only on the blood vessels. Vascular cell-to-cell communication is in part accomplished by an intricate system involving endothelial cell regulation of vascular smooth muscle (VSM) cell tone partly via the release of signaling molecules that move through gap junctions (GJ). In addition, endothelial–endothelial cell communication also contributes to vascular tone regulation via GJ-modulated vasodilator production [6–9].

GJs are tightly packed clusters of intercellular channels between endothelial–endothelial cells and endothelial–VSM cells that couple cells both electrically and metabolically [10, 11]. GJ-mediated intercellular communication (GJIC) directly connects the cytoplasm of adjacent cells in order to facilitate the passive and directional diffusion of ions, small signaling molecules (e.g. Ca^{2+} and IP3) and second messengers (cAMP and cGMP) from one cell interior to another (Fig. 11.1) [1, 3, 12]. The coupling of neighboring cells permits relatively fast acting, coordinated intercellular signaling important for vascular homeostasis [13], e.g. paracrine interactions through an extracellular pathway.

Normal pregnancy-induced hemodynamic changes are associated with vascular adaptations in the maternal cardiovascular system including profound reductions in systemic and uterine vascular resistance with the most dramatic changes seen in the uterine vascular beds [14–16].

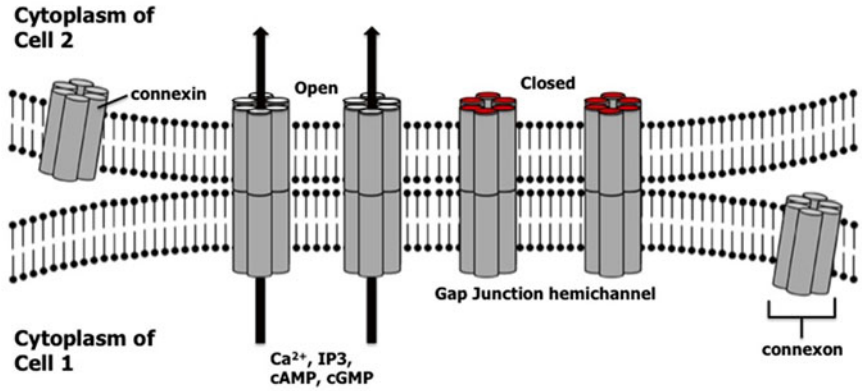

Fig. 11.1 GJ structure and intercellular communication. Illustration of GJ channels, which connect the intercellular environments of two neighboring cells and facilitate the passive and directional diffusion of small ions, signaling molecules, and secondary messengers. Membrane proteins called connexins hexamerize to form a GJ hemichannel called a connexon. Connexins configure on adjacent cell membranes and connect, permitting cell–cell communication

These vascular adaptations are dependent upon endothelial cell adaptations that play a critical role in the modulation of vascular resistance and thus uterine blood flow (UBF) through elevations in the potent endothelial-derived vasodilators prostacyclin (PGI_2) and nitric oxide (NO) [15–18]. Additionally, it was recently recognized that cell-signaling processes that were shown to regulate sustained elevations in these vasodilators include the enhancement of cell–cell connectivity via GJs in pregnancy [19, 20]. These mechanisms may have clinical relevance since endothelial cell dysfunction has been shown to be a critical and central event in the pathophysiology and progression of preeclampsia (PE) [21–24]. Moreover, impaired and mutational alterations in the expression and/or function of GJs are related to a wide variety of pathological conditions including hypertension [25, 26]. We hypothesize that if these negative changes in GJs manifest during gestation, this will be associated with gestational diseases such as PE with intrauterine growth restriction (IUGR). Therefore, identifying factors that regulate GJIC and their physiological mechanisms of action are relevant and significant to understanding the function of GJs and GJ-related diseases.

In this review, we integrate the available evidence for connexin hemichannels acting as a pathway for paracrine intercellular communication and their role in signaling. We evaluate and summarize information related to: (1) GJ structure and regulation; (2) second messenger regulation of GJ phosphorylation and formation; (3) pregnancy-induced changes in cell-signaling; and (4) the role of uterine arterial endothelial GJs in gestation.

2 GJ Structure and Regulation

GJs are formed by members of the connexin (Cx) gene family that is composed of 21 isoforms in humans [27, 28]. Connexins are four pass integral membrane proteins that hexamerize to form a GJ hemichannel called a "connexon" (Fig. 11.1). A GJ is formed when a collection of connexons meet head-to-head with connexons in an adjacent cell forming cell-to-cell channels. Different connexin isoforms yield channels with different conductance, permeability, and regulatory control properties [3, 29, 30]. There are four different connexin isoforms Cx37, Cx40, Cx43, and/or Cx45 found in the vasculature depending on the vessel type [30, 31]. The expression of connexins also is not always uniform within blood vessels since they are made up of several cell types.

For example Cx45 is observed only in the VSM cells [32] whereas Cx37, Cx40, and Cx43 have been reported to be present in both VSM and endothelial cells [33–35]. However there are conflicting data on the localization and expression of Cx37 in the aorta, pulmonary artery, coronary artery, and uterine artery smooth muscle cells [6, 34, 36–38]. A consistent observation is that Cx43 is the predominate connexin isoform found in more than 34 tissues and 46 cell types [39] including uterine tissues [30, 31, 40]. Understanding the distribution and regulation of GJ proteins is important in elucidating their conductive and gating properties for cell–cell communication.

Connexins are highly regulated on multiple levels both by alterations in gene expression [41], epigenetic mechanisms [41], and also post-translation modifications via phosphorylation on the C-terminal domain where sites for protein–protein interaction are present [3]. Phosphorylation of Cx43 is seen at multiple specific regulatory sites (serine, threonine, and tyrosine residues) that can control trafficking, assembly, and degradation [3, 27, 42, 43]. Throughout its life cycle, Cx43 is differentially phosphorylated [44–48], with most phosphorylation events on serine regulatory sites [48–51], which affects the rapid turnover of connexins and drives channel gating [3, 52]. The activation of cyclic nucleotides (cAMP and cGMP) and kinases such as protein kinase A (PKA) [53–55], protein kinase C (PKC) [56, 57], p34cdc2/cyclin B kinase (p34cdc2) [50], casein kinase 1 (CK1) [58], mitogen-activated protein kinase (MAPK) [59, 60] and pp60src kinase [45, 61] as well as protein kinase inhibitors such as proto-oncogene tyrosine-protein kinase Src, can alter Cx43 phosphorylation and lead to assembly or degradation of Cx43 GJ channels [52, 58, 62–66]. For example, Cx43 channels conductive states can be driven via phosphorylation by either PKA or by PKC, which respectively increases [54] or decreases [42] gap junction communication. However, the identification of which protein kinase(s) specifically mediates cyclic nucleotide-induced signaling and the characterization of GJ-related signaling events downstream from kinase activation remains incomplete.

3 Second Messengers Regulation of Gap Junction Phosphorylation and Formation

The cyclic nucleotides, cAMP and cGMP, are second messengers that are involved in a variety of cellular processes including increasing GJ protein assembly [54, 55, 67–70] (Fig. 11.2). It is widely accepted that cAMP-mediated signaling in endothelial cells, osteocytes, and osteoblast cells are involved in the up-regulation of Cx43 [19, 55, 67–71]. cAMP may regulate Cx43 expression acutely by the rapid phosphorylation and trafficking of Cx43 to the cell membrane or chronically by increasing Cx43 gene transcription [41]. Elevated cellular cAMP typically leads to increased GJIC and the number and size of GJ plaques [53, 72] in a process termed "enhanced assembly" in which GJ channels are formed [54]. These cAMP events are mediated via cAMP-dependent protein kinase (PKA) mechanisms that are associated with an increased phosphorylation state of the Cx43 protein that regulates increased connexin export to the plasma membrane, assembly into GJs, and gating. Although cAMP-mediated signaling in endothelial cells are involved in the up-regulation of the level of Cx43 within gap junctions [55, 67–70], virtually nothing is known about the role of cGMP on GJIC. In many tissues, cAMP and cGMP levels are interrelated and the effects of either cAMP or cGMP elevations can be difficult to differentiate due to their regulation by phosphodiesterase activity and cross-talk [73]. Only in one report was there an indication that 8-Br-cGMP decreased gap junctional conductance in cardiac tissue [74], suggesting cGMP may be working to inhibit GJ function. While PKA has been shown to function to increase GJIC, whether cGMP-dependent protein kinase PKG has a direct, an indirect (potentially via cAMP levels) or no effect on Cx43 phosphorylation in the endothelium is unknown.

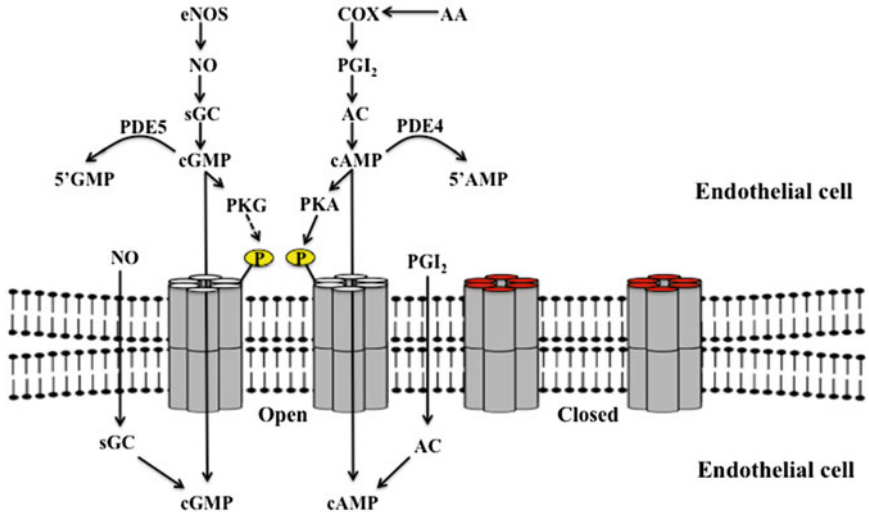

Fig. 11.2 Signal transduction pathway for PGI_2/cAMP/PKA and NO/cGMP/PKG systems for endothelium–endothelium communication. In the vascular system, PGI_2 and NO are produced by the endothelium. PGI_2 is synthesized from AA by the COX pathway. PGI_2 activates adenylate cyclase, leading to increased production of cAMP, whereas the degradation of cAMP is catalyzed by phosphodiesterase 4 (PDE4), which converts cAMP to inactive 5′AMP. cAMP activates PKA to phosphorylate and open gap junction channels to enhance cell–cell communication. NO is synthesized from eNOS and activates sGC, yielding increased levels of cGMP, whereas the degradation of cGMP is catalyzed by PDE5, which converts cGMP to inactive 5′GMP. cGMP activates PKG to phosphorylate and may open gap junction channels and enhance cell–cell communication. Adenylate Cyclase (AC); Arachidonic Acid (AA); Cyclooxygenase (COX); cyclic AMP (cAMP); cyclic GMP (cGMP); endothelial NO synthase (eNOS); Phosphodiesterase 4 (PDE4); Phosphodiesterase 5 (PDE5); Prostacyclin (PGI_2); soluble guanylatecyclase (sGC)

3.1 PKA Phosphorylation of Cx43 Enhances GJIC

Inhibition of PKA via H89 (PKA inhibitor) eliminated enhanced GJ assembly [54]. Therefore the assembly of GJs that are enhanced by cAMP is thought to be mediated via cAMP-dependent protein kinase (PKA). Studies by TenBroek and coworkers have indicated that serines 364/365 (S364/365) at the C-terminal domain of Cx43 [69] are important phosphorylation sites for cAMP-enhanced GJ assembly. Since PKA-mediated phosphorylation at S364 increases the total number and conductance of GJs [75–77] and GJ assembly was abrogated by Cx43 mutations at S364, these data clearly suggest that phosphorylation of S364 may be a prerequisite for enhanced assembly [69]. However, because Cx43 is a poor substrate for PKA as demonstrated in vitro, there is considerable controversy as to whether PKA directly phosphorylates Cx43 or whether it activates other kinases that perform this task [70, 78, 79]. The possibility of direct PKA phosphorylation cannot be excluded because purified PKA was able to phosphorylate the C-terminal region of wild-type Cx43 to a low level in vitro, but not a Glutathione *S*-transferases (GST)-construct in which S365, S368, S369 and S373 were mutated to alanine. It is also known that PKA can partly act independently of cAMP [80–82]. cAMP-independent PKA effects are thought to occur via the "Exchange Protein Directly Activated by cAMP" (Epac) signaling pathway that leads to activation of Rap, a small molecular weight GTPase of the Ras family, which has also been implicated in GJ regulation [80–82].

4 Pregnancy-Induced Changes in Cell-Signaling

Endothelium-dependent relaxations of the systemic and uterine vasculatures during gestation rely on the up-regulation of the biosynthetic

processes for the potent vasodilators, PGI_2 and NO, during gestation. These profound gestational increases in endothelial production of PGI_2 and NO are responsible for the downstream induction of the respective substantially elevated levels of arterial cAMP [83–89] and cGMP [18, 85, 87–96]. The importance of cAMP and cGMP, as well as GJs, in mediating cell-signaling events and thus cell-to-cell communication were described in detail above, however herein we focus on their role(s) in vasodilation and cardiovascular adaptations seen during gestation. Pregnancy-induced endothelial cell adaptations help to maintain the dramatically increased UBF that facilitates ample oxygen and nutrient delivery to the growing fetus.

4.1 Prostacyclin and cAMP

Vasodilatory prostanoids (i.e. PGI_2 and PGE_2) normally function in an autocrine or a paracrine fashion and exert their physiological effects on various vascular cell types. The endothelium is the primary source of PGI_2 production in uterine vessels, however it is also produced by VSM cells [13]. Pregnancy is directly associated with mechanisms that contribute to the significant rise in uterine secretion of PGI_2 including elevations in endothelium-derived enzymes [13, 17, 97–99] such as the prostanoid producing enzymes $cPLA_2$ [100], cyclooxygenase [13, 97], and PGIS [101–104]. Vascular-derived PGI_2 and PGE_2 is synthesized under basal conditions and in response to various stimuli, such as cytokines, growth factors, mechanical strain, as well as estrogens, and regulate multiple functions including smooth muscle contraction/relaxation [17, 24, 71, 101–103]. However, the central role of endogenous vasodilator prostanoids (PGI_2 and/or PGE_2) in acutely maintaining UBF during gestation is questionable since in vivo experiments infusing the cyclooxygenase inhibitor, indomethacin, lowered prostaglandin production by 70–80 % without significantly decreasing UBF [103, 105, 106]. Another interpretation of these results is that there are multiple other vasodilators (e.g. NO, CNP, EDHF, etc.) [92, 94] elevated in pregnancy and through redundant mechanisms perfusion is maintained by them as the prostaglandins are reduced, suggesting a coordinated co-regulation servo-mechanism. Redundant vasodilatory mechanisms therefore would confer an evolutionary advantage for the developing fetus. Previous studies have shown the role of the prostanoids produced by the utero-placental unit and its vasculature during pregnancy are functioning to reduce the uterine and systemic vasoconstrictor effects of angiotensin II [103, 107] and also norepinephrine [103, 105, 106, 108, 109]. The effects of PGI_2 in vascular cells are mediated by the classic PGI_2/cAMP/PKA pathway (Fig. 11.2). Similarly, the prostanoid PGE_2 activates adenylate cyclase to increase cAMP and has been shown to stimulate GJ function and Cx43 expression in osteoblast-like cells [19, 20].

From a clinical and translational perspective, PGI_2 is quite important. There are decreased PGI_2 plasma concentrations and urinary metabolite concentrations [110–113] prior to the onset of clinical symptoms of PE. Placental PGI_2 is present early in the gestation and increases considerably between 6 and 12 weeks of human pregnancy during the time when a dramatic decrease in systemic and uterine vascular resistance are observed. Women with PE are also considerably more sensitive to infusions of exogenous angiotensin II compared to normal pregnant women showing elevated pressor responses [114, 115]. It has also been reported that the normal pregnancy insensitivity to angiotensin II is abrogated by treatment with a cyclooxygenase inhibitor [88, 103, 107, 115, 116]. This further illustrates the role PGI_2 plays in uterine vasculature in normal pregnancy.

4.2 Nitric Oxide and cGMP

The vascular effects that increase UBF during gestation are partly mediated by the rapid production of the potent vasodilator NO via elevations in endothelial nitric oxide synthase (eNOS) expression and its activation [18, 93, 99, 117, 118]. Consistent with previous reports [17, 18, 98, 99, 118–120], we have recently shown that uterine artery endothelium (UAendo) total eNOS was elevated during the follicular phase and pregnancy

[6]. The reported stimulatory phosphorylation site serine 635 (S635), an index of enzyme activity in UAendo [121, 122], was also elevated by pregnancy suggesting that both expression capacity and activity of eNOS are increased to accommodate increases in UBF. Additionally, we reported a pregnancy-associated adaptation in nonreproductive OAendo which showed increases in both P^{635}eNOS and total eNOS [6], the latter confirming our previous observation [18]. These data and others suggest that systemic and uterine resistance vessels show greater endothelium and NO-mediated vasorelaxation in pregnant vs. nonpregnant animals [90, 123], and in women [124]. Similarly, elevations in shear stress, which is the most powerful physiologic mechanical stimulus of endothelial NO production [125], is an additional mechanism that is associated with rises in UBF and likely exacerbate the rises in eNOS and NO [6, 125]. Shear stress, which is the tangential frictional force exerted on the surface of endothelial cells, acutely regulates vascular tone by altering the production of vasoactive mediators by endothelial cells through simulating the expression and phosphorylation activation state of eNOS, the latter controlled via posttranslational modifications [126–128]. NO exerts its physiological effects through the NO/cGMP signaling cascade. The NO/cGMP/PKG signaling cascade is increased in response to growth factors, vasoactive peptides, and other stimuli and have been shown to effect differentiation/proliferation of VSM [129–131]. Additionally, Yao et al. have reported that activation of the NO/cGMP pathway increases GJIC and Cx43 expression acting through PKA activity [132] suggesting possible cross-talk between cyclic nucleotides and their respective protein kinases via phosphodiesterases.

Endothelium-dependent relaxation acting via the NO-cGMP pathway is inhibited in systemic aortic vessels of IUGR in rat pregnancies [133]. Likewise, placental expression of eNOS is reduced in various pregnancy conditions associated with IUGR in humans [134]. Studies have reported correspondingly reduced urinary nitrite/nitrate (NO metabolites) in subjects with PE [135], although plasma levels were not different from controls. Still, in pregnancies with associated IUGR, Schiessl et al. also observed reduced plasma nitrite/nitrate and cGMP [136]. A dramatic elevation of this cyclic nucleotide is observed in normal pregnancy occurring in the uterine venous drainage of pregnant sheep, i.e. adjacent to the uterine horns with placentation [94], suggesting uterine vascular NO output in particular is profoundly elevated at this time.

5 Uterine Arterial Endothelial Gap Junctions in Gestation

Since, endothelial cells express various combinations of Cx37, Cx40, Cx43, and/or Cx45[30, 31], which provide the potential of diversity in GJ conduction and regulation [3], it is important to study the changes in expression and distribution of connexins during pregnancy. We recently reported the expression of both Cx37 and 43 in the uterine artery. However, Cx43 protein is much more abundant within the uterine artery [6, 7, 37, 125] and myometrium where it accumulates until parturition and is necessary for the synchronization of electrical and metabolic activities of uterine smooth muscle contractions during delivery [40]. Thus the distribution of connexins in reproductive tissues may play a critical role in the conductance and gating of signaling molecules.

5.1 Distribution of Connexin Proteins in the Uterine Artery Endothelial Cell Model In Vitro

In our previous in vitro culture studies using a validated ovine uterine artery endothelial cell (UAEC) model derived from nonpregnant (NP-UAEC) or pregnant ewes (P-UAEC), we detected Cx43, but not Cx37 or Cx40 in UAECs [7]. Using the same ovine UAEC passage 4 culture model, we demonstrated that the presence of functional Cx43 is specifically required for ATP-induced Ca^{2+} associated eNOS activation in vitro [7] (Fig. 11.3). Using short peptide mimicking sequences that correspond to specific short Cx sequences, GJ channels were blocked with the inhibitory Gap peptide GAP27 specific to Cx43 [(43,37) Gap27], which selectively blocked the normal pregnancy enhanced Ca^{2+} responses to ATP [7]. However this

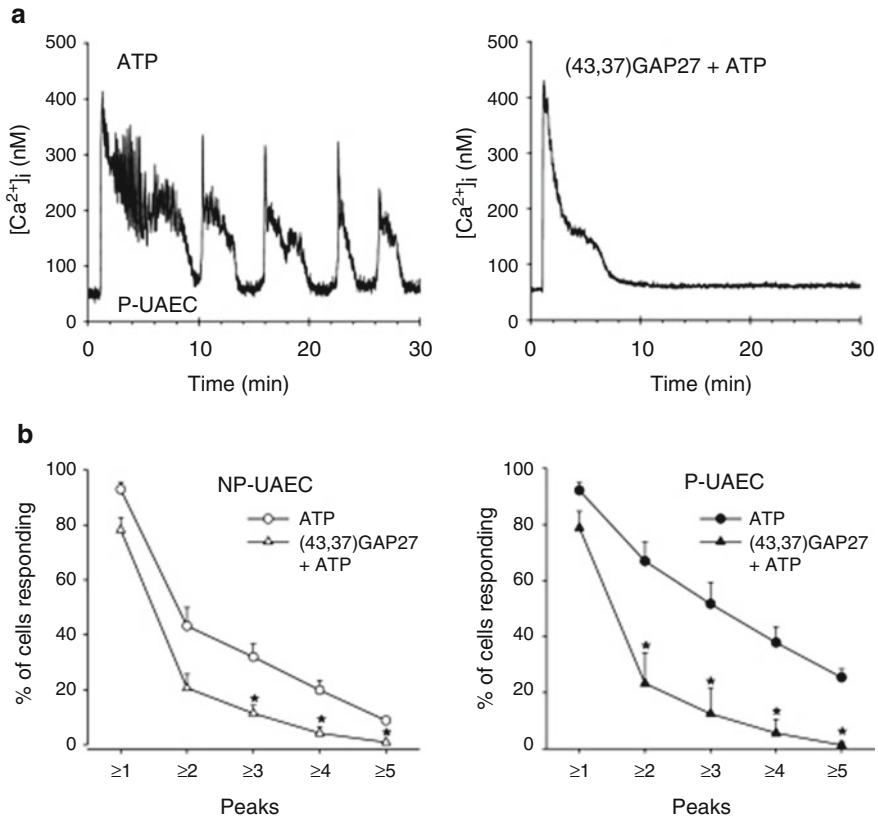

Fig. 11.3 GAP27 inhibition of $[CA2+]_i$ burst activity in confluent uterine artery endothelial cells from the pregnant state (P-UAECs). (**a**) P-UAECs treated with 100 μM ATP show the expected $[Ca^{2+}]_i$ burst activity (*left*), but pretreatment of 300 μM [43,27] GAP27 prevents all burst activity except for the initial $[Ca^{2+}]_i$ response (*right*). (**b**) Quantification of $[Ca^{2+}]_i$ burst activity shows that although P-UAECs (*right*) initially demonstrate a greater percentage of cells with burst activity than NP-UAECs (*left*), application of [43,37] GAP27 inhibits both down to a common level. Data are means ± SEM n = 5–7 dishes, with approximately 60 observations per dish. Significant difference between ATP alone and [43,37] GAP27 plus ATP response is shown by $*P < 0.05$ [7]. Data originally reported by Yi et al. [7] (Reprinted with permission from the Society for the Study of Reproduction). Inhibitory Gap peptide GAP27 specific to Cx43 [(43,37) GAP27]; Uterine Artery Endothelial Cells from the pregnant sheep (P-UAECs); Uterine Artery Endothelial Cells from non-pregnant sheep (NP-UAECs)

was only seen in the P-UAECs, but not in NP-UAECs, thereby demonstrating Cx43 is vital to pregnancy specific vasodilatation programming as previously defined [17].

5.2 Distribution of Connexin Proteins in the UA Endothelium and VSM In Vivo

Recently we also determined if the pregnancy specific changes observed in our in vitro model [7] were also seen in vivo [6] and thus have physiologic relevance in pregnancy. In this study, we utilized an in vivo ovine model restricting pregnancy to a single uterine horn [6, 137–139]. In Fig. 11.4 we show that Cx37 and 43 were both expressed and upregulated by the physiologic state of pregnancy in UAendo and/or uterine artery vascular smooth muscle (UAvsm) [6]. Moreover, UAvsm Cx37 and Cx43 were elevated by the follicular phase, but even more so by pregnancy; a physiologic state of high estrogen [15, 120, 125, 140, 141]. The nongravid unilateral side also showed elevations in UAvsm Cx37 and Cx43, demonstrating that systemic circulating hormones

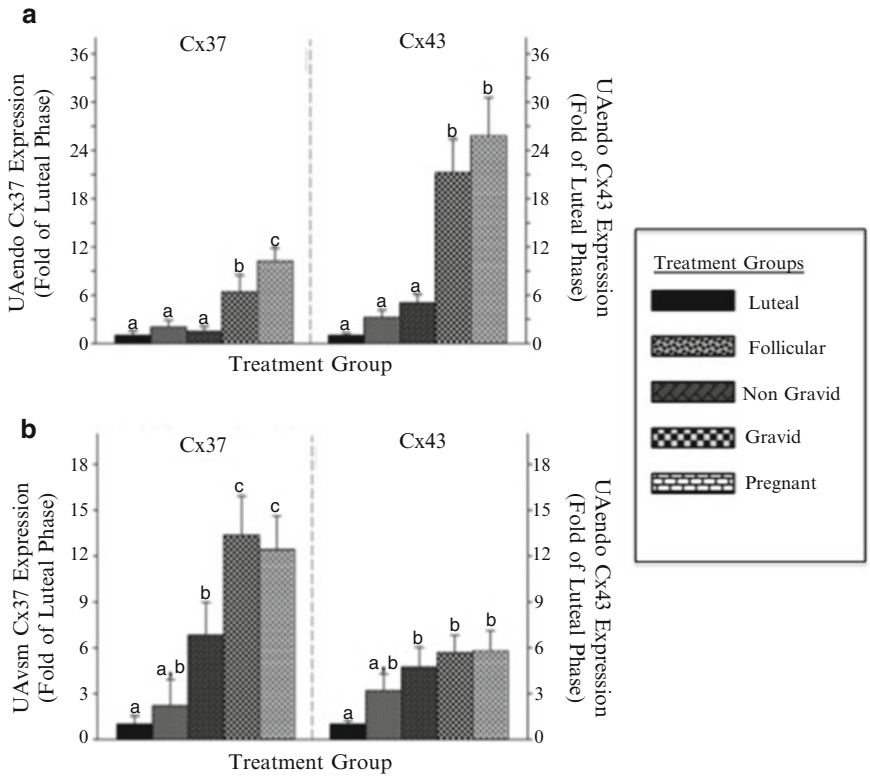

Fig. 11.4 Connexin 37 and Connexin 43 protein expression in ovine (**a**) uterine artery endothelium (UAendo) and (**b**) vascular smooth muscle (UAvsm) from luteal, follicular, nongravid/gravid unilateral pregnant, and control pregnant ewes. Uterine horns were ligated laterally before breeding, therefore restricting pregnancy to a single uterine horn yielding one horn that was gravid while the other was nongravid [6, 137–139]. Western Blot analysis comparing relative levels of connexin 37 and 43 in (**a**) UA endo and (**b**) UAvsm obtained from luteal (n = 8), follicular (n = 8), unilateral (nongravid vs. gravid; n = 15), and pregnant (n = 23) sheep. Data shown from these treatment groups are expressed as means ± SEM fold of Luteal. Different letters denote differences ($P < 0.05$). This figure is partly adapted from data originally reported [6]

(e.g. estrogen) may partly regulate VSM connexins. This is consistent with another smooth muscle cell type in the myometrium, in which Cx43 is up-regulated when endogenous estrogen is elevated in labor [142]. Cx43 is classically categorized as a "contraction associated protein" elevated in labor when the estrogen/progesterone ratio is elevated [40, 142]. The pregnancy-associated fold changes of Cx37 were similar in UAendo and UAvsm suggesting that Cx37 synthesis may be co-regulated and we proposed this is the basis for the formation of myoendothelial GJs for heteromeric cell communication between UAendo and UAvsm [8, 9]. By contrast, pregnancy-induced Cx43 increases were much greater in UAendo than in UAvsm [8, 9] and specifically support a role for endothelial–endothelial homomeric cell communication.

5.3 Expression of Cx43 in Systemic vs. Uterine Arterial Endothelia

The physiologic importance of GJ expression is perhaps best evaluated when examining the specificity of the local uterine vs. systemic effects of pregnancy. We previously reported that the uterine vasculature is affected in many aspects to a much greater extent by pregnancy and estrogen

treatments than nonreproductive vasculatures [17, 18, 98, 99, 118–120]. In studying the omental arteries (OAs) and renal arteries (RAs) as prototypic vessels important for blood pressure regulation, we found that both OAendo and RAendo Cx37 and Cx43 levels remained unchanged to both treatments, demonstrating that gestational endothelial adaptations were specific to uterine, but not systemic vasculature [6].

5.4 The Role of Pregnancy-Enhanced eNOS Activity/NO Production in Association with Gap Junction Function

As described above, it is known that cAMP and possibly cGMP up-regulate Cx43 expression and the enhanced production of NO by the UAendo is critical to vascular adaptations to pregnancy. However, what is not known is if the corresponding local UAendo changes in the expression and distribution of connexins play a role in further reinforcing enhanced in vivo eNOS activation and NO production during normal gestation. We demonstrated that Cx43, but not Cx37, specifically modulate ATP-stimulated Ca^{2+}-mediated eNOS activation evident in ex vivo UAendo, demonstrating physiologic functional significance [6]. These data are consistent with our observations shown in passage 4 cultured P-UAECs, but not NP-UAECs (Fig. 11.3), that Cx43 was a prerequisite requirement for ATP-mediated Ca^{2+} bursts-associated NO production [7]. We showed using ex vivo isolated UAs that this ATP-stimulated pregnancy programmed burst pattern for Ca^{2+}-mediated NO production were specific to Cx43 and are seen ex vivo and thus under physiologic conditions [6], rather than in vitro conditions [7] (Fig. 11.3). In addition, [43,37] Gap27 pretreatment of UAendo in vessels from pregnancy and P-UAECs converted the ATP-stimulated Ca^{2+} and NO response to ones that were identical to those we had reported from nonpregnant Luteal or Follicular UAendo and NP-UAECs [143] (Fig. 11.3b).

6 Discussion and Perspectives

Collectively, we have presented data supporting the notion that Cx43 expression and function are involved in the endothelial adaptations needed to increase cell–cell communication during pregnancy. We have shown that Cx43 is crucial for local Ca^{2+}-mediated eNOS activation and NO production by the UAendo [6]. In addition, we suggest a role for prostanoids such as PGI_2 in stimulating Cx43 expression [19, 20]. We also suggest an important physiologic mechanistic role for connexins and elevations in UA shear-stress to maintain uterine perfusion via NO and PGI_2 during ovine pregnancy [6, 125]. These pregnancy-induced endothelial cell adaptations are critical in gestation since their dysfunctions are found in disorders of pregnancy such as PE with IUGR which is seen in 5–13 % of all pregnancies [21]. Notably, PE with IUGR are associated with reduced UBF causing significant maternal and fetal morbidity and mortality as well as greater susceptibility and earlier onset of future cardiovascular disease in both the mother and baby [21].

GJs have a significant role in regulating vasodilatory pathways that modulate numerous cardiovascular functions including increasing and maintaining UBF during gestation. The connection between endothelial dysfunction, reduced PGI_2 and NO biosynthesis, and reduced UBF in PE has been previously reported [110–113, 132]. In this review we suggest that the normal physiologic rises in UBF and thus UA shear stress, Cx43 and eNOS phosphorylation states are increased via local mechanisms only in the uterine vessels adjacent to the uterine horn that contain a feto-placental unit. Understanding the mechanisms regulating UA function gives us greater understanding of the specific mechanisms controlling normal UBF during gestation which may function abnormally in PE. Local steroid hormones or growth factors produced by the placenta may modulate mechanisms controlling UBF. These are locally secreted into the uterine venous blood

and reach the tissues via arterial-venous shunts [144] or the lymphatic drainage [145, 146] in order to cause unilateral vasodilation and vascular remodeling. We recently reported that UAendo ATP-induced eNOS activation and NO production is Ca^{2+}-mediated and has an obligatory requirement for Cx43 [6]. Additionally, the local production of prostanoid enzymes as well as PGI_2 by the endothelium may play a role in increasing UBF through the stimulation of cAMP associated Cx43 expression and assembly as well as through the regulation of the responses to vasoconstrictors that are elevated in gestation [103, 105, 106, 108, 109]. Thus even under conditions of uterine space limitations and placental insufficiency, uterine perfusion is partly maintained at control levels via the co-regulation of Cx43, prostanoids, and eNOS for more robust PGI_2 and NO production. The end result is to maintain UBF for nutrient and oxygen delivery and thus fetal growth in an albeit comprised *in utero* environment.

Acknowledgements *Funding Sources*: Supported by National Institutes of Health grants R25-GM083252 (BCA—ML Carnes, PI); and HL49210, HL87144, HD38843, and HL117341 (RRM, PI).

We wish to thank S. Omar Jobe, Mayra B. Pastore, Mary Y. Sun, Amanda Hankes, Rosalina Villalon Landeros, Jayanth Ramadoss, Jill M. Kock, Gladys E. Lopez, Terrance M. Phernetton, Jason L. Austin, Cindy Goss, and Tim Taylor for their assistance with these studies and manuscript preparation. This work was partial fulfillment of Bryan Ampey's Ph.D. degree for the Endocrinology Reproductive Physiology Training Program.

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Effect of Preeclampsia on Placental Function: Influence of Sexual Dimorphism, microRNA's and Mitochondria

12

Leslie Myatt, Sribalasubashini Muralimanoharan, and Alina Maloyan

Abstract

In pregnancy fetal growth and development occur in a sexually dimorphic manner. Male and female fetuses respond differently to the intrauterine environment with males disproportionately suffering from perinatal morbidity and mortality. We have demonstrated placental dysfunction and sexually dimorphic responses in pregnancies complicated by severe preeclampsia. Production of cytokines and apoptosis in the male placenta is heightened relative to that of the female placenta. We also find increased expression and stabilization and a sexual dimorphism in expression of the transcription factor HIF-1α, but a defect in binding to the hypoxia response element with corresponding reduced expression of HIF-1α target genes including VEGF and Glut-1. HIF-1α is involved in crosstalk with the redox sensitive transcription factor NFκB in regulation by cytokines, reactive oxygen species and expression of inflammatory genes. We find increased placental expression and DNA binding of NFκB and a sexually dimorphic response suggesting a role for NFκB in placental dysfunction with preeclampsia. Placental mitochondrial complex III activity and complex I and IV expression are reduced and alterations in mitochondrial morphology are found in preeclampsia and are linked to the hypoxamir miR-210. We propose that with severe PE placental HIF-1α is stabilized by excessive ROS, inflammation and relative hypoxia. This increases the expression of miR-210 in the placenta causing repression of mitochondria-associated target genes, potentially leading to mitochondrial and placental dysfunction. This placental dysfunction may lead to a fetal programming effect that results in disease in later life.

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L. Zhang and C.A. Ducsay (eds.), *Advances in Fetal and Neonatal Physiology*, Advances in Experimental Medicine and Biology 814, DOI 10.1007/978-1-4939-1031-1_12,

Keywords
Placenta • Sexual dimorphism • Preeclampsia • Mitochondria • Inflammation • Oxidative stress • Fetus • Pregnancy • Obesity

1 Introduction

The syndrome of preeclampsia (PE) is defined by hypertension and significant proteinuria developed at or after 20 weeks of gestation in previously normotensive women and which resolves postpartum [1]. It is a multisystem disorder, which complicates 5–8 % of pregnancies worldwide [2] and is associated with significant fetal and maternal morbidity and mortality. Severe PE is associated with fetal growth restriction, indicating placental dysfunction, and with preterm birth and perinatal death. Several mechanisms have been suggested to play a role in the pathogenesis of PE including an abnormal immune response, defective placentation, relative placental hypoxia or ischemia and oxidative/nitrative stress [3]. This leads to an exaggerated maternal inflammatory response [4] and generalized maternal endothelial cell activation, the causes of which are still uncertain but thought to be triggered by angiogenic or other factors released from the placenta [5] that damage the maternal endothelium [6, 7]. In particular, our studies have focused on the effect of preeclampsia on placental function as a mediator of fetal programming. Recently, developmental programming of cardiovascular disease, potentially mediated by placental dysfunction, has been suggested to occur in offspring of preeclamptic women [8–10]. We summarize our recent studies on placental function in PE, revealing a sexually dimorphic effect on inflammation and apoptosis and the novel role of micro RNA's in regulation of placental mitochondrial function.

2 Sexual Dimorphism in Pregnancy

In "normal" pregnancy and development fetal growth occurs in is a sexually dimorphic manner with the male and female fetus responding differently to the intrauterine environment. Male fetuses grow faster and are usually larger than females [11]. However, a male fetus is more at a risk of poor outcome than the female fetus in association with placental insufficiency, PE, infection, IUGR, preterm delivery [11] and more late stillbirths associated with pre-gestational diabetes [12]. A Norwegian population-based study of 1.7 million singleton births clearly identified that preterm delivery and perinatal mortality and morbidity are dominated by the male sex [13]. While some reports suggest that PE is more prevalent with male fetuses [14], the Norwegian study shows an increased incidence of PE at <37 weeks gestation with a female fetus, but which may reflect the fact that male fetuses are delivered earlier due to other problems [15] and may not therefore stay *in utero* to allow the mother to develop PE. Sex specific adaptation of the placenta may be central to the differences seen in fetal growth and survival. Male fetuses reputedly try to maximize growth *in utero*, a strategy that places them at risk in an adverse environment [12] and may lead to increased incidence of adverse perinatal outcomes, including preterm birth, placenta previa, premature lung development; in contrast, females were shown to be more sensitive to maternal asthma [16–19]. Females, however, may adapt to the adverse intrauterine environment in an attempt to survive further maternal insults and ensure survival. The female neonate can more readily adapt to ex utero life even when delivered in a highly immature state at mid gestation, an effect possibly mediated by *in utero* adaptations to an adverse environment prior to delivery [20]. This may then relate to their risk of developing disease in adult life where differences in incidence to various diseases are clearly documented. The sex of the fetus also seems to be able to affect maternal physiology, an effect potentially mediated via the placenta. The male fetus is associated with a

more vasoconstricted state in the maternal microcirculation and greater endothelial dysfunction of preeclamptic women compared to those with female fetus [18].

3 Role of the Placenta in Sexually Dimorphic Events in the Fetus

What underlies the sexual dimorphism in fetal growth and development and response to an adverse intrauterine environment? The placenta functions as a key regulator of fetal growth and development by facilitating nutrient supply to and waste removal from the fetus and secretion of peptide and steroid hormones that regulate fetal growth and development. Alterations in placental function have the ability to mediate fetal programming [21, 22]. There is burgeoning evidence that these roles of the placenta can be regulated in a sexually dimorphic manner. Microarray analysis has shown distinct sexually dimorphic differences in gene expression in the human placenta. In particular immune genes were expressed at higher level in female placenta compared to male [23]. Expression of 59 genes were changed in the placenta of women with asthma vs. no asthma with a female fetus compared to only 6 genes changed in those with asthma but a male fetus [24]. Hence gene expression in the placenta also responds to maternal inflammatory status in sex-dependent manner [24–27] with differences seen in placental cytokine expression, insulin-like growth factor pathways and the placental response to cortisol in relation to an adverse maternal condition (asthma). Changes in diet also provide distinctive signature of sexually dimorphic genes in placenta with expression generally higher in genes in female than male placentas [28]. Yeganegi et al. have shown that male placenta has higher TLR4 expression and a greater production of TNFα in response to LPS than the female placenta, which may underlie the propensity to preterm birth in males [29]. The mechanisms underlying such changes remain unknown; but evidence from other complicated pregnancies links sex differences to gonadal steroids. Immune function in adults is known to be regulated in a sex-specific manner as determined by differential effects of estrogen and testosterone [30]. There have been reports that women with PE have increased plasma testosterone levels compared to those of healthy pregnant women, with significantly higher levels in male- than in female-bearing preeclamptic pregnancies [31–34]. At the same time, the placental levels of aromatase, a rate-limiting enzyme converting androgens to estrogens, varied depending on fetal sex: it was much higher in the preeclamptic placentas with female than male fetuses [35]. Aromatase can be downregulated by TNFα, hypoxia, insulin and leptin, which mirror the actual conditions of the placenta in the context of maternal obesity [33, 36–38].

4 Sexual Dimorphism in the Placenta with Preeclampsia

We have reported elevated levels of TNF-a, IL-6 and IL-8 in preeclamptic placentae compared to normotensive controls, and a sexual dimorphism in expression of cytokines with male preeclamptic placenta showing much higher levels of cytokines than female preeclamptic placentas [39] (Fig. 12.1). Reinforcing our previous work showing increased placental oxidative and nitrative stress during PE and significant alterations in antioxidant defenses [40] we found significant increases in ROS levels defined by DCF staining in placenta from preeclamptic compared to normotensive pregnancies [41]. Oxygen tension has a major role in placental development [42] and human cytotrophoblast proliferation [43]. The transcription factor hypoxia-inducible factor-1α (HIF), which is expressed in the villous cytotrophoblast and decreases with gestational age in normal pregnancies [44], plays a role in the regulation of trophoblast function [45]. Hypoxia is reported to stimulate expression of a number of angiogenic proteins including endothelin, VEGF and Flt-1 [46] possibly via the action of HIF-1α [47]. Increased placental HIF-1α has been previously observed in pregnancies complicated by PE [48, 49], however, a defect in the oxygen sensing mechanism was also seen in early onset PE such that HIF-1α was not responsive to hypoxia [49].

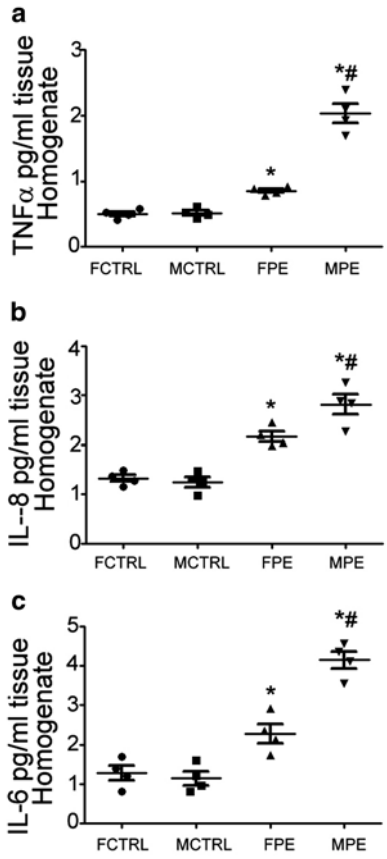

Fig. 12.1 Production of cytokines and chemokines by male and female placentas of normotensive and preeclamptic pregnancies. (**a**) TNFa, (**b**) IL-8 and. (**c**) IL-6 levels in CTRL and PE placentas. N = 5 in each group. *$p < 0.05$ vs. FCTRL and MCTRL. #$p < 0.05$ compared to FPE. Reproduced from Muralimanoharan et al. [39] with permission from Elsevier

We recently found increased placental HIF-1α protein levels with severe PE at term, but no change in HIF-1α mRNA [41] indicating that HIF-1α protein is stabilized. In addition to hypoxia, inflammatory cytokines, estrogen and reactive oxygen species (ROS) stabilize HIF-1α during normoxia in a number of tissue types including placenta [50]. Hence the increased ROS may be responsible for the stabilization of otherwise short lived HIF-1α in placenta seen during PE [50]. HIF-1α is thought to bind to the consensus HRE and activates transcription of downstream targets such as VEGF and GLUT-1. However, in the placenta with severe PE we found low binding of HIF-1α to the HRE and thus significantly lower levels of VEGF and GLUT-1 mRNA levels with PE compared to normotensive controls [41]. This aberrant response of placental cells to a hypoxic or inflammatory insult may lead to tissue damage underlying the pathology observed in PE. We also revealed a sexually dimorphic effect as we have recently shown that levels of HIF-1α were significantly higher in male preeclamptic placentas compared to both female preeclamptics and to normotensive male and female controls (Fig. 12.2). VEGF protein levels were significantly lower in preeclamptic placenta and still lower in male preeclamptic compared to female [39].

Increased trophoblast apoptosis has been observed in placentas from pregnancies complicated by PE and thought to contribute to the pathogenesis of this condition [51]. Early studies found no difference in Bcl-2, Bcl-xL, Bax and Bak expression in placental villi of preeclamptic vs. normotensive placentas [52], but others reported the expression of Bcl-2 to be less abundant in syncytiotrophoblast from severe preeclamptic placentas [53]. The increased trophoblast apoptosis with PE is thought to be the result of placental oxidative stress, in part triggered by hypoxia [54] which decreases expression of Bcl-2 (anti-apoptotic) while increasing the expression of p53 and Bax (pro-apoptotic) [55]. We observed an increase in apoptosis in preeclamptic placenta, which was significantly greater in the placentas of males compared to females, again indicating sexual dimorphism [39]. The increased expression of the apoptotic proteins p53 and p53 upregulated modulator of apoptosis (PUMA), as well as Bax, activated caspase-3 and caspase-9 together with significantly lower expression of Bcl-2 in preeclamptic placentas are consistent with the increased apoptosis. In addition, we have found that PUMA, Bax and Bcl-2 were changed in a fetal sex-dependent manner with significantly greater expression in the male preeclamptic placenta [39].

There is a growing body of evidence that HIF-1α can also be activated through inflammation-related factors that include cytokines (IL-1b and TNFα) with NFκB as the key link that drives cytokine cellular signaling [56].

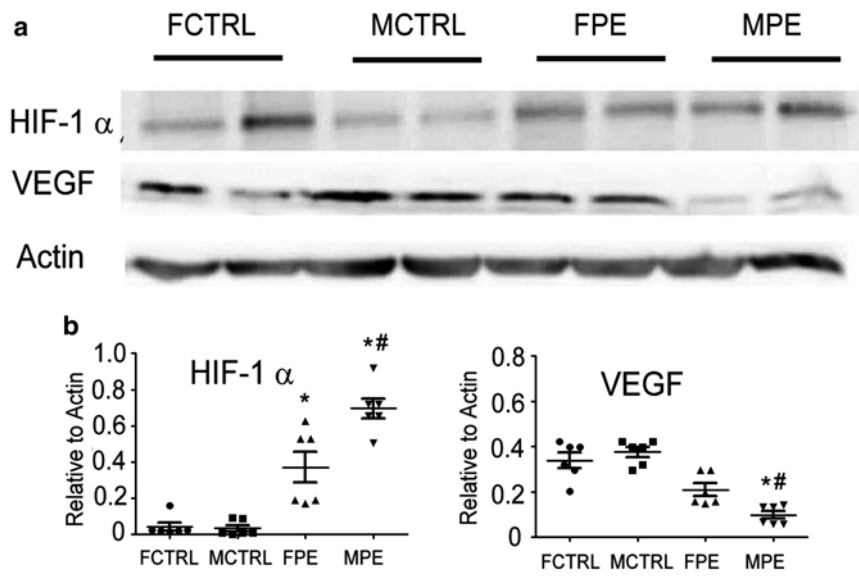

Fig. 12.2 Expression of HIF-1a and VEGF in the placental villous tissue of normotensive and preeclamptic pregnancies. (**a**) Representative Western blots showing expression of HIF-1a and VEGF. Actin expression was used as loading control. (**b**) Scatter plot showing the quantification of HIF-1a and VEGF. N = 5 in each group. *p < 0.05 compared to FCTRL and MCTRL. #p < 0.05 compared to FPE. Reproduced from Muralimanoharan et al. [39] with permission from Elsevier

There is a crosstalk between hypoxia and inflammation in placenta: HIF-1α activates NFκB [57], NFκB controls HIF-1α transcription [57], and HIF-1α activation may be concurrent with inhibition of NFκB [58]. NFκB is a redox-sensitive transcription factor regulating a battery of inflammatory genes, and has a variety of different effects in numerous pathological states [59]. In most cells, NFκB is found in the cytoplasm in its inactive form, bound to inhibitory proteins. Many extracellular stimuli, including bacterial lipopolysaccharide, viruses, oxidants, inflammatory cytokines, and immune stimuli, can activate NFκB. Once activated, it binds to regulatory DNA elements in the promoter regions of inflammatory and immune response genes, such as those encoding pro-inflammatory cytokines, chemokines, enzymes relevant for inflammation, and adhesion molecules [60]. Vaughan and Walsh reported a marked increase in NFκB activity in preeclamptic placentas as well as in cultured trophoblasts exposed to either hypoxia or inflammation or both [61].

In addition to sexual dimorphism in pro-inflammatory cytokine production and apoptosis in the placenta with PE [39], we also found an increase in the expression and binding activity of NFκB p65 in the preeclamptic placentas compared to CTRL with much higher levels in male preeclamptic compared to female (Fig. 12.3). This may suggest that increased inflammatory and trophoblast cell death observed in the placenta of preeclamptic pregnancies are, at least partially, induced by NFκB p65, further emphasizing the role of inflammation in the etiology of PE. Further studies are required to understand the mechanism(s) underlying the sexual dimorphism in inflammatory responses and the involvement of NFκB.

5 Inflammation, Oxidative Stress and Mitochondrial Function

The inflammation and oxidative stress of normal pregnancy are heightened in PE [62] with placental mitochondria, an important source of reactive oxygen species (ROS), contributing to generation of oxidative stress [63]. Mitochondria are the major oxygen consuming organelles, play a

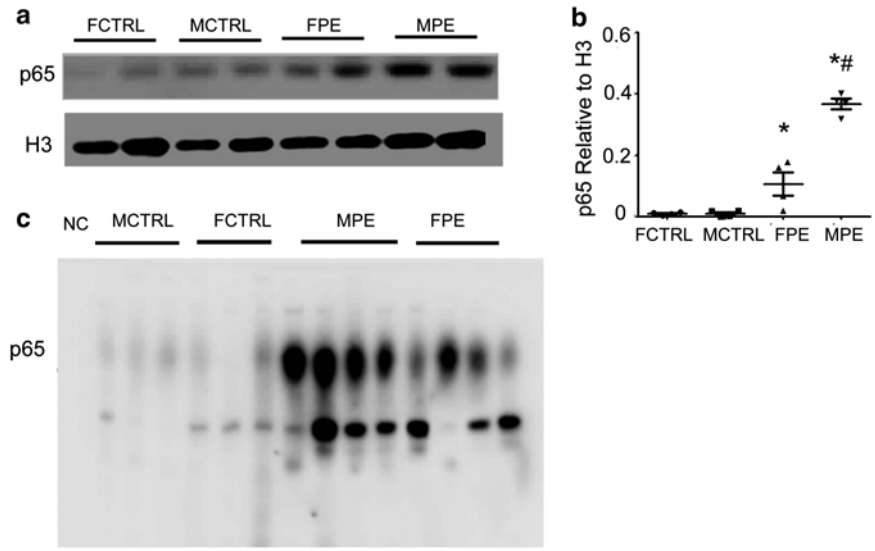

Fig. 12.3 NFkB p65 expression and binding to DNA in placentas from normotensive and preeclamptic pregnancies. (**a**) Representative Western blots and (**b**) quantification bars showing expression of NFkB p65 expression in nuclear fraction of CTRL and PE villous tissue. Expression of histone H3 was used as loading control. (**c**) EMSA showing binding of nuclear protein extract to the consensus DNA binding site of p65. N = 5 in each group. *$p < 0.05$ compared to FCTRL and MCTRL. #$p < 0.05$ compared to FPE. Reproduced from Muralimanoharan et al. [39] with permission from Elsevier

crucial role in sensing the cellular oxygen concentration [64] and are the main source of endogenous ROS in most mammalian cell types [65]. Of the oxygen consumed by mitochondria, up to 5 % is converted to ROS as byproducts of oxidation-reduction reactions in the respiratory chain. When dysfunctional, mitochondria generate excessive amounts of ROS which may be involved in the triggering of PE and IUGR [66, 67].

Obesity, a major health issue in both the developed world and in developing countries [68], is associated with increased morbidity in pregnancy per se, but is also associated with programming of offspring for disease in adult life [69]. Obesity increases the risk of development of PE, thought to be due to the pre-existing low grade inflammation and oxidative stress of obesity [70]. An increase in reactive oxygen species (ROS) and reduction in the oxidative capacity of brown adipocytes results in impaired thermogenesis, and has been linked to diet-induced obesity [71]. Wilson-Fritch et al. demonstrated down-regulation of approximately 50 % of gene transcripts encoding mitochondrial proteins in adipose tissue in a rodent model with the onset of obesity [72]. In adults, obesity is associated with compromised mitochondrial function [73] suggesting this may be a mechanism linking obesity to PE.

Mitochondrial oxidative phosphorylation is a key energy source for placental function [74]. Several studies have shown that the increasing metabolic activity of placental mitochondria results in excessive production of ROS leading to oxidative stress, which may be exaggerated in pregnancies complicated by PE, IUGR and maternal obesity [75–80]. Thus, mitochondrial abnormalities and ROS formation could be part of a vicious cycle and represent a central mechanism of placental dysfunction in these disease states. Mitochondrial function in other metabolic tissues (e.g., liver, heart and brain) appears to be regulated by sex hormones [81–85]. The involvement of placental mitochondrial dysfunction in the pathogenesis of PE has been described in patients with mitochondrial disorder patients [67] and since reinforced [86], the exact mechanism remains unclear.

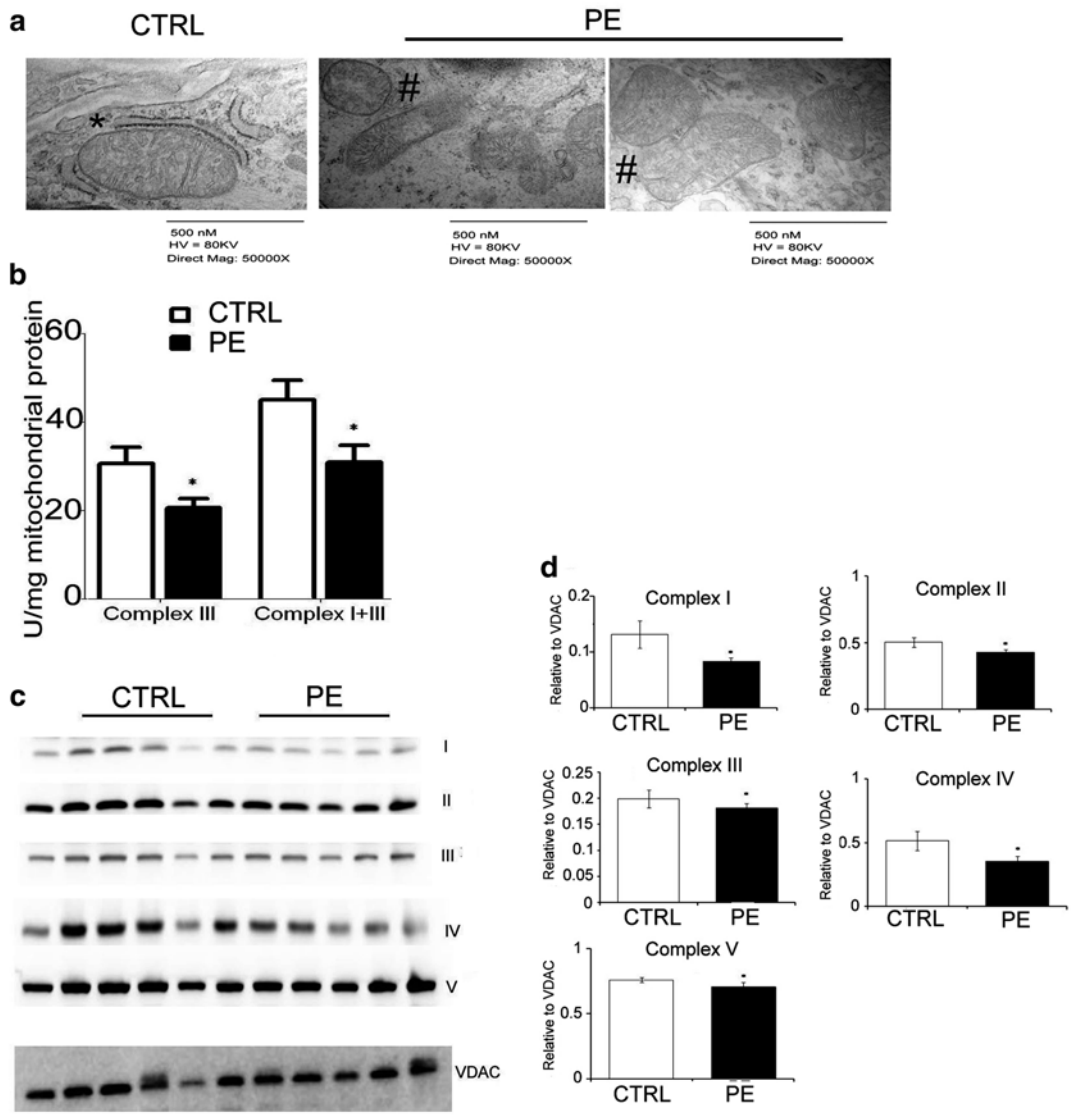

Fig. 12.4 Mitochondrial dysfunction in preeclampsia. (**a**) Electron micrograph illustrating the morphological changes in the mitochondria of control and preeclamptic placenta (magnification = 50,000). *Hash* indicates mitochondrial swelling and broken cristae in placentas from PE and *asterisk* showing intact mitochondria form CTRL placenta. (**b**) Mitochondrial complex activity measured calorimetrically in the isolated mitochondria from flash frozen villous tissue of control and preeclampsia. (**c**) Immunoblots showing expression of mitochondrial complex proteins. VDAC was used as loading control. (**d**) Band intensity of the mitochondrial complexes is normalized to the intensity of VDAC Values shown are mean ± SEM, *$p < 0.05$ vs. control, ($n = 6$ each group). Reproduced from Muralimanoharan et al. [41] with permission from Elsevier

Mitochondria exist as dynamic networks and alterations in mitochondrial morphology during apoptotic cell death, fragmentation of the network and the remodeling of the cristae have been reported [87, 88]. We have reported significant morphological abnormalities at the ultrastructural level, together with reduction in complex III activity and reduction in the protein expression of complexes I and IV in the preeclamptic placentas [41] (Fig. 12.4). Reduction in complex activity suggests altered electron flow through complex III and perhaps damage to other complexes, which

may contribute to an increase in ROS production. The scope and nature of the decreased activity in complex III in PE remains to be defined.

6 MicroRNA and Placental Function

Regulation of cell proliferation, mitochondrial metabolism, oxygen sensing and apoptosis in placenta has been recently found to be regulated by small (22 nucleotide) non-coding RNAs-microRNAs (miRNAs) [89]. MiRNAs are highly conserved, regulatory molecules that play an important role in the post-transcriptional regulation of gene expression by promoting RNA instability or translational inhibition [90]. Enquobahrie et al. [91] have shown eight differentially expressed placental miRNAs in pregnancies complicated by PE with miR-34C, 139 and 328 being downregulated and miR-210 upregulated [91]. Upregulation of placental miR-210 has been also described in preterm and severe PE [92–96]. Zhang et al. found that elevated miR-210 during PE suppresses trophoblast cell migration and invasion by targeting Homeobox-A9 (HOXA9) and Ephrin-A3 (EFNA3) [97]. MiR-210 is known to be involved in the hypoxic response and has been shown to be over-expressed in a HIF-1α-dependent manner in many types of tumors. It is purported to be involved in the shift of tumor metabolism from oxidative phosphorylation to glycolysis (Warburg effect) [98]. In addition, a mechanistic link between miR-210, HIF-1α, mitochondria-associated target proteins, and mitochondrial function has been identified in cancer cells [99, 100]. MiRNAs are now recognized as essential mediators of numerous cellular processes [101] with a number of hypoxia-related miRNAs (hypoxamirs) such as miR-23, miR-24, miR-26, miR-107 and miR-210 being identified that display significant upregulation in hypoxia [102]. MiR-210 expression is under direct control of HIF-1α and is downregulated in HIF-1α knockout cell lines [103] and is robustly induced in the hypoxic state in several tissues [104]. Interestingly miR-210 is involved in mitochondrial dysfunction in various types of cancer [105] with miR-210 delivery under normal oxygen conditions being able to inhibit mitochondrial energy production, impair oxygen consumption [106], induce lactate accumulation [99, 100], alter mitochondrial membrane potential and disrupt mitochondrial structure in cancer cells [107].

7 miR-210 and Placental Mitochondrial Function

We recently tested the hypothesis that mitochondrial dysfunction seen in the placenta of pregnancies complicated by PE is mediated by over-expression of miR-210. We observed a twofold increase in the expression of miR-210 during PE and strong correlation between miR-210 expression and hypoxic markers in our patient cohort [41], confirming that miR-210 expression was likely driven by hypoxia in vivo, as previously shown in other solid tumors.

We subsequently searched for targets of miR-210 involved in mitochondrial function. MiR-210 targets ISCU in trophoblast cell lines [93] possibly by targeting the transcript coding for ISCU, which facilitate the assembly of iron–sulfur clusters that are incorporated into enzymes involved in energy production, including mitochondrial respiratory complexes [106]. ISCU are a prosthetic group that promotes electron transport and oxidation-reduction in mitochondria cluster assembly protein [108]. Reduced expression of ISCU mediated by miR-210 contribute to a decrease in the activity of the TCA cycle (through aconitase targeting) and the electron transport chain (through complex I destabilization) [106]. Mir-210 regulates the expression of ISCU, a key factor in the assembly of Fe-S cluster subunits of complexes I, II and III in the mitochondria of cancer cells [100]. Indeed we found a reduction in placental ISCU mRNA expression during severe preeclampsia (Fig. 12.5). Repression of ISCU by miR-210 could alter the stoichiometry and function of complexes in electron transport and oxygen consumption leading to an increase in ROS production and decreased protein expression of complex I and activity of complex III as corroborated by our data.

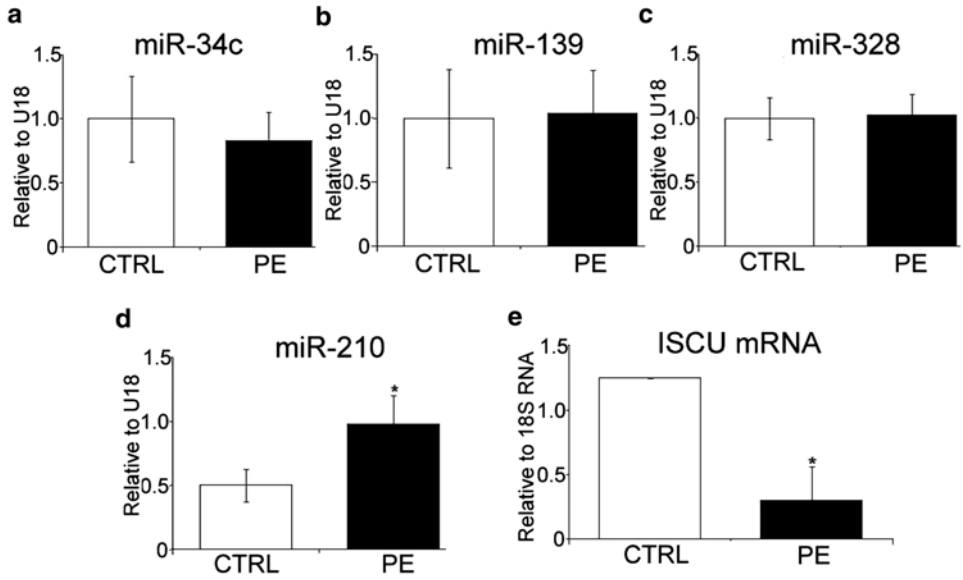

Fig. 12.5 Expression of miRs and target gene in villous tissue. (**a**) Expression of miR-34C (**b**) miR139, (**c**) miR-328 (**d**) miR-210, normalized to U18. (**e**) mRNA expression of ISCU normalized to18SRNA measured in the villous tissue of control pregnancies and those complicated by preeclampsia. Values shown are mean ± SEM, *$p < 0.05$ vs. control, ($n = 6$ each group). Reproduced from Muralimanoharan et al. [41] with permission from Elsevier

To explore the role of miR-210 in placental mitochondrial respiration we conducted gain- and loss-of-function experiments using cultured primary trophoblast. Mitochondrial function of isolated trophoblast was determined by extracellular flux measurements, sequentially adding pharmacological inhibitors of the respiratory chain similar to previous approaches [109]. Overexpression of miR-210 caused a 50 % reduction in maximum respiration and 60 % reduction in reserve (spare) capacity of trophoblast cells [41]. Furthermore, miR-210 inhibition rescued the mitochondrial respiration in the presence of Deferoxamine (DFO) which chemically simulates hypoxia. When cells are subjected to stress, mitochondria are capable of drawing upon their reserve capacity to serve the increasing energy demands for maintenance of organ function, cellular repair or ROS detoxification [110]. This suggests that mir-210 acts to regulate mitochondrial function during hypoxia to prevent the cells depleting their spare capacity in order to preserve ATP production. We propose that short-term hypoxia treatment does not alter cellular ATP production, since we did not find a significant effect on the ATP coupled respiration. However, long-term hypoxia or continuous cycles of ischemia/reoxygenation may result in further depletion of mitochondrial spare capacity and an inability of the cells to maintain ATP production resulting in excessive ROS production and increase in mitochondrial damage. Based on our *in vivo* and *in vitro* functional studies, we suggest that miR-210 exerts a major influence on placental mitochondrial function during PE.

8 Summary

We have demonstrated placental dysfunction in pregnancies complicated by severe preeclampsia and clearly show sexually dimorphic responses, with the response of the male placenta being heightened relative to that of the female placenta, both in production of cytokines and in apoptotic responses. We also show increased expression and stabilization of the transcription factor HIF-1α, together with a sexual dimorphism in HIF-1α expression but a defect in binding to the hypoxia response element and corresponding reduced

expression of HIF-1α target genes including VEGF and Glut-1. HIF-1α is involved in crosstalk with the redox sensitive transcription factor NFκB in regards to their regulation by cytokines, reactive oxygen species and their effect on expression of inflammatory genes. We find increased expression and DNA binding of NFκB with preeclampsia and again a sexually dimorphic response. This perhaps underlies a role for NFκB in placental dysfunction with preeclampsia. We also find mitochondrial dysfunction in preeclampsia, evidenced by reductions in complex III activity and complex I and IV expression and alterations in mitochondrial morphology. We have also demonstrated a link between the increases in the hypoxamir miR-210 and placental mitochondrial dysfunction in preeclampsia and have identified the mitochondrial targets of miR-210 as ISCU.

Thus, we propose a model that with severe PE placental HIF-1α is stabilized by different factors including excessive production of ROS, inflammation and relative hypoxia. This, in turn, increases the expression of miR-210 in the placenta causing repression of mitochondria—associated target genes, potentially leading to mitochondrial and placental dysfunction. This placental dysfunction may lead to a fetal programming effect that results in disease in later life.

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Altitude, Attitude and Adaptation

13

Dean A. Myers and Charles A. Ducsay

Abstract

The fetus has the extraordinary capacity to respond to stress during development, which, in a large part, is mediated by the hypothalamo- pituitary-adrenal (HPA) axis. Hypoxia represents a significant risk to fetal homeostasis and can occur in a wide range of settings including maternal smoking, preeclampsia, preterm labor and high altitude. To study fetal adaptation to chronic, gestational hypoxia, we developed a model of high-altitude, long-term hypoxia (LTH) in pregnant sheep. We discuss the role of LTH on the HPA axis and potential programming of adaptive responses. LTH causes significant activation of the hypothalamic paraventricular nucleus (PVN) and anterior pituitary. In marked contrast, there is an adaptive inhibition in the adrenal, thus balancing the potentially maladaptive centrally mediated responses to LTH. Additionally, we discuss effects of LTH on adipose tissue development. LTH enhances leptin production, which in turn has a regulatory role on the adrenal cortex. Importantly, LTH also has a significant impact on programming of adipose tissue function. Together, our studies show that LTH induces a number of adaptive responses in the ovine fetus. Although they may be beneficial during fetal life, these adaptations could prove to be deleterious in the postnatal period and adulthood.

Keywords

Hypoxia • Fetus • Cortisol • Hypothalamus • Adipose

Supported by National Institutes of Health Grants PO1HD31226, R01HD51951

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L. Zhang and C.A. Ducsay (eds.), *Advances in Fetal and Neonatal Physiology*,
Advances in Experimental Medicine and Biology 814, DOI 10.1007/978-1-4939-1031-1_13,

1 Dr. Ducsay's Perspective

I first met Dr. Longo at a meeting in Oxford in 1984 and we had a long discussion about fetal physiology. At the time I was doing research on preterm labor at the Oregon Regional Primate Research Center. This was a truly serendipitous meeting, since later that year, Dr. Longo sent out an announcement that the Center was recruiting a reproductive physiologist. Fortunately I got the position and arrived in Loma Linda in December of 1985. Under Dr. Longo's leadership, I was able to establish an independent research program and receive NIH funding. His mantra was "Do good science and I'll stay out of the way." I continued my work on preterm labor, but one day, a simple conversation with Dr. Longo changed my research direction. He mentioned that he had a plan to take pregnant sheep to high altitude and study the effects of long term hypoxia (LTH) on fetal development. Although I hadn't worked with sheep since graduate school, this seemed like a bad idea. The early work of Mont Liggins, John Challis and others clearly showed that stress (i.e. activation of the fetal hypothalamic-pituitary-adrenal (HPA) axis) would lead to preterm delivery. Well here was Dr. Longo, planning on taking sheep to 13,000 ft, reducing fetal PO_2 by 25 %. This sounded stressful to me; enough so that I said as diplomatically as I could "Dr. Longo, you are going to have preterm lambs strewn all over the side of that mountain. It should be a great model for preterm labor!" Surprisingly, the ewes did not delivery prematurely. This led to our early observations that, in the late gestation LTH fetus, fetal cortisol levels were similar to normoxic controls under basal conditions. However in response to a secondary stressor such as hypotension [1] or umbilical cord occlusion [2] LTH fetuses mounted an enhanced cortisol response. Together, these data suggested that the fetal hypothalamo-pituitary adrenocortical stress axis had adapted to the long term stress of LTH to prevent what I had predicted: an early stress induced maturation of the HPA axis and a premature rise in fetal plasma cortisol, which in this species, drives parturition.

2 Dr. Myers' Perspective

During my years as a post-doctoral fellow in Dr. Peter Nathanielsz' laboratory at Cornell, Dr. Longo was one of those individuals at the annual SGI meeting that was larger than life. At the SGI meeting, he always seemed to be surrounded by all the other 'big names', a true champion in the perinatal field. It wasn't until many years later, as a Professor at the University of Oklahoma Health Sciences Center, that I would get to know Dr. Longo personally- thanks to his high altitude sheep! I was talking to Dr. Charles Ducsay from Loma Linda while at an SGI meeting in Washington DC. Charley was telling me about the high altitude fetal sheep preparation that they were working with and my first comment, as someone who had been studying the fetal sheep HPA axis for a decade and a half, was that the hypoxic stress of altitude should cause these sheep to deliver early! Over lunch later that day, he showed me the data from the LTH fetuses and then the response of these fetuses to an acute stressor and I was truly perplexed. We started to discuss the data that my laboratory was presenting at the SGI meeting on maturation of proopiomelanocortin (POMC) processing to ACTH in the fetal anterior pituitary and its regulation by the hypothalamus and how this data might provide a mechanism in the adaptation shown by the LTH fetuses. Charley invited me to give a seminar at Loma Linda, to meet the members of the Center for Perinatal Biology and discuss further potential collaboration on the LTH model. While at Loma Linda for what was to become the first of many, many visits, I was able to finally meet and have a chance to talk science with Dr. Longo. By this time, Charley and I were already discussing my participation on Charley's project on the resubmission of the NIH Program Project grant that Dr. Longo served as Program director. Dr. Longo fully supported our collaboration and my participation with the Center. Over the years of productive collaboration since those early days, Dr. Longo has made me feel fully at home, often remarking that I'd become part of the furniture of

the Center. It's truly amazing that his insightful concept of high altitude pregnant sheep would have proven so fruitful for the Center and so well recognized for its contributions to fetal and maternal adaptation to long-term hypoxia.

3 The Fetal Hypothalamo-Pituitary-Adrenocortical (HPA) Stress Axis

During pregnancy, the fetus is exposed to a wide range of intrauterine perturbations, indeed stresses, that pose mild to severe threats to fetal health and survival. However, the developing fetus has an amazing ability to respond and adapt to a wide range of potential insults, particularly as its homeostatic mechanisms mature as term gestation approaches. One of the major fetal defense mechanisms is the hypothalamo-pituitary-adrenocortical (HPA) axis. As in adults, the fetal HPA axis, via cortisol, plays a key role in the capacity of the fetus to respond to, and survive these adverse intrauterine events (stressors).

The ovine fetus has been used extensively to study the process of maturation as well as function of the HPA axis. During the final third of gestation, the ovine HPA axis undergoes a slow maturation, during which it gains an increasing capacity to respond to acute stresses. In addition, as the maturation progresses fetal plasma cortisol concentrations undergo an exponential rise culminating at term [3]. Indeed, the preterm glucocorticoid surge is a hallmark of all late gestation mammalian fetuses and essential for critical organ maturation needed to survive the transition to extra uterine life. Maturation of the HPA axis occurs at all levels, with increased expression of the ACTH secretagogues (corticotropin releasing hormone [CRH] and arginine vasopressin [AVP]) in the hypothalamic paraventricular nucleus [4, 5] as well as increased anterior pituitary expression of the ACTH precursor, proopiomelanocortin [6, 7]. This is coupled with its enzymatic processing to ACTH [8], and increased expression of steroidogenic enzymes in the adrenal cortex [9–11], in particular, the rate-limiting enzymes, CYP17 and CYP11A1. The seminal research of the late Dr. Tom McDonald and his colleagues using stereotaxic lesions of the fetal PVN definitively showed that the fetal PVN was essential for the maturation of both the anterior pituitary corticotrope as well as the adrenal cortex [12, 13]. In addition to the late gestation cortisol rise, the increased capacity of the developing fetus to mount a cortisol response to a secondary stressor reflects increased functionality of all aspects of the HPA axis, and is also dependent upon the PVN.

While the preterm rise in fetal plasma cortisol is essential for organ maturation, basal (non-stressed) plasma levels of fetal cortisol are maintained within relatively narrow limits for a given gestational age. Glucocorticoids, like cortisol, are well known for their ability to induce cellular differentiation (hence organ maturation) and therefore, oppose growth. Studies using maternal synthetic glucocorticoid treatment have clearly shown fetal growth restriction in a wide variety of species including rodents [14, 15] sheep [16, 17] and humans [18]. Similarly, episodes of acute stress resulting in repeated elevations in fetal cortisol have comparable effects on fetal growth. In sheep and other ruminants, fetal cortisol also plays an essential role of inducing parturition via activation of placental CYP17 expression leading to a decline in progesterone and increase in estradiol [19]. Thus, in these species, the HPA axis provides a mechanism via which a chronically stressed fetus, during the latter portions of pregnancy, can induce both maturation and birth, allowing it to escape the adverse intrauterine environment and increasing chances of survival. In marked contrast, fetal cortisol does not play an active role in parturition in non-human primates. Here, chronic intrauterine stressors lead to elevated basal and stress-induced bouts of fetal cortisol that impair fetal growth and ultimately hinder its chances of survival post birth. This in turn may lead to life long effects, hence programming, as so elegantly predicted by the late Dr. David Barker with his 'fetal origins' hypothesis.

Hypoxia is a common fetal perturbation, indeed stressor, which in later stages of gestation can activate the fetal HPA axis. Hypoxia can be considered either acute, ranging in duration from

minutes to a few hours, or the so-called chronic hypoxia which is several hours to days in length. In addition, the severity of the hypoxia can vary, from mild to moderate (decreases in PO_2 of ~10–50 %) to severe (50 % or greater). Hypoxic conditions can also be either episodic or sustained. As one might predict, continued exposure to severe hypoxia is often lethal or has lasting deleterious impacts on the fetus and its developing organs, in particular the CNS. Fetuses are commonly, and perhaps routinely, subjected to mild to moderate hypoxia over the course of gestation, in particular in the situations of maternal smoking, preeclampsia, preterm labor, obesity and high altitude. During latter stages of gestation, fetal exposure to moderate sustained or episodic hypoxia in sheep typically results in a robust acute fetal HPA response as well as an advanced maturation of the fetal HPA axis resulting in early delivery of a growth restricted fetus [4, 20]. Indeed, in this model, as one might expect, hypoxia increased expression of CRH and AVP in the PVN, enhanced POMC expression and elevated fetal plasma ACTH leading to a premature maturation of the adrenal cortex and cortisol production [4, 21].

However, what is the impact of sustained fetal exposure to moderate hypoxia initiating *prior to* period of HPA maturation? In the situations that are commonly associated with moderate long-term (i.e. sustained) hypoxia (LTH) described above, the fetus is exposed to hypoxia prior to maturational phase of homeostatic defense mechanisms. As discussed in the opening paragraphs, Longo and colleagues at Loma Linda developed a model of high altitude (3,820 m, Barcroft Laboratory, White Mountain Research Station, Bishop, CA) induced LTH from approx. 40 days of gestation onward. Fetal PO_2 is maintained at ~18 mmHg (normal is ~23 mmHg) thus creating a moderate hypoxic state for the duration of gestation. Indeed, this is a true state of *gestational hypoxia*. Since hypoxia, and in particular, moderate hypoxia, is a potent fetal stressor it is remarkable that these fetuses do not exhibit growth restriction or fetal acidosis, and the pregnancies are of normal duration. Our studies have focused on the impact of this LTH on the developing HPA axis and the adaptive mechanisms that have obviously been invoked to permit normal growth and maturation of the fetus in this adverse intrauterine environment.

3.1 HPA Adaptation to the High Life

Our initial studies into the function of the HPA axis in the late gestation LTH fetus were simply quantifying basal ACTH and cortisol concentrations and exploring the HPA response to secondary stressors (severe acute hypotension or umbilical cord occlusion [UCO]). Curiously, since these fetuses had been exposed to LTH since early gestation, resting fetal plasma cortisol concentrations were not different from normoxic control fetuses at the same gestational age of 136–141 days gestation (dG; term is ~145 dG). Thus, the LTH fetus preserves the exponential rise in fetal plasma cortisol during late gestation that is necessary for organ maturation and parturition and helps to explain the lack of growth restriction observed in these fetuses. Also, as predicted by the cortisol concentrations, immune-reactive (IR) ACTH concentrations in the plasma of the LTH fetuses were not different from controls. Thus, the initial indications were that the LTH fetus adapted such that they no longer responded to moderate hypoxia as danger or stress signal.

However, this concept was soon belied as we found that the response of the LTH fetus to secondary stressors, both hypotension and UCO, was resoundingly different compared to normoxic control fetuses. In response to these acute secondary stressors, the plasma cortisol response was significantly elevated vs. control while the plasma IR-ACTH concentrations achieved were similar, indicative of a change in the adrenocortical response to ACTH. However, an alternative explanation existed that centered on ACTH. While plasma IR-ACTH remains relatively constant over the final third of gestation during the period of ACTH-dependent adrenocortical maturation, the "bioactivity" of plasma IR-ACTH increases during this period [22]. Unlike adults where circulating IR-ACTH consists almost

entirely of the mature 39 residue ACTH peptide, in fetal sheep, IR-ACTH represents ACTH as well as POMC and partially processed POMC in the form of ACTH-precursors such as the so called 22 kDa proACTH in significant quantities [23, 24]. Both POMC and 22 kDa proACTH have been shown to inhibit ACTH-induced cortisol production in ovine fetal adrenocortical cells [25]. During late gestation there is a progressive PVN-dependent maturation of anterior pituitary corticotrope function including the processing of POMC toward a more adult like profile. Thus, LTH could alter the function of the HPA axis in the fetus at either the level of the hypothalamus and/or anterior pituitary or at the adrenal cortex in its capacity to respond to ACTH.

To explore the initial possibility, we addressed POMC expression and processing in the anterior pituitary as well as the concentrations of ACTH and major ACTH precursors (POMC and 22 kDa proACTH) in LTH fetal plasma, both unstressed and in response to secondary stressors in the LTH fetus. In the anterior pituitary, POMC processing to ACTH was enhanced, as were signs of increased secretion of ACTH (less ACTH stores) [26]. These changes were accompanied with sustained POMC expression. In accordance with our findings at the level of the anterior pituitary, fetal plasma concentrations of ACTH were higher in response to a secondary stress in the LTH fetuses, consistent with the increased cortisol observed during this stimulus. However, unexpectedly, we also observed that basal plasma ACTH was elevated as well in the LTH fetus, despite these fetuses maintaining normal ontogenic concentrations of cortisol in the unstressed state. We also noted that the ratio of ACTH to ACTH precursors was increased, reflective of an enhanced bioactivity of fetal plasma IR-ACTH. Thus, although basal IR-ACTH levels were similar between the LTH and control fetuses, this IR-ACTH was composed of more ACTH and less precursor.

These findings immediately led us to explore our second hypothesis: namely that the adrenal cortex had altered its sensitivity to ACTH accounting for the noted differences in the cortisol response to a secondary stressor. However, we now had to modify our hypothesis. Rather than an increased sensitivity to ACTH, it appears that the adrenocortical cells have adapted to LTH via the capacity to seemingly decrease their sensitivity to circulating ACTH (thus maintaining the normal ontogenic maturation and late gestation rise in fetal plasma cortisol). This seemed to occur while the cells still maintained the capacity to respond to a secondary stress induced increase in ACTH. The latter could be partially answered by the above noted increase in plasma ACTH in the LTH fetus in response to acute stress, but the adaptations accounting for the maintained basal cortisol production in the face of two-fold increased basal plasma ACTH seemed contradictory and somewhat enigmatic. We initially explored expression of key genes that govern various aspects of adrenocortical cortisol synthesis including response to ACTH (the ACTH/melanocortin 2 receptor [MC2R]), delivery of cholesterol to the inner mitochondrial membrane via StAR, where cholesterol is cleaved by CYP11A1 to pregnenolone (first rate limiting step in cortisol synthesis) and subsequent downstream enzymes (CYP17, CYP21, CYP11B1). Despite the elevated basal ACTH, expression of MC2R, CYP11A1 and CYP17 was approximately 50 % less in the LTH fetal adrenal cortex providing one mechanism via which these fetuses escaped the effects of the noted excess plasma ACTH. Although StAR mRNA was not different in the LTH fetal adrenal cortex, the 30 kDa 'spent' form of the protein was elevated indicative of an enhanced delivery of cholesterol to CYP11A1, which could help account for the maintained basal production of cortisol.

Based on our findings, the fetal HPA axis responded to LTH as a chronic stressor as might be predicted with a wide range of responses. These included increased CRH and AVP at the level of the hypothalamic PVN (Myers and Ducsay, unpublished observations), enhanced POMC processing to ACTH in the anterior pituitary, as well as enhanced basal ACTH levels [26] and enhanced ACTH in response to an acute secondary stressor [1, 2]. At the same time however, the fetal adrenal exhibited a unique adaptive response to prevent premature maturation of the cortex that would result in early birth of a

growth-restricted fetus. Yet, these fetuses retained an enhanced cortisol response to an acute secondary stressor, perhaps reflective of a need for these fetuses 'living on the edge' to mount a greater stress response. Deciphering the mechanisms of how the fetal HPA axis adapted to LTH resulting in these responses, in particular the counterintuitive changes noted in the adrenal cortex, was clearly the next step. Identifying the factors mediating these changes could be translated to human pregnancies where a chronically hypoxic, growth restricted fetus was at risk to limit the deleterious actions of sustained moderate hypoxia.

3.2 The Search for the Great Mediator

The adaptive response at the adrenal cortex to LTH clearly involved both intracellular mechanisms as well as extracellular mediators of the hypoxic environment. One such factor is nitric oxide (NO) and we have recently reviewed the role of NO in the regulation of adrenal steroidogenesis in response to LTH [27]. Another novel potential mediator that caught our eyes was the adipose polypeptide hormone, leptin. Leptin had been reported as a physiological suppressor of the HPA axis in adults at both the level of the PVN as well as directly at the adrenal cortex. Both the long, or active splice variant of the leptin receptor (ObRb) as well as short variant (ObRa) of the leptin receptor were found to be expressed in the adult adrenal cortex. Further, leptin had been reported to suppress both ACTH stimulated CYP11A1 and CYP17 expression. In rodent adrenals, leptin also acts to limit corticosterone production via effects on StAR and PBR proteins.

Leptin circulates in fetal sheep, albeit at lower levels compared to adults and in sheep, like humans [28], is primarily produced by the developing adipose tissue. Intracerebral infusion of leptin to late gestation fetal sheep limits both the amplitude and mean levels of ACTH and cortisol pulses. Further studies in fetal sheep by McMillen and colleagues demonstrated that intravenous leptin infusion inhibited the fetal HPA axis, suppressing both the prepartum rise in ACTH and cortisol [29]. The effects were reduced as term pregnancy approached. Based on these pharmacological findings of an effect of exogenous leptin on the fetal HPA axis, we initially asked whether leptin was even elevated in the LTH fetus. Indeed, leptin is a hypoxia inducible gene and hypoxia plays a role in adipose expansion and angiogenesis. We reported that fetal plasma leptin as well as perirenal adipose expression of leptin were elevated in the late gestation LTH fetus [30]. In addition, adrenocortical expression of the ObRb was increased in the adrenal cortex of the LTH fetus. Thus, we hypothesized that leptin was poised to be a potential mediator of the adaptive responses we observed in the HPA axis of the LTH fetus.

To explore leptin as a possible mediator of the effects of LTH that we observed on the fetal HPA axis, and in particular on the fetal adrenal cortex, we performed a 4 day infusion of an ovine leptin receptor antagonist to LTH and normoxic control fetal sheep starting at 139 dG [31]. Surprisingly, during the infusion period, the leptin receptor antagonist had no effect on plasma ACTH or cortisol in either the LTH for normoxic control fetuses compared to saline infused controls for either group. Also curious, the leptin receptor antagonist had no effect in the control fetuses on either CYP11A1 or CYP17 expression, even though STAT3 phosphorylation (STAT is a key component of the leptin receptor signaling pathway) was suppressed. However, in the LTH fetal adrenal, CYP11A1 and CYP17 expression was restored to levels similar to control fetuses. This study emphasized a key finding on the role of leptin in both normal function of the late gestation ovine fetal HPA axis: namely that endogenous leptin may not be playing a physiological role in regulating the maturation of the fetal HPA axis since prior studies used pharmacological levels of leptin [29, 32]. Alternatively, leptin may play a role earlier in gestation, in particular when the fetal adrenal cortex undergoes its approximately mid-gestation loss of cortisol production despite maintained ACTH levels. The latter is supported by our observations that younger fetuses at ~120–130 dG have elevated ObRb compared to near term fetuses [33].

A second major discovery from our studies with the leptin antagonist infusion [31] was that while leptin appears to be a mediator of the adaptation observed at the level of the fetal adrenal cortex to LTH, at least in terms of CYP expression, it is not the only mediator since no effect was observed on either ACTH or cortisol levels. Perhaps the most intriguing finding from these series of studies in the LTH fetus was that fetal perirenal adipose, which initiates its differentiation at approximately mid gestation, may be subject to hypoxic modification of function beyond simply increasing production of leptin. Indeed considering the susceptibility of developing adipose and other organs involved in metabolism for programming for later obesity in the offspring by a variety of intra-uterine stressors or maternal conditions, we felt compelled to further explore the impact of LTH on the developing adipose in the ovine fetus.

3.3 Perirenal Fat- Which Color Does LTH Prefer: Brown, Beige or White?

The perirenal fat deposit in sheep and human fetuses is largely considered a brown fat deposit, characterized by high expression of uncoupling protein-1 (UCP1; [34–36]. UCP1 catalyzes adaptive thermogenesis in brown adipose deposits by dramatically increasing the proton conductance of the inner mitochondrial membrane [37]. Expression of UCP1 peaks during the final week of gestation [38, 39], assuring effective thermoregulation for the newborn extra-uterine environment via non-shivering thermogenesis. In addition to its expression, UCP1 is activated at birth, accompanied by elevated lipolysis and mobilization of available fat stores.

Since we observed that LTH results in the upregulation of perirenal adipose leptin expression leading to elevated fetal plasma leptin [30], we determined if any other brown or white adipose genes were affected by development under these conditions of sustained moderate developmental hypoxia. At 136–139 dG, we noted a significant elevation in expression of UCP1. In addition, we noted increased expression of deiodinase 2 (DIO2; catalyzes the conversion of T_4 to T_3), 11β hydroxysteroid dehydrogenase I (HSD11B1; catalyzes the conversion of cortisone to cortisol), PPAR , and PGC1α, all hallmarks of the brown fat phenotype [30]. In support of LTH enhancing the functionality of this brown fat deposit in the ovine fetus, we also noted increased expression of the β3 adrenergic receptor (AR) and transcription factors NRF2 and mtTFA, the latter of which regulate expression of genes governing mitochondrial function [40]. However, we have not found evidence of increased mitochondrial numbers in the perirenal fat of the LTH fetus (Myers, DA and Ducsay, CA, unpublished observations). Thus, LTH may simply increase the activity of existing mitochondria in this fat store. Based on these observations, it appears that the LTH environment is preparing these fetuses to more efficiently generate non-shivering thermogenesis in the post-natal environment.

The mechanism(s) governing the increased brown adipose tissue (BAT) phenotype of the perirenal fat deposit in the LTH fetus is presently unknown. Since exogenous glucocorticoids enhance expression of UCP1 and adrenalectomy prevents the late gestation increase in expression of UCP1 in the ovine fetus [38, 41, 42], the noted increase in HSD11B1 may facilitate local metabolism of cortisone to cortisol, representing one mechanism for the enhanced BAT phenotype in the LTH perirenal adipose deposit. Similarly, T_3 is a known mediator of the BAT phenotype [38] and DIO2 expression is elevated in the perirenal adipose of the LTH fetus. Thus, these two tissue specific responses in the perirenal adipose in response to LTH may allow this tissue to increase its BAT phenotype without systemic increases in cortisol or thyroid hormone which would deleteriously impact fetal growth and organ function with potentially life long consequences. An increase in sensitivity to catecholamines via the increased βAR3 expression may play a role in the adaptive response of the perirenal fat to LTH [38, 43].

The fetal perirenal adipose depot, while considered to have a brown fat phenotype due to its high level of UCP1 and other BAT associated genes, is not typical of brown fat since it consists

of a mixed population of both multilocular and unilocular fat deposits. Unilocular fat is typical of white fat as opposed to the classic dense multilocular nature of the classic brown fat deposits, and similarly is less vascular. Further, fetal perirenal fat expresses leptin at levels typical for white fat and the expression of leptin is equally distributed in both unilocular adipocytes as well as multilocular adipocytes in this fat store. UCP1 immunostaining appears similarly distributed in fetal perirenal fat. However, other genes that are hallmarks for brown fat are highly expressed in late gestation fetal perirenal fat including the transcriptional co-activators, PRDM16 and PGC1α, and the transcription factor PPAR , as well as genes such as DIO2 and CIDEA, which are highly expressed in brown fat [44, 45]. In adults, some white adipose tissues can express high levels of UCP1 upon either cold exposure or activation of cAMP signaling pathways e.g., adrenergic stimulation [46, 47]. In addition to UCP1 expression there is an increased multilocular appearance of these fat deposits during the 'browning' process. However, while BAT is derived from a myf-5 lineage i.e., muscle cell/BAT precursor, [48–50], these fat deposits are derived from the classic white adipose tissue (WAT) lineage [48–50]. The brown fat like cells in these fat deposits have been referred to as "Beige" or "Brite" cells, and the process via which UCP1 expression is increased has been termed "Beiging" [48–50]. Considering that the perirenal fat rapidly loses its expression of UCP1 and other phenotypic BAT genes (e.g., PGC1α) post birth [45], perirenal fat may represent a true beige deposit and not the classic myf-5 derived BAT.

The increased BAT phenotype of the perirenal fat during late gestation in response to LTH raises an intriguing question considering that increases in BAT in experimental animals is associated with a leaner phenotype while a loss in BAT is associated with obesity and its related metabolic disorders [48, 51, 52]. Thus, our findings have implications for the LTH offspring post-birth in terms of both sensitivity to fat deposition and metabolism if the BAT phenotype of the perirenal fat is maintained. This is especially relevant considering the dramatic increase observed in childhood and adult obesity and metabolic disorders. The '*fetal origins of adult disease*' hypothesis of Barker [53–55] purports that so-called adverse intrauterine environments can cause permanent epigenetic imprints in the embryo/fetus, predisposing it to susceptibility to a variety of diseases, including obesity and metabolic disorders *later in life*. Indeed, maternal under nutrition during gestation is linked to obesity and type II diabetes later in life [54, 56]. However, few studies have focused on fetal origins of *childhood obesity* or of equal importance, *early changes in adipose function* that can predispose an individual to obesity, metabolic disorders and cardiovascular disease.

An increased risk of obesity and metabolic syndrome has been reported among children born from gestational diabetic or obese mothers [57]. Further, children of obese mothers or mothers with gestational diabetes have a greater neonatal fat mass [58]. A study by Oken et al. [59] reported that the rate for 'risk for obesity' was 17.1 % with a 9.7 % 'obesity' rate in children of obese mothers, compared to 14.2 % and 6.6 %, respectively, in children of non-obese mothers. Thus, while maternal obesity (gestational and pre-gestation obesity) is strongly linked as a causative factor in childhood obesity [58], it is apparent that not all children born from obese women will develop obesity [58], and a significant population of children from non-obese/diabetic mothers are at high risk or will develop childhood obesity. This emphasizes that other factors contribute to the programming of childhood obesity. Therefore, we propose that fetal hypoxia, via its impact on developing abdominal fat, predisposes the offspring to early fat deposition.

In studies in the LTH fetus, we found that in addition to BAT phenotypic genes, genes governing WAT expansion and function are also upregulated by LTH (e.g., PPAR , HSD11B1). Thus, we were intrigued as to which phenotypic adaptation, if any, was maintained in the LTH lamb post birth. Surprisingly, by 14 days post birth, the LTH lambs lost the brown fat phenotype significantly compared to the normoxic control lambs [60]. This included a decrease in UCP1 as well as

the transcriptional regulators of the brown fat phenotype, PGC1α and PRDM16. However, PPAR and PPARα expression as well as HSD11B1 expression was similar in the perirenal fat of the LTH and control lambs at 14 days post-birth. Considering that brown and/or beige fat is thought to be protective of adiposity, it appears that the in utero LTH environment may have impacted the perirenal fat in such a manner to favor fat deposition/expansion (PPAR and HSD11B1) rather than lipid metabolism (UCP1). It will be of considerable interest to see how these lambs respond if provided access to a restricted diet or a diet high in fat and or carbohydrates and to further follow the changes in gene expression in perirenal fat stores as well as visceral and subcutaneous fat.

4 Perspectives/Conclusions

The fetus has the ability to successfully respond to acute stressors. As we have detailed above, it also responds to long-term stressors like high altitude hypoxia. It does so with an "attitude" towards adaptation with a balance between upregulation of the hypothalamic-pituitary axis and a down regulation of adrenal responsiveness at the basal level. One of the mechanisms involved in this adaptation is activation of adipose tissue and enhanced production of leptin. This regulation of basal cortisol is crucial to survival and may also have the added advantage of enhancing the brown fat phenotype in anticipation of birth into a potentially hostile environment. However, in the transition from fetal to neonatal life, there is a shift to an enhanced white fat phenotype that has the potential to result in enhanced adiposity in later life. At present, the mechanism(s) of this unintended consequence of fetal adaptation to altitude remains to be elucidated. However, the results of these studies strengthen the hypothesis that LTH plays a key role in fetal programming with effects long after birth. Dr. Longo always closes his letters and emails with the phrase: "persevere." It is remarkable that in his LTH sheep that the fetus has done just that!

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The Separation of Sexual Activity and Reproduction in Human Social Evolution

14

Scott Morin, David Keefe, and Frederick Naftolin

Abstract

In industrialized societies the progression of natural selection has been determined and in many cases superseded by social evolution. In the case of reproduction, there has been a decline and delay of childbearing without diminished sexual activity. While this has value for these societies, there are penalties associated with barren cycles. These include increases in endometriosis and breast and genital cancer. There also are associated issues regarding population movements that fill the "vacuums" left by underpopulation. These matters are of more than passing interest as we cope with unintended consequences of Man's dominance over the environment and other life forms.

Keywords

Evolution • Reproduction • Cancer • Endometriosis • Environment • Demography • Genetic engineering

1 Introduction

The fundamental theory of Darwinian (physical-adaptive) evolution states that species adapt to their ecological niche through the selection of traits that confer survival advantage. The result is natural selection of better-adapted strains to the exclusion of less well-adapted strains. This constant competition is based on one all-encompassing goal and its mechanism—the passage of one's genes to the next generation through chromosomal recombination and other mechanisms that occur during reproduction. Through natural selection mechanisms that drive reproduction are highly developed, very sensitive to evolutionary pressures and deeply woven into the genetic fabric of each individual. In humans, reproduction is accomplished through sexual activity. In support of reproduction there have evolved a set of physical and behavioral systems of penalty and reward that encourage sexual behavior, reward fertile cycles, support

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L. Zhang and C.A. Ducsay (eds.), *Advances in Fetal and Neonatal Physiology*,
Advances in Experimental Medicine and Biology 814, DOI 10.1007/978-1-4939-1031-1_14,

pregnancies, safe parturition and lactation/nurturing of offspring, at least until they are of reproductive age. There are penalties for barren cycles and failed reproduction [1–5]. These pro-reproductive adaptations have been honed over hundreds of thousands of years of evolutionary pressure and are among the most fundamental characteristics [6, 7]. However, there are at least three examples of deviation from this narrow construct of reproduction as a cardinal attribute; eusociality/inclusive fitness, reproductive altruism and human social evolution.

1.1 Eusociality/Inclusive Fitness

Natural selection follows many routes to success. One, termed eusociality, is a permanent stratification of behavior into reproductive (queens) and non-reproductive members (helpers and workers) within a clade of insects, that results in the most success for the group [8]. This kind of purposeful sterility was commented upon as early as the mid-nineteenth century, by Charles Darwin "… one special difficulty, which at first appeared to me insuperable, and actually fatal to my whole theory….[I]t can be shown that some insects and other articulate animals in a state of nature occasionally become sterile; and if such insects had been social, and it had been profitable to the community that a number should have been annually born capable of work, but incapable of procreation, I can see no very great difficulty in this being effected by natural selection. But I must pass over this preliminary difficulty. The great difficulty lies in the working ants differing from both the males and the fertile females in structure, as in the thorax, and in being destitute of wings and sometimes of eyes, and in instinct….I can see no great difficulty in any character becoming correlated with the sterile condition of certain members of insect-communities: the difficulty lies in understanding how such correlated modifications of structure could have been slowly accumulated by natural selection. This difficulty, though appearing insuperable, is lessened, or, as I believe, disappears, when it is remembered that selection may be applied to the family, as well as to the individual, and may thus gain the desired end…" [9] (Quoted by Hunt) [10]. In this discussion, Darwin's insights foreshadowed the possibility that higher forms might develop strategies with reproductive traits that fostered the survival of the species. This includes rewards and penalties that maintain a clear path to natural selection ("inclusive fitness").

Others have studied and illuminated eusociality and inclusive fitness among insects [8, 11–13]. And, the rules governing eusociality are currently under consideration as possibly analogous behaviors among higher forms [14–17]. Although no primates have been reported that show the stratification based on irreversible sterility that are found in insects; rather, it has been pointed out that reproductive altruism, i.e. elective abandonment of reproduction for the good of the group has many parallels to eusociality [18]. While a case might be made that genetic infertility, as in Turner Syndrome, or the anovulation that accompanies metabolic syndrome, are akin to eusociality. However, these have not been proven to be adaptive or contribute to the good of the group.

1.2 Sociobiology, Group Selection and Reproductive Altruism, Including "The Grandmother Effect."

There has been a persistent thread of interest about intragroup behavior in higher forms, including humans, which might have a basis in natural selection and contribute to the success of the species [19]. There are some similarities to eusociality [14, 20]. In general, this line of observation has been in regard to the contribution of related or unrelated third parties who furnish food, and otherwise protect the at-risk offspring [13, 21–25]. Other forms of reciprocity that support the group have been likened to sociality among humans [18]. While the benefits of direct protection and provision are well-established, their actual impact on evolution remains to be proven. In fact, some have made the argument that forms of altruism could have adverse effects with time [26]. Recent studies have attributed

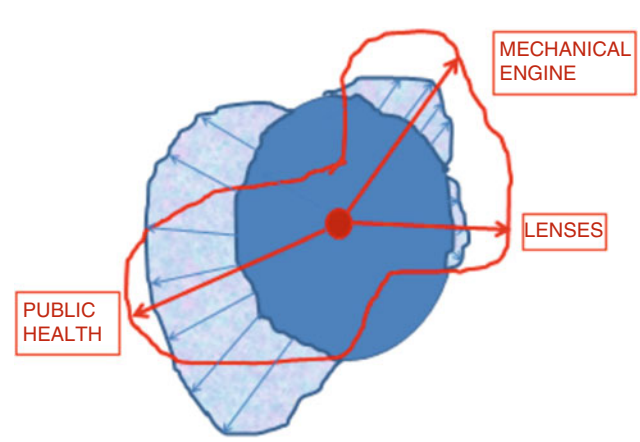

Fig. 14.1 Social Evolution as the driver of Natural Selection. Although Natural Selection (*dark blue area*/perimeter) has greater inertial mass, it is driven to fill ecological niches (*light blue area*) exposed by Social Evolution (examples—*red perimeter* and *arrows*). As evolved forms flow into these niches the context of evolution changes. In this manner, Social Evolution has become the zeitgeber of human evolution

molecular/genetic contributions to the offspring. Trans-generational maternal and grand-maternal effects are an exciting new vision made possible through the development of molecular biology and genetics. It will be interesting to revisit the field in future. For the moment, however, insufficient data are available to materially contribute to the present discussion [13, 14, 27, 28].

In summary, aside from the recent appearance of Human Social Evolution (social evolution), natural selection has been working throughout history and has set the table for both the emergence of social evolution and for Man's willful control of his and her home and environment. The results have been truly historic and presage elements of human evolution and of the willful control of reproduction.

Social Evolution—During evolution adaptations have evolved that allow higher forms to thwart others that are comparatively stronger, faster or otherwise more physically fit. The most consequential of these adaptations in hominids is the development of a large and complex prefrontal cortex of the brain made up of closely packed gyri. This furnishes a remarkable surface area:volume ratio in which Homo sapiens houses the neurons that enable the intellectual capacity that has allowed Man to best competitors through brainpower rather than physical fitness. This outcome has also led to the transition from dependence solely on slow, stepwise physical-adaptive-evolution as we could be steered by technological advancement and the social systems that allow us to gain from the remarkable advances over the environment, competitors and disease. To be sure, physical-adaptive evolution and its dependence on reproduction continues, but it has often been side-stepped or pulled along by the development of novel ecological niches in the wake of contributions during social evolution, Fig. 14.1.

Management of Man's pre-eminence has necessitated the development of social systems that allowed co-existence of humans, cooperative efforts and activities that largely escape the confines of physical-adaptive evolution. Social evolution generally progresses in a series of solutions to problems that do not require natural selection, just the preparation of intellectual stepping stones. In this manner, there is no reasonable expectation of a failure or even slowing of social evolution. Social evolution is sensitive to its surroundings. For example, although humans early on used their mental prowess in agrarian pursuits,

groups developed that capitalized on their intellect first to improve agriculture and later to construct tools and machines to replace animal and human labor; the industrial revolution was born [29]. The power of industrialization and the need for protecting the environment gave birth to modern methods of public health; management of waste, development of systems to furnish clean water at great distances, and public health measures such as vaccines, antibiotics and hygiene [30, 31]. With the resultant freedom from early death because of acute illnesses, out-breeding became increasingly common and a source of lineage-dependent social evolution [32].

In summary, while physical-adaptive evolution remains a force, it is slow, random and rarely a match for the intellectual agility and products of the prefrontal cortex. Rather, the industrial revolution and the development of modern public health measures signaled temporal ascendancy of social evolution over natural selection. To be sure, for many reasons there has been an uneven development of this transition; agrarian societies remain the least affected by social evolution and populate large portions of today's world. However, even this reflects the impact of the industrial revolution that has furnished agricultural tools, pesticides and economical fertilizers that enable more modern agricultural lifestyles. So far since the march of industrial progress has given developing populations demographic license but not relief from poverty and they are overflowing into the industrialized world. This becomes especially obvious as the birth rates fall in the latter societies [33].

1.3 Social Evolution, Demography, the Role of Women, Sexual Activity and Reproduction

Freedom from fear of competitors among the other species, the use of surrogates and machines to compensate for lack of evolutionary fitness and the ability to prevent and treat illness has resulted in unheard of ventures by Man. The success of social evolution has given "survival of the fittest" a different meaning as the exclusion of unfit individuals has been overruled by the ability of Man to side-step natural selection. Among its other results, this has repurposed reproduction and made it a matter of choice in industrialized societies [34] despite the penalties of barren cycles (see below). This pivotal movement in modern society has occurred without evidence of reduced sexual activity. In fact, since sexual activity triggers the reward system that drives reproduction, regardless of the reproductive rate it is not likely that sexual activity will slake in the foreseeable future [35].

The control of reproduction and the spread of market economies have been accompanied by the emergence of women in the workforce, and more self-determination than existed for females under physical-adaptive evolution [36]. More recently the availability of assisted reproductive technologies has allowed even more tailored family-spacing and fostered the ascendancy of women in industrial societies through delay of childbearing to fit career goals [37].

Though sex without reproduction may be seen as adaptive in the context of social evolution, it is maladaptive in terms of physical-adaptive evolution. The shift to barren cycles is a form of antagonistic pleiomorphism in which costs of low reproductive productivity are exacted on women and may result in adverse outcomes. Since men continue to practice sexual behavior, they seem to have escaped the evolutionary penalties of declining populations, except in (less productive) agrarian societies that require workers. Further, decreased reproduction in response to social evolution slows natural selection.

2 Large-Scale Genetic Engineering Has Enabled the Ascendency of Social Evolution

The forces of Darwinian evolution are incremental, requiring both a niche to fill and characteristics to fill it [9]. Generally, adaptive traits lead to reproductive fitness and maladaptive traits are

deselected if they impair competition for reproduction. Under social evolution the development of advanced cognitive function has changed this hierarchy of evolutionary forces. In all but the most agrarian societies, classical Darwinian laws of natural selection no longer are the only drivers of survival and reproductive success. After early evidences of increased intelligence [38], the effects on hominids were limited due to problems in communication and the inability of large populations to be impacted by the results of new applications. With out-migrations from Africa, the impact of superior skills and tools was felt in ever larger areas; there was consolidation of populations, hybrid vigor and the filling of ecological niches by groups that did not rely on physical-adaptive mechanisms to dominate their environs. Starting with inventions such as agriculture, humans moved on to astronomy, architecture, seamanship, gunpowder, non-feudal social and political systems, public health measures and the industrial revolution, all of which shifted the playing field to favor non-agrarian societies [39–41].

One mainspring of these changes was the ability for Man to overrule Darwinian evolution through massive, clinical genetic engineering; for example the industrial revolution was spurred by the steam engine and other machines that negated the importance of personal strength. In another example, ground lenses improved the vision of individuals previously disadvantaged by their poor eyesight. In this case, those who had been limited by their inferior vision and were previously unable to compete for resources and reproduction were made fit and able to pass their lineage to the next generation. On a larger scale, wide employment of public health measures spared individuals susceptible to environmental infections and toxins to overcome these dangers regardless of their natural fitness [42]. Vaccination eradicated or significantly reduced the impact of diseases which had killed or severely debilitated individuals prior to their opportunity to reproduce [43]. The development of antibiotics allowed treatment of infections that were once fatal, and, the discovery and use of agents like insulin extended the life of diabetics into the reproductive period. Thus, without prejudice, previously unfit traits that were on the way to being excluded were *pari-passu* retained and are now on the rise [44]. Thus, social evolution need not concur with natural selection, and might address niches whose presence might not have been forecast in past millennia, see Fig. 14.1.

In summary, modern Man is engaging in genetic engineering on the grandest scale imaginable. These activities were neither planned (eugenics) nor directly genomic (gene manipulation). Many who would have been excluded by Darwinian "survival of the fittest" have been spared and/or rendered capable of reproducing. Physical-adaptive evolution remains at play, but the forces of societal change act much more swiftly; in most cases, it takes centuries to select for a beneficial physical trait while the genetic pool may be altered by economic or geopolitical forces in a single generation. Thus, the development of the human condition has been moved by social, economic, and geopolitical forces; social evolution.

3 Antagonistic Pleiomorphism and Penalties of Sexual Activity Without Reproduction

For males the imperative to engage in aggression against other males in order to impregnate more females is unaffected by the separation of sexual activity from reproduction. On the contrary, for women, social evolution has incurred penalties related to infertile ovarian/endometrial cycles. In addition to the energy and hemoglobin costs of extra cycles, these include pre-menstrual syndrome [5], endometriosis [4], and breast, endometrial and ovarian cancer [1–3]. Reinforcing this point, these conditions are all prevented by pregnancy and by contraception that blocks ovulation and/or cause endometrial atrophy [45, 46].

To some degree, the development of Assisted Reproductive Technologies has mitigated the psychological impact of delayed pregnancy. However, it appears that this form of genetic

engineering has not been without penalties; there is an increase in age-related congenital abnormalities, especially Down Syndrome and a newly appreciated role of aging males in conditions such as the autism spectrum [47].

4 Other Impacts of Separating Sexual Activity from Reproduction

Aside from public health measures, in industrial societies few advances in technology have had a more profound societal impact than effective, safe and easily available contraceptives. This is especially true for contraception managed by women [36]. Their ability to regulate childbearing has opened the way to entry, and a more profound engagement, in the workforce [48]. The widespread use of contraception also has been accompanied by the acceptance of an unprecedented separation between male-female relations and reproduction, especially in the industrialized societies. This has extended beyond the family unit. The average age of first intercourse for women in the US remains around the time of puberty while the age at first pregnancy continues to rise [49]. Social and medical resolution of unwanted pregnancies are generally available, though this remains a vexing personal and societal issue [50, 51]. Sexual relationships are common among couples with no expectation of, or future plans for, reproduction. As these relationships have become more common, they have also become more accepted as normal in much of the industrialized world. This is exemplified by the shift in attitudes regarding children and marriage; only 41 % of American adults now report that children are very important to a successful marriage, a decline from 65 % in 1990 [52]. As well, the relaxation of the pressure to maximize reproduction has cast a different light on same-sex relationships and has led to a more open public dialog about homosexuality and gay and lesbian relationships and legal rights [53].

5 Antagonistic Pleiomorphism: Challenges and Opportunities Surrounding the Changing Role of Women in Industrialized Societies

When previously adaptive behavior becomes maladaptive, it may be termed antagonistic pleiomorphism. Often the change is a result of environmental shifts' resulting in formerly adaptive behavior being stranded as a kind of atavism, and no longer adaptive [54]. In the present case, sex with reproduction is adaptive in a Darwinian context, but may not satisfy goals driven by social evolution. This, then, is an example of antagonistic pleiomorphism related to the emergence of social evolution. We have mentioned how this shift has played out in the role of women in industrial societies. The effects of this shift in responsibility for the planning, management and outcome of reproduction will not easily be absorbed by the slower process of physical-adaptive evolution. Rather, it appears that women/couples will have to adapt to their new options regarding reproduction's involvement in contemporary society rather than adapting for natural selection. For example, women have used it to enlarge their role in political, corporate and individual activities. The results have been impressive, as has already been seen throughout the world [55, 56].

During physical-adaptive evolution the anchoring of women to the family unit, plus pregnancy-related illnesses and maternal mortality pushed women into the background of most societies. Women had little control over their reproductive lives, and were relegated to childbirth and childrearing. Especially in non-industrialized societies, women have been treated as chattel and widely abused. We have seen the two extremes of genital mutilation for "purity" and children and women being sold into sex work coexisting. There is the proxy use of women in "honor killings" for the benefit of male-dominated

tribal societies. These all remain open wounds related to tribalism, one of the most basic characters of natural selection. In fact, many unreconstructed societies have continued to suppress the emergence of women. Female literacy lags well behind male literacy in a large number of countries. Literacy rates have been demonstrated to correlate with contraceptive use in the developing world [57, 58]. And, wherever rape is used as a tool for social dominance, the advancement to a state with equal rights and opportunities for each sex remains challenging. Social evolution has the promise to cleanse Mankind of such practices. The emergence of females to an equal footing with their male counterparts is linked to their control of reproductive function. And, through the process of social evolution and managed childbearing the role of women has substantially improved in most industrialized societies. Females now comprise the majority of college graduates in the United States every year and are the breadwinners in 41.4 % of American families [59]. Women have risen to the head of state in many countries. These advances have been hallmarks of social evolution and are entwined with the separation of sexual activity from reproduction. Further, the cost of childrearing in non-agrarian societies today often necessitates two incomes. As a result, couples often delay childbearing with the use of contraception in an effort to obtain sound financial footing prior to attempting to reproduce. In some cases, the increased focus on financial stability and the associated delay in attempts to conceive result in stress and difficulties with subfertility [60]. More couples are now utilizing assisted reproductive technologies as a means to help conceive after missing their most fertile window, which further contributes to acceptance of the separation of reproduction and sexual activity.

Does sociobiology offer leads about the evolutionary impact of these matters? Behavior of lower forms may have indicated what man could do despite the reproductive imperative of natural selection, including reproductive- and other forms of altruism, and, departures from evolutionary norms had already been shown to have immediate and long-term effects, including sociological punishments for this straying from the norm, that are presently under study [61–63].

6 Conclusions

Although the flora and fauna of the world remain responsive to the principles of Darwinian evolution, in humans the tempo and requirements of natural selection have been overtaken by social evolution and its consequences. Social evolution now opens ecologic niches in the plant and animal kingdoms that accelerate the impact of Man. But, the characteristics and alacrity of agrarian and industrialized populations to adopt change differs. Perhaps the main differences are bound up in the attitude toward reproduction as a sustaining attribute, the role of women in society and the demographic and wealth shifts that have brought about the separation of sexual activity from reproduction. Review of the rewards and penalties of this situation shows an impressive undercurrent of personal penalties related to delay or cessation of reproduction. Examples include higher rates of mood disorders, endometriosis, infertility and cancer. There also are societal consequences, for example, the continued pace of sexual activity in the absence of reproduction also has impacted social structures based on monogamous relationships.

Above all, it should be obvious that factors underlying such matters as the separation of sex from reproduction must be viewed in the context of the moving stage that is the evolving world. These developments must be seen as talismans of the need to better understand our biological and social surroundings. Diminished populations in industrialized regions plus the threat of food shortages, climate change and increasing disparities in the distribution of resources all forecast increasing encroachment of migration out of impoverished agrarian areas by unsophisticated populations. But, this will only happen after the indigenous populations strip their areas of

vegetation and edible/valued species. The losses of diversity and of the flora that protect our environment are fraught with terrible consequences. It is time to "stop, look and listen," as we were taught to beware of oncoming traffic. It is necessary to perform the research and employ the remedies that will ensure that there will be something to bequeath our successors [64].

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The Influence of Growth Hormone on Bone and Adipose Programming

15

Anita M. Oberbauer

Abstract

In utero growth hormone exposure is associated with distinct immediate growth responses and long term impacts on adult physiological parameters that include obesity, insulin resistance, and bone function. Growth hormone accelerates cellular proliferation in many tissues but is exemplified by increases in the number of cells within the cartilaginous growth plate of bone. In some cases growth hormone also potentiates differentiation as seen in the differentiation of adipocytes that rapidly fill upon withdrawal of growth hormone. Growth hormone provokes these changes either by direct action or through intermediaries such as insulin-like growth factor-I and other downstream effector molecules. The specific mechanism used by growth hormone in programming tissues is not yet fully characterized and likely represents a multipronged approach involving DNA modification, altered adult hormonal milieu, and the development of an augmented stem cell pool capable of future engagement as is seen in adipose accrual. This review summarizes findings of growth hormone's influence on in utero and neonatal cellular and metabolic profiles related to bone and adipose tissue.

Keywords

Growth hormone • Adipose tissue • Insulin-like growth factor • Bone growth • Small-for-gestational age

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L. Zhang and C.A. Ducsay (eds.), *Advances in Fetal and Neonatal Physiology*, Advances in Experimental Medicine and Biology 814, DOI 10.1007/978-1-4939-1031-1_15,

1 Fetal Programming and Growth

It is well recognized that the hormonal and environmental milieu in which the fetus develops greatly impacts health and disease susceptibility in later life [1, 2]. Early intrauterine nutritional and environmental stressors or endocrine challenges may all program the fetus such that adult physiology is altered. For instance, metabolic bone diseases are purported to arise from prenatal undernourishment [3]. Evidence likewise exists for neonatal metabolic programming for obesity. Small for gestational age (SGA) offspring, often arising as a consequence of malnourished mothers, is also correlated with adult hypertension, glucose intolerance, insulin resistance, type 2 diabetes, dyslipidemia, reduced bone mass, and reduced bone strength. Differential fetal programming occurring at the gene level with methylation, gene silencing, and other epigenetic modifications permit adult onset disorders associated with SGA neonates (reviewed in [4]).

2 Role of GH-IGF in Normal Growth and Development

Postnatally, the growth hormone (GH) insulin-like growth factor (IGF) axis is the predominant endocrine pathway that modulates overall body growth [5, 6]. In utero, GH plays a much reduced role as compared to postnatal development with other hormones assuming more prominent fetal roles: insulin, IGF-I, IGF-II, glucocorticoids, and thyroid hormones being essential in normal fetal growth [1]. Yet it is known that GH influences fetal growth. For instance, when mice transgenic for a GH construct are exposed to the transgene stimulus, hence elevated GH, in utero, they exhibit a 12 % reduction in birth weight [7] and calves born to dams given exogenous bovine somatotropin (GH) have reduced birth weights [8, 9]. Insulin levels of calves born to GH treated dams also tended to be reduced at birth [9] reflecting additional influences due to GH.

In a cohort of infants having low birth weight, at 1 year of age IGF-I levels were not associated with being born SGA. Likewise, when 17 years of age, there were no associations between IGF-I levels and being born SGA [10]. There was however a trend in children born SGA to have lower circulating GH as young adults [10] indicating persistent effects by early developmental experiences on GH secretion.

Interestingly, despite the early growth compromise, approximately 90 % of SGA births have accelerated growth in the first two postnatal years and achieve a final height that is within the normal range [11, 12]. However the remaining 10 % of SGA births do not exhibit catch-up growth indicating sustained impairment of the GH-IGF endocrine axis suggesting fetal programming of the axis as a consequence of early growth dysfunction [13].

Evaluating early effects of GH on inflammation and immune competence also suggests a direct role of GH in fetal and early neonatal development. Treating SGA rat pups with exogenous GH during the pre-weaning growth stage reverses the persistent negative effects on immune function that accompanies SGA [14]. The ability of GH to reverse chronic inflammatory conditions suggests that GH can restore epigenetic changes evoked by uterine growth perturbations. The responsiveness of the neonate demonstrates the plasticity of postnatal development.

As noted above, postnatal development is highly regulated by GH. For instance, in GH transgenic mouse models, mouse pups born and nursed by dams exposed to elevated GH grow approximately 50 % faster than contemporary pups nursed by dams with normal circulating GH [15] and transgenic pups stimulated to express GH likewise show significant growth enhancement. In normal rodent development, the composition of gain is invariant such that each unit of gain is composed of the same proportions of water, lipid, protein and ash across the entire growth phase, despite the speed of accrual [16]; under conditions of elevated GH, composition of protein gain exceeds that of the other components [17] indicating GH repartitions nutrients preferentially.

3 Programming Linear Bone Growth by GH

In addition to protein accretion, another key growth parameter controlled by GH is linear bone elongation and bone remodeling. A consequence associated with SGA neonates is reduced bone mass and strength in adulthood reflecting programming of this axis [18]. As noted above, the vast majority of SGA births achieve normal height within the first 2 years [11, 12] demonstrating competence of the growth plate chondrocytes regulating linear growth. Early therapeutic provision of GH to SGA neonates having sufficient GH enhances the velocity of bone growth transiently but only for the duration of GH treatment [4]. Similar acceleration effects of GH on linear growth rates have been reported for rodents. As seen in children, transient high GH levels in mice increased growth rate but did not alter overall outcome on bone length [7]. Importantly, for rodents the developmental stage of exposure is critical to bone elongation more so than duration of exposure [19]. Specifically in the mouse, provision of GH between birth and 28 days is most influential on bone elongation and adult size. Initiating the elevated GH beyond 28 days of age increases growth but not to the extent realized with earlier exposure despite the presence of a functional growth plate. At the cellular level, GH accelerates bone growth by hyperplasia as opposed to growth plate chondrocyte hypertrophy [19].

Calves and rodents exposed to elevated GH in utero have reduced birth weights. In mice, bone lengths are reduced postnatally and catch-up growth is not detected by weaning (22 days postnatal) but if GH is provided after birth then the postnatal exposure to GH compensates for the in utero growth inhibition reversing the effect and overall bone length is restored [7]. In another model that altered fetal GH, treatment of pregnant sows with beta hydroxyl beta methylbutyrate significantly enhanced GH and IGF-I in the piglets [20]. In piglets from the treated dams, femur lengths remained significantly reduced at 6 months of age though measures of bone strength were improved. Taken together, these data indicate exposure of the fetus to elevated GH represses linear bone into adulthood.

4 Programming Adiposity by GH

Offspring from mothers who are undernourished during pregnancy exhibit SGA at birth and adult onset obesity, insulin resistance, hypertension, and metabolic dysfunction. These conditions can be ameliorated by providing exogenous GH at early postnatal stages with GH treatment improving cardiovascular function and reversing endothelial dysfunction [14, 21]. For example, in rats, the adiposity that typically accompanies adulthood in offspring of undernourished mothers can be reversed by provision of GH treatment preweaning ([22] and reviewed in [23]). Caution is warranted however as provision of GH to neonates can result in the development of insulin resistance [24].

Elevated GH in adult mice significantly increases circulating insulin with the levels remaining elevated even after reducing circulating GH levels demonstrating insulin resistance [25]. Insulin resistance is observed in SGA prepubertal children (reviewed in [4]) and Jensen et al. [13] suggest that those that do not experience catch-up growth may be even more at risk for type 2 diabetes. Concern surrounds therapeutic GH treatment of SGA children because GH reduces insulin sensitivity potentially exacerbating the risk of diabetes. Although insulin levels in children born SGA return to normal levels following cessation of GH treatment [4], the SGA children who have been treated with GH are not yet old enough to fully evaluate consequences of GH treatment on adult carbohydrate metabolism.

Insulin resistance promotes the retention of lipid stores thereby contributing to adult onset obesity reflecting. The elevated insulin, as a consequence of the elevated GH, may also induce lipoprotein lipase further promoting adipose storage. Prenatal adipocyte differentiation with enhanced lipogenic and adipogenic capacity also promotes adult obesity as seen in SGA offspring [26].

White adipose tissue in humans and rodents possess a reservoir of precursor cells that can continue to differentiate [27, 28]. In a GH transgenic mouse model in which the GH transgene can be directly and specifically regulated to increase circulating GH, elevated GH either in utero or postnatally results in increased adipose depot size and lipid stores [7]. The adiposity was due to both greater adipocyte numbers as a result of hyperplasia and differentiation driven by GH and enlarged cellular lipid content [25, 29].

A possible mechanism explaining GH contributions to the development of SGA, and thus future adiposity, is the impact of GH on maternal leptin levels. Elevated GH can drive adipocyte differentiation increasing maternal adipose storage potential thereby increasing overall level of leptin in circulation. Notably, concomitant with enhanced adipose content driven by elevated GH, in transgenic mice transiently exposed to elevated GH, associated with the elevated leptin levels is elevated NPY gene and protein expression suggesting leptin resistance [30]. However, maternal exposure to GH also drives the development of fetal adiposity as seen in sheep treated with GH. Although leptin levels were reduced in both the dam and the fetus as a response to treatment, adipose was significantly amplified [31] indicating the direct effect of GH on adipose metabolism.

An additional mechanism of potential GH programming lies in its effects on membrane lipids. Elevated GH alters cellular membrane characteristics to create a more unsaturated lipid profile [32]. Membrane desaturase pathways activated in response to GH cause a net flux through the lipid metabolism pathways to generate eicosanoids [33]; many of the changes are consistent with the inflammation conditions observed in chronic health disturbances associated with the obesity and other adult sequelae of SGA [24].

5 GH Programming of Leptin

Leptin, first identified in adults, has a clear role in postnatal and adult energy metabolism (reviewed in [34]). The fetus too is exposed, in varying degrees, to leptin derived from the maternal circulation, the placental, and the fetus itself. Leptin is known to effect pre- and postnatal development with autocrine/paracrine mechanisms preferred over the endocrine system [34]. Leptin regulates brain and bone development in the fetus [35], controls intrauterine and periuterine growth, and programs appetite drive in the neonate to facilitate rapid body weight accrual [36] indicating that in fetal metabolism, leptin has an analogous role as it does in the adult. Fetal exposure to leptin is also intimately involved in fetal programming of adult satiety including that of hunger-mediated ingestive behavior. Specifically, reduced neurotransmission of leptin signals of energy storage adequacy during neonatal life chronically alter adiposity and food intake regulation at later ages [36, 37].

Leptin levels correlate with birth weight: SGA infants have low leptin levels while large for gestational age babies born to mothers with diabetes have high leptin concentrations [38]. This association appears to be independent of the GH/IGF endocrine axis reviewed by [36] although GH can impact maternal and fetal leptin levels as detailed below.

Despite the knowledge of leptin's involvement, the specific mechanistic role of leptin in fetal life is less well defined especially in light of the varied sources of leptin. As an example, insulin and IGF-I concentrations are directly correlated with leptin in newborn [39] but the correlation does not answer the mechanistic question of whether leptin is directing fetal growth or the leptin is a mere reflection of adipose accrual in the growing fetus since fetal adipose is significant source of leptin [40]. It is also known that leptin in fetal circulation can be regulated by circulating insulin [41] and dams with elevated insulin influence the leptin levels in the fetus. Further, leptin derived from the placenta may influence fetal growth by influencing placental function.

Maternal leptin levels increase over the course of pregnancy while the fetus during the final trimester of gestation contributes ever increasing amounts of leptin as the fetal white adipose depots enlarge [36]. The placenta synthesizes leptin with the majority entering maternal circulation though the role of placental leptin in the

fetus varies by species (reviewed [34]) as does the placenta's permeability to leptin. In the sheep maternal and fetal leptin levels correlate with nutritional plane of the dam but that does not hold true for primates [40]; the rat placenta is more similar to the sheep with leptin permeable to the placenta [42].

Elevated maternal leptin in mice correlate with reduced placental and fetal weights of their offspring [43]. In contrast, human offspring born to mothers with gestational hyperleptinemia are born with higher leptin levels and are at risk of being large for gestational age [36]. These mothers also had elevated insulin that increases placental leptin synthesis leading to greater exposure of the fetus [44]. In a prospective human study designed to assess the impact of maternal weight gain during pregnancy on offspring body weight, it was found that cord leptin was positively associated with birth weight and excessive weight gain during pregnancy was directly associated with cord hyperleptinemia [45]. In a separate prospective human study, elevated maternal leptin levels accompanied SGA births [46] indicating that elevated maternal leptin reflected impaired fetal growth. Thus the influence of maternal leptin in human fetal development remains indeterminate.

Circulating neonatal leptin, for human infants, is associated with numerous fetal growth indicators including body weight, bone length as reflected in crown rump length, adiposity, and bone mineral density (reviewed in [34]). Low leptin in SGA neonates correlates with elevated leptin and leptin resistance at adulthood. The initial function of neonatal leptin resistance may be permissive for the catch-up growth seen in the majority of SGA offspring [47]. In neonatal pigs and humans exhibiting nutritionally induced SGA birth weights, nutritionally adequate diets during the postnatal period facilitates catch-up growth though there is a greater proportion of lipid accumulation than would accompany normal developmental growth [48].

In rats, restricting maternal dietary protein to induce intrauterine growth restriction and SGA pups, these SGA pups, when adults, have excess abdominal adipose depots [49]. Further, as adults, the fat depots exhibit altered gene profiles for genes associated with lipid metabolism and adipose accrual upregulated. Interestingly, neonatal provision of exogenous leptin to SGA piglets and rat pups reverses the adipocyte proliferation induced by intrauterine growth restriction [37, 48]. Excessive calories in the neonate creating "maternal diet-induced obesity", leads to leptin resistance in adulthood optimizing conditions favorable for adult onset metabolic disease [50]. McMillen et al. [51] reported that fetal leptin can modify adipocyte metabolism leading to disrupted appetite regulation of the neonate culminating in programmed leptin resistance, enhanced adiposity, and adult obesity. It is also known that leptin can drive adipocyte differentiation. The action of leptin and leptin resistance in the neonate clearly requires additional study.

Leptin resistance occurs when the receptors for leptin fail to transmit adequacy of energy stores. It is speculated that leptin resistance, programmed during fetal and neonatal life, represents impaired maturation of the neural circuits informing the body of energy stores [48]. The reduced leptin levels in SGA infants are suspected to disrupt normal neuronal programming and adult regulation of appetite. Supporting this concept is that provision of leptin to SGA rodent pups can normalize adult metabolism [37]. Increased expression of fetal neuropeptide-Y (NPY), leptin's receptor, may also contribute to the obesity that follows SGA [26, 51].

Dysregulation of GH early in life can increase adipocyte proliferative and differentiative potential thereby predisposing an individual to elevated leptin levels that would impact adult life. In GH transgenic mice with a regulated promoter enabling transient exposure to excessive GH, mice transiently exposed to elevated GH during early postnatal development rapidly become obese upon withdrawal of the elevated GH; accompanying the adiposity is an overall increase in circulating leptin [25]. In these animals, each adipocyte expresses less leptin such that on a per gram of adipose basis leptin secretion is reduced but the cumulative amount is greater due to the overall enhanced adipose storage. It is hypothesized that the reduced leptin expression at the

adipocyte level represents a GH induced insulin resistance and reduced adipocyte glucose utilization. At the hypothalamus, elevated leptin corresponds to enhanced transcript and protein expression of NPY [30]. Mice in which the GH remains elevated also exhibit reduced leptin expressed on per gram of lipid [29].

Whether GH directly alters leptin in utero or whether it is through a secondary mechanism remains unknown. However, GH can drive adipocyte differentiation enabling future adiposity, and potentiate insulin and leptin resistance. Enlarged adipose depots will synthesize more leptin further exacerbating the leptin resistance. Taken together, altered perinatal leptin levels can affect fetal programming and ultimately potentiate adult metabolic disorders.

6 Conclusion

Growth hormone directly impacts bone length and strength postnatally while substantive evidence exists to demonstrate a significant role of GH in utero with adult consequences. Similarly GH modulates adipose development pre- and postnatally at both the proliferation and differentiation stages, including adipose membrane involvement. Growth hormone may exert its programming influence through leptin and persistent effects on its action. Additional studies are necessary to characterize the contribution of GH and the mechanistic changes that mediate the fetal programming observed.

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The Fetal Cerebral Circulation: Three Decades of Exploration by the LLU Center for Perinatal Biology

16

William J. Pearce

Abstract

For more than three decades, research programs in the Center of Perinatal Biology have focused on the vascular biology of the fetal cerebral circulation. In the 1980s, research in the Center demonstrated that cerebral autoregulation operated over a narrower pressure range, and was more vulnerable to insults, in fetuses than in adults. Other studies were among the first to establish that compared to adult cerebral arteries, fetal cerebral arteries were more hydrated, contained smaller smooth muscle cells and less connective tissue, and had endothelium less capable of producing NO. Work in the 1990s revealed that pregnancy depressed reactivity to NO in extra-cerebral arteries, but elevated it in cerebral arteries through effects involving changes in cGMP metabolism. Comparative studies verified that fetal lamb cerebral arteries were an excellent model for cerebral arteries from human infants. Biochemical studies demonstrated that cGMP metabolism was dramatically upregulated, but that contraction was far more dependent on calcium influx, in fetal compared to adult cerebral arteries. Further studies established that chronic hypoxia accelerates functional maturation of fetal cerebral arteries, as indicated by increased contractile responses to adrenergic agonists and perivascular adrenergic nerves. In the 2000s, studies of signal transduction established age-dependent roles for PKG, PKC, PKA, ERK, ODC, IP3, myofilament calcium sensitivity, and many other mechanisms. These diverse studies clearly demonstrated that fetal cerebral arteries were functionally quite distinct compared to adult cerebral arteries. In the current decade, research in the Center has expanded to a more molecular focus on epigenetic mechanisms and their role in fetal

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L. Zhang and C.A. Ducsay (eds.), *Advances in Fetal and Neonatal Physiology*,
Advances in Experimental Medicine and Biology 814, DOI 10.1007/978-1-4939-1031-1_16,

vascular adaptation to chronic hypoxia, maternal drug abuse, and nutrient deprivation. Overall, the past three decades have transformed thinking about, and understanding of, the fetal cerebral circulation due in no small part to the sustained research efforts by faculty and staff in the Center for Perinatal Biology.

Keywords
Fetal calcium metabolism • Fetal cerebral circulation • Fetal endothelium • Fetal hypoxia • Fetal signal transduction

1 Introduction

Through the 1970s, interest in the fetal cerebral circulation grew steadily. The foundational studies of Rudolf and colleagues [1] had firmly established the idea that cardiovascular homeostasis was negotiated very differently in the fetus than in the adult. A broad variety of reviews also advanced the idea that patterns of CNS damage in the human infant due to hypoxia and trauma were highly dependent on gestational age [2] and exhibited very different patterns of injury than observed in adults [3]. Interest in the fetal cerebral circulation was further stimulated by a growing number of cases involving open-heart surgery in human fetuses and neonates [4]. Together, these clinical priorities motivated numerous basic science studies of the fetal cerebral circulation. In 1971, Zamenhof and colleagues laid the groundwork for modern epigenetics with their studies of the trans-generational effects of maternal food restriction on brain growth in rat offspring [5]. The elegant and ground-breaking studies of Nuwayhid and colleagues clearly established that responses to adrenergic and cholinergic agonists were very different in the fetal and adult pulmonary circulation [6], and that in turn, autonomic regulation of the heart and lungs exhibited many unique characteristics in fetuses compared to adults [7]. Further work by Su and colleagues reinforced the view that adrenergic, cholinergic, and serotonergic vascular neuroeffector mechanisms were markedly different in fetal and adult arteries [8, 9]. Together, these early basic science studies set the stage for a rapid expansion of studies of the fetal cerebral circulation.

2 The 1980s: Studies of Cerebral Hypoxia and Autoregulation

As the decade of the 1980s opened, clinical interest in the fetal cerebral circulation was growing rapidly. In his now classical review in the New England Journal of Medicine, Volpe laid out the main features and issues related to neonatal intracranial hemorrhage [10]. Other prominent reviews focused on the increased incidence of intraventricular hemorrhage in premature infants [11] and the heightened vulnerability of the immature cerebral circulation to hypoxia and ischemia [12], and even strokes [13]. Whereas the progression of many of these insults to neonatal hypoxic-ischemic encephalopathy was well recognized [14], no mechanisms responsible for the unique vulnerability of the fetal brain were clearly identified. As recognized in several major reviews, the lack of understanding of the etiology of fetal brain injury was attributed to the fact that most studies of this category of pathophysiology had been conduced in autopsy specimens, many of which were in advanced stages of disease [15].

With this realization came a new motivation to examine the structure and function of the fetal cerebral circulation using animal models.

Among the first investigators to use animal studies to implicate compromised cerebrovascular regulation as a cause of neonatal hypoxic-ischemic encephalopathy were Robert & Susan Vannucci [16], whose neonatal rat model of hypoxic ischemia has now been used in more than 1,000 publications. About the same time that the Vannucci model was being established, a fetal lamb model was introduced [17] that offered many advantages, the most notable of which was that it enabled chronic instrumentation. With the fetal lamb model, many important findings quickly accrued, include the connection between fetal asphyxia and vasogenic brain edema [17], the reduced efficiency of cerebral autoregulation in the fetus ([18–20] and the vulnerability of fetal cerebral autoregulation to hypoxic insults [21]. Within this context, the Ashwal, Longo team at LLU were among the first to use the microspheres technique in a chronically catheterized fetal lamb preparation to simultaneously measure cerebral perfusion to more than 30 brain regions to establish that cerebral blood flow was highly heterogeneous in the fetal brain, and that vasodilatory responses to acute hypoxia lasted long after normoxemia was restored [22, 23]. The Ashwal, Longo team at LLU went on to establish the fundamental responses of the unanesthetized fetal cerebral circulation to hypercapnia, acidosis, hypotension [24], evoked auditory potentials [25], and calcium channel antagonists [26]. Together, these studies demonstrated that the fetal cerebral circulation was preferentially distributed to the brain stem, exhibited an attenuated hypercapnic reactivity, exhibited excellent coupling between local cerebral metabolism and local perfusion, and autoregulated over a very narrow range of blood pressures, compared to adults.

The burst of studies of fetal autoregulation in the 1980s focused attention on fetal cerebrovasculature and its unique contractile characteristics. To better understand how the contractility of fetal cerebral arteries contributed to overall flow-metabolism coupling the fetal brain, the pial window technique was adapted for use in neonatal piglets and used in many studies to reveal that pial arteries received a functional sympathetic innervation at birth [27], were highly reactive to hypoxia and hypercapnia [28, 29], and were highly dependent on prostanoid metabolism for many vascular responses [30]. Despite the in vivo advantages of the pial window technique, however, this approach did not provide a clear definition of pial vascular reactivity independent of the adjacent brain tissue; all applications of exogenous substances influenced both the pial vasculature and the underlying brain parenchyma. For this reason, interest in isolated cerebral arteries, studied in vitro, grew rapidly. Although the study of isolated adult cerebral arteries began in the 1970s [31–33], it was not until the mid-1980s that fetal cerebral arteries were studied in vitro [34]. These early studies illustrated that the contractility of fetal cerebral arteries was highly dependent on gestational age such that responses to vasodilator prostanoids decreased with fetal age, whereas vasoconstrictor responses increased [34]. The first contribution in this area from LLU came from the team of Ashwal and Pearce [35], which was the first to demonstrate direct vasodilator effects of acute hypoxia on fetal cerebral arteries that varied with artery size and age.

3 The 1990s: Growing Interest in Fetal Cerebral Vascular Biology

3.1 Effects of Pregnancy on Cerebral Arteries

The rising interest in fetal cerebral arteries in the early 1990s quickly translated into a parallel interest in the effects of pregnancy on maternal cerebral arteries, particularly at LLU. The team of Hull, Longo, and Pearce produced a series of contributions that revealed that pregnancy depressed reactivity to NO in maternal extracerebral arteries, but elevated it in cerebrals [36]. Further studies detailed the parallel effects of pregnancy on cGMP synthesis and endothelium-dependent relaxation [37] and also revealed that

pregnancy-induced increases in contractility were depressed by chronic hypoxia [38]. Other studies advanced the idea that human placental arteries were responsive to exogenous NO, but not to most endothelium-dependent vasodilators [39]. These early publications helped form a background against which additional studies of fetal cerebrovascular structure and function could be performed.

3.2 Effects of Postnatal Maturation on Cerebral Artery Structure and Contractility

The initial studies of isolated fetal cerebral arteries at LLU examined the effects of maturation on artery structure and function and were the first to measure wall thicknesses and active wall stresses in these small arteries [40]. These findings established that fetal carotid arteries matured both structurally and functionally much sooner than did cerebral arteries, and that fetal cerebral arteries had much smaller smooth muscle cells, had much larger extracellular space, and had greater water content than did adult cerebral arteries [41]. Interestingly, fetal arteries were more compliant, but more reactive to stretch [42] and aminergic agonists [43] than were adult arteries. This increased reactivity appeared attributable, at least in part, to a greater sensitivity to calcium in fetal cererbral arteries, as determined by indirect measurements [44]. This series of studies also introduced the ideas that cerebrovascular maturation decreases binding affinity for norepinephrine, and more so in 4th branch than 2nd branch middle cerebral arteries [45]. Parallel studies were also the first to show that maturation right-shifted dose-response relations for ATP-sensitive potassium channel activators, indicating that fetal cerebral arteries exhibited unique and highly reactive electrophysiological characteristics compared to adult arteries [46]. In addition, fetal cerebral arteries exhibited a depressed reactivity to electrical transmural stimulation, indicating that the cerebral sympathetic perivascular innervation was probably not fully functional at term [47]. Equally important, these early functional studies also suggested that cerebral arteries from the fetal lamb were a good model for studying the structural and functional characteristics of human infant cerebral arteries [48].

3.3 Effects of Postnatal Maturation on Signal Transduction in Cerebral Arteries

Owing in large part to the many publications in the early 1990s that the vascular endothelium was a major determinant of vascular reactivity [49], early studies of fetal cerebrovascular signal transduction at LLU focused on endothelium dependent responses, including reactivity to exogenous NO donors [50], which was greater in immature than in mature cerebral arteries. Corresponding measurements of rates of cGMP synthesis and turnover further revealed that basal cGMP was more than 5× greater in newborn than adult arteries, that rates of cGMP synthesis were 2× greater in newborn than adult arteries, and that rates of cGMP degradation were 50 % greater in fetal than adult arteries [51]; clearly a major component of age-related differences in endothelium-dependent relaxation was attributable to corresponding differences in cGMP metabolism.

Another focus of work at LLU in the 1990s was on calcium biology in the fetal cerebral vasculature. Initial studies suggested that for equivalent active stresses, immature cerebral arteries required greater calcium uptake than did adult arteries [52, 53]. This greater reliance on calcium uptake appeared due to reduced sensitivity to IP3 despite elevated levels of IP3 receptors in fetal compared to adult cerebral arteries [54, 55]. Subsequent measurements of myofilament calcium sensitivity in permeabilized preparations suggested that calcium sensitivity was greater in fetal than adult cerebral arteries, particularly following activation of G-protein coupled receptors [56, 57]. Companion studies further demonstrated that 5HT-induced contraction of both fetal and adult cerebral arteries relied on activation of Rho-kinase but not PKC or PKA [58]. For norepinephrine-induced

contractions, the influx of extracellular calcium through L-type calcium channels was also critically important for contraction, particularly in fetal cerebral arteries [59].

3.4 Effects of Hypoxia and Ischemia on the Fetal Cerebral Circulation

In regards to pathophysiology, the majority of studies of the fetal cerebral circulation at LLU in the 1990s focused on the effects of hypoxia. Whereas acute hypoxia clearly had the capacity to relax isolated fetal cerebral arteries [60] through endothelium-independent mechanisms [61], chronic hypoxia dramatically altered the structure and contractility of both fetal and adult cerebral arteries and attenuated the vasodilator efficacy of NO [62, 63]. In relation to norepinephrine, hypoxia also decreased adrenergic receptor density and NE-induced IP3 mobilization [64], but upregulated pre-synaptic adrenergic reactivity while simultaneously depressing post-synaptic adrenergic reactivity [65]. In relation to serotonin, hypoxic effects were less pronounced than for norepinephrine and were attributable largely to decreases in 5HT-receptor density [66].

Another area of focus of hypoxia studies at LLU in the 1990s was on the role of parenchymal ornithine decarboxylase as a sensor and/or mediator of the cerebral effects of hypoxia [67]. These unique studies revealed that acute hypoxia increases ornithine decarboxylase activity and polyamine concentrations in fetal brain [67] through pathways that can also be activated by administration of CO to the mother [68]. Interestingly, these effects of hypoxia could be elicited even in newborn brain slice preparations [69], and appeared to be mediated by oxygen radicals [70], indicating the fundamental cellular nature of the effects of cerebral hypoxia.

At the whole animal level, work at LLU in the 1990s focused increasingly on the effects of cerebral ischemia [71]. Effort was invested to develop a middle cerebral artery occlusion model in SHR pups [72] and this preparation enabled elucidation of a neurotoxic role for NO following transient focal cerebral ischemia in the immature brain [73]. Subsequent studies of nNOS biochemistry revealed that nNOS abundance was heterogeneously distributed throughout the fetal brain, and that nNOS cofactor levels were not present at saturating conditions [74]. Correspondingly, cofactor supplementation studies indicated that local ischemic vulnerability was strongly influenced by both nNOS abundance and cofactor availability, and more so in immature than in mature brain [75, 76]. A key conclusion of these studies was that age-related and regional differences in ischemic vulnerability could be explained, at least in part, by differences in overall nNOS activity.

4 The 2000s: Calcium, cGMP, PKC, and Fetal CBF

The year 2000 ushered in an era of renewed enthusiasm for studies of cerebral development and maturation. Early studies revealed the presence of a novel fatty-acid binding protein in fetal brain with potential involvement in neuronal differentiation and axon growth [77]. Within the fetal vasculature, the expression of the key contractile protein smooth muscle alpha-actin proved to increase significantly as a function of maturity [78]. Systematic studies identified all four known classes of potassium channels in fetal cerebral arteries and their involvement in both norepinephrine-induced [79], and 5HT-induced [80], contractions. Detailed studies of fetal and adult cerebrovascular BK channels further revealed that these channels were more sensitive to calcium in the fetus than in the adult [81], due at least in part to differential phosphorylation by PKG and PKA [82]. Together, these findings helped explain why BK channel activity was inherently greater in fetal than adult cerebral arteries [83].

At the level of G-protein coupled receptors, studies from the Longo lab revealed that the alpha-2 subtype of adrenergic receptor was chiefly prejunctional in both fetal and adult cerebral arteries, but the fetal cerebral arteries also expressed a significant post-synaptic population of these receptors [84]. For serotonin, ERK

activation appeared as a significant downstream component of 5HT receptor activation, particularly in fetal arteries [85]. Incubation with dexamethasone also exhibited a time-dependent ability to attenuate 5HT-induced contractions through a cyclooxygenase-dependent pathway in fetal arteries [86]. Conversely, incubation with dopamine enhanced contractile responses to 5HT [87], suggesting that vasoactive compounds commonly administered to neonates in the NICU may have conflicting effects on cerebrovascular contractility.

4.1 The Role of Calcium in the Functional Maturation of Fetal Cerebral Arteries

Exploration of the contractile role of calcium in fetal and adult cerebral arteries proceed at a rapid pace throughout the 2000s. Studies of NE-induced contractions demonstrated less reliance on calcium release from the sarcoplasmic reticulum, and far greater reliance on calcium influx in fetal compared to adult cerebral arteries [88]. Direct measurements of intracellular calcium mass using isotope tracer methods revealed that fetal cerebral arteries have significantly less IP3-releasable calcium than adults, and that the ryanodine-releasable pool is relatively small in both age groups [89]. Correspondingly, immunoblotting measurements demonstrated that L-type calcium channel density was greater in fetal than in adult cerebral arteries [90]. From a functional perspective, simultaneous measurements of wall calcium with Fura-2 photometry and artery diameter suggested that myofilament calcium sensitivity was lower in term fetal than adult cerebral arteries [91]. Because this result contradicted previous measurements indicating greater calcium sensitivity in immature arteries [56], this finding was very interesting. Ultimately, this inconsistency led to the hypothesis that not all smooth muscle cells in the artery wall are of the same phenotype, contractility, and calcium sensitivity. Thus, membrane permeabilization as used in the earlier study [56] would "clamp" calcium in all smooth muscle cells, regardless of phenotype. In contrast, Fura-2 photometry would record calcium signals from all cells, both contractile and non-contractile. This important hypothesis motivated detailed studies of smooth muscle phenotype in the cerebral artery wall, and has since appeared to be correct [92, 93]; compared to adult cerebral arteries, fetal cerebral arteries appear to contain a larger proportion of smooth muscle cells in the synthetic phenotype which exhibit a relatively high basal calcium that changes little in response to agonist stimulation.

To further explore relations between calcium concentration, myosin light chain phosphorylation, and contractile force, studies by Nauli et al. measured all three variables simultaneously again using membrane permeabilized preparations [94]. These interesting studies enabled discrimination between "thick filament regulation," which determined the relation between cytosolic calcium concentration and myosin light chain phosphorylation, and "thin filament regulation", which determined the relation between myosin light chain phosphorylation and contractile force. With this approach, fetal cerebral arteries exhibited greater overall calcium sensitivity (calcium vs. force) than did adult cerebral arteries, even though calcium was less able to promote myosin light chain phosphorylation (thick filament regulation) in fetal compared to adult cerebral arteries. Conversely, the relation between phosphorylated (activated) myosin light chain and contractile force (thin filament regulation) was markedly upregulated in fetal compared to adult cerebral arteries. To test the physiological importance of these differences, reliance on calcium influx for myogenic tone in 2nd branch middle cerebral arteries was explored and found to be significantly greater in immature than mature cerebral arteries [95]. Separate experiments in which myofilament calcium sensitivity was measured directly in membrane permeabilized preparations corroborated the earlier findings [96], confirmed that fetal arteries exhibit depressed thick filament regulation and enhanced thin filament regulation, and suggested for the first time that myogenic stretch is more tightly

coupled to myosin light chain phosphorylation in adult than in fetal arteries [97]. Owing to the important age-related differences in myosin light chain phosphorylation observed in fetal and adult arteries, further experiments were carried out to measure MLCK activity, in situ using a custom-built, rapid freeze apparatus [98]. Consistent with previous results, MLCK activity was markedly less in adult than in fetal arteries. Further studies in membrane permeabilized 2nd branch middle cerebral arteries were the first to confirm that immature cerebral arteries contain a greater proportion of non-contractile smooth muscle, and as a consequence rely more on myofilament Ca(2+) sensitization and Ca(2+) influx to maintain myogenic reactivity than do adult cerebral arteries [99].

4.2 Roles of PKC and cGMP in Cerebrovascular Maturation

In light of the important age-related differences in cerebrovascular calcium handling indicated by studies in the Center for Perinatal Biology, a variety of further studies were initiated to better understand what mechanisms were responsible, with particular emphasis on cytosolic kinases. In turn, inhibition of PKC augmented NE-induced IP3 and calcium responses in adult, but not fetal, cerebral arteries whereas PKC stimulation increased calcium in fetal but not adult arteries, suggesting a critical role of PKC in age-related differences in cerebrovascular calcium handling [100]. Application of the ERK inhibitor U0126 potentiated phenylephrine-induced contractions in fetal but not adult arteries, indicating an age-dependent role for ERK in modulation of contractility [101]. Subsequent studies revealed that PKC activation preferentially activated ERK2 in fetal arteries, and ERK1 in adult arteries, further implicating ERK in age-related differences in contractility [102]. This study also suggested that PKC activation increased Rho-kinase in fetal arteries, but activated CPI-17 and caldesmon in adult arteries; again, cytosolic kinases appeared to play a major role in age-related differences in cerebrovascular contractility.

Another kinase studied extensively in the 2000s was guanylate cyclase, which is the intracellular "effector" for many of NO's actions within vascular smooth muscle [103]. A series of systematic experiments by Nauli et al. [104] demonstrated that maturation of cerebral arteries attenuated the ability of cGMP to promote vasorelaxation. Further studies revealed that soluble guanylate cyclase was more abundant [105], and that cGMP was more potent as a vasorelaxant [106], in fetal than adult cerebral arteries. Further studies in membrane permeabilized preparations showed that cGMP attenuated myofilament calcium sensitivity more effectively in fetal than adult cerebral arteries [107]. Detailed studies of relaxation rates indicated that upon stimulation with NO, cGMP concentrations rise more rapidly in immature than mature cerebral arteries due to greater rates of phosphodiesterase activity in adult arteries [108]. Additional studies showed that maturational increases in endothelial vasodilator capacity [109] were attributable to age-dependent increases in NO release secondary to elevated eNOS specific activity [110] and abundance [111]. Together, these studies strongly implicated both guanylate cyclase and PKC as major determinants of age-dependent cerebrovascular reactivity.

4.3 Effects of Hypoxia on Fetal and Adult Cerebral Arteries

Studies of cerebrovascular pathophysiology also continued throughout the 2000s with a particular emphasis on hypoxia. Morphometric measurements indicated that chronic hypoxia increased endothelial cell density and smooth muscle cell size in fetal cerebral arteries, but had opposite effects in adult cerebral arteries [112]. Measurements of 5HT receptor density, binding affinity and IP3 generation showed that 5HT was more efficiently coupled to IP3 synthesis in fetal than adult arteries [113] and that acute hypoxia decreased 5HT receptor density and binding affinity much more in adult than in fetal arteries [114]. For norepinephrine contractions, chronic hypoxia depressed cytosolic calcium and overall

contractility much more in adult than in fetal arteries, due in part to attenuation of potassium-channel mediated relaxation in fetal arteries [115]. In other studies, chronic hypoxia decreased cerebrovascular nNOS abundance, due presumably to decreased innervation by nitridergic nerves, particularly in immature arteries [116]. Ex vivo neurophysiological studies also demonstrated that chronic hypoxia can markedly attenuate calcium-induced calcium release, SERCA function, and subsequent norepinephrine release in fetal sympathetic neurons [117].

Regarding the NO/cGMP pathway, studies in the late 2000s indicated that chronic hypoxia depressed NO release via reduced eNOS specific activity without decreasing eNOS abundance [118]. In parallel, hypoxia also decreased soluble guanylate cyclase activity in fetal but not adult arteries without a change in mRNA abundance for guanylate cyclase [119]; hypoxia clearly affected some aspect of mRNA translation, suggesting the possible involvement of microRNAs. Overall, chronic hypoxia inhibited NO-induced vasodilation in both adult and fetal ovine cerebral arteries via decreased sGC activity [120]. Together, these results emphasized that chronic hypoxia induces a variety of both adaptive and maladaptive effects in fetal cerebral arteries including increased protein content, decreased IP3 synthesis and IP3 receptor expression, increased 5HT receptor affinity, decreased activity of ATP- and calcium-sensitive potassium channels, and decreased calcium-dependent myosin phosphorylation [121, 122]. As a whole, these findings led to the important hypothesis that fetal responses to chronic hypoxia may "program" the fetal cerebral circulation and thereby alter its function throughout postnatal life.

4.4 Fetal Cerebral Blood Flow, In Vivo

To compliment the many studies conducted using isolated cerebral arteries, Center faculty invested considerable effort to develop and test contractile mechanisms through measurements of cerebral blood flow in intact, unanesthetized, chronically instrumented fetal sheep [123]. This novel method helped illustrate the involvement of NO in hypoxic vasodilatation of fetal cerebral blood flow [124], the ability of only 4 min of umbilical cord occlusion to ablate cerebral autoregulation [125], the absence of ischemic preconditioning responses in fetal cerebral circulation responses to repeated cord occlusions [126], the indirect role of prostanoids in fetal cerebrovascular responses to hypoxia [127], the effects of hypercapnia on cerebral oxygenation in the fetus [128], the positive effects of maternal oxygen supplementation on fetal cerebral oxygenation [129], and the ability of the fetal brain to adapt to chronic hypoxia through modification of oxygen extraction [130]. Similar studies have also demonstrated the ability of maternal caffeine to decrease fetal cortical oxygen tensions [131], the potential effects of maternal hypocapnia, CO_2 supplementation, and hypercapnia on fetal cerebral oxygenation [132, 133], and finally, the tight coupling between ECoG state with cerebral oxygen consumption and cerebral blood flow in the term fetal brain under both normoxic and hypoxic conditions [134]. As a whole, this group of studies helps depict the fetal cerebral circulation as a highly dynamic system capable of adaptation and homeostasis mediated by mechanisms unique to the fetus. Clearly, the relation between cerebral metabolism and cerebrovascular resistance is tightly coupled in the fetal brain, but is mediated by mechanisms quite distinct from those in the adult cerebral circulation.

As an adjunct to studies of the fetal lamb cerebral circulation, efforts to develop new animal models to explore stroke and ischemia in the immature brain have also continued [135], although with less activity than during the 1990s. In terms of relevance to the human infant cerebral circulation, the Angeles group employed MRI techniques to assess brain injury in human infants. Most interestingly, NICU infants treated with opioids during the first week of life demonstrated less brain injury and better long-term neurologic outcomes than infants not treated. This study has important implications for the long-term effects of neonatal stress and pain, and suggest the possible involvement of epigenetic mechanisms [136].

5 The Current Decade: The Work Continues

Many of the research programs initiated in the 2000s remain active in the current decade. New ultrastructural studies of cerebrovascular morphology have revealed dramatic changes in the size and shape of the smooth muscle cells in fetal cerebral arteries, further suggest that cerebral arteries contain a structurally highly heterogeneous population of smooth muscle cells, and indicate that development of the extracellular matrix during late fetal development is highly dynamic [137]. Studies comparing patterns of gene expression in fetal, newborn, and adult cerebral arteries point to dramatic activity in genes regulating cell proliferation, growth, and assembly pathway genes during fetal development, but decreased activity in genes governing mitogen-activated protein kinase-extracellular regulated kinase, actin cytoskeleton, and integrin-signaling pathways [138]. Among the genes so regulated were those controlling expression of alpha-adrenoceptors; in fetal cerebral arteries both the alpha-1B and alpha-1D adrenergic receptors were detected and observed to participate in contractile responses to adrenergic agonists [139].

Concerning the effects of chronic hypoxia, a recent microarray study has identified 38 fetal vascular genes upregulated more than twofold, and 9 genes downregulated more than twofold, in response to hypoxia [140]. Although the functional implications of these changes in gene expression remain unclear, these findings constitute an excellent foundation for further work. For example, hypoxic changes in the genes regulating expression of PKC may help to explain the observations that hypoxia potentiates PKC-mediated contractions more in adult than fetal arteries, but inhibits ROCK contribution to PKC-mediated contractions more in fetal than adult arteries [141]. These studies are ongoing.

Recently, another avenue of investigation has opened in the Center regarding cellular mechanisms of response to hypoxia, with particular emphasis on VEGF. In organ culture VEGF can alter the expression of contractile proteins, contractility and smooth muscle phenotype [142]. Correspondingly, the vascular remodeling induced by chronic hypoxia [143] may involve the effects of VEGF on smooth muscle differentiation [144]. Given that hypoxic increases in circulating VEGF levels are transient, it is particularly interesting that the long-term effects of hypoxia on smooth muscle phenotype appear to be mediated, at least in part, by an increased abundance of VEGF receptors in arterial smooth muscle [93]. Importantly, long term hypoxia through the actions of VEGF also transforms smooth muscle expression of myosin heavy chain informs, which helps explain the corresponding changes in contractility, particularly in fetal arteries [92]. Hypoxic activation of the VEGF pathway may also help explain the attenuating effects of hypoxia on PKG-mediated activation of BK channels in fetal arteries [145]. From many perspectives, VEGF appears to be a master coordinator of numerous vascular responses to chronic hypoxia, and perhaps other stresses as well [146].

Another new area of investigation in Center is focused on epigenetic mechanisms of fetal adaptation. In response to maternal food restriction (50 % total caloric intake of paired controls), a recent microRNA survey revealed dozens of types that were upregulated and downregulated with a broad variety of target genes [147]. Most interestingly, the patterns of change in microRNA were not the same in day-old offspring as in 12-months old offspring, suggesting that maternal food restriction imposes some form of epigenetic programming throughout the fetal genome. In a more targeted study, maternal protein restriction induced in fetal brains an increased mRNA expression of angiotensinogen and angiotensin converting enzyme-1 (ACE-1), with a decrease in mRNA levels of angiotensin II type-2 (AT2) receptors [148]. This study also produced the very interesting result that the promoter regions of the ACE-1 gene were hypomethylated, and miR27a and miR27b, which influence the translation of ACE-1 mRNA, were also upregulated. Again, these results strongly suggest that fetal stress translates through epigenetic mechanisms into major alterations of cerebral structure and function.

How these mechanisms influence cerebrovascular structure and function is a topic of ongoing investigation, but abundant reports in the recent literature strongly suggest the involvement of epigenetic mechanisms in most major cerebrovascular pathologies [149].

6 Overview

The past three decades have transformed thinking about, and understanding of, the fetal cerebral circulation. Overall, it is clear that fetal cerebral arteries are in many ways structurally and functionally quite different than adult cerebral arteries. More importantly, the mechanisms that govern the composition and contractility of cerebral arteries are highly dynamic and involve multiple mechanisms operating at all levels of organization. At the whole animal level, circulating vasotrophic factors such as VEGF and angiotensin mediate the effects of environmental influences such as hypoxia and food restriction on cerebrovascular growth and differentiation. In general, the proliferative effects of these vasotrophic factors are more pronounced in fetal than adult smooth muscle. Many other growth factors are undoubtedly involved in these processes, and are under active investigation. At the tissue level, cerebrovascular smooth muscle alters membrane populations of receptor and ion channels, cytosolic kinases, and calcium handling, all of which culminate in a heightened sensitivity to aminergic agonists, decreased capacity for calcium release, and increased reliance on calcium influx for contraction in fetal compared to adult cerebral arteries. At the cytosolic level, myofilament calcium sensitivity and soluble guanylate cyclase are upregulated in the fetus but calcium release and PKC appear to be upregulated in the adult, at least in relation to their roles in NE-induced contractions. At the molecular level, fetal and adult tissues express very different patterns of genes and microRNAs, which help to explain why both structural and contractile protein compositions are so different in fetal and adult cerebral arteries. The functional implications of these differences remain under active investigation.

As for most scientific programs of research, studies of the vascular biology of the fetal brain have produced more questions than answers. That said, it is clearly a worthy topic of continued investigation, particularly as recent work suggests that smooth muscle plays a critical role not only in regulating blood flow, but in the responses to, and recovery from, cerebral injury [150]. Without doubt, the Center for Perinatal Biology will continue their adventures in this arena, and with all the new genetic and analytical tools becoming available, the next 30 years might be even more interesting than the last.

Acknowledgements The work summarized in this manuscript was supported by USPHS Grants HL54120, HD31266, HL64867, NS076945 and the Loma Linda University School of Medicine.

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17 Placental Vascular Defects in Compromised Pregnancies: Effects of Assisted Reproductive Technologies and Other Maternal Stressors

Lawrence P. Reynolds, Pawel P. Borowicz, Chiara Palmieri, and Anna T. Grazul-Bilska

Abstract

Many factors negatively affect pregnancy establishment and subsequent fetal growth and development, including maternal factors such as nutritional stress, age, body mass index, and genetic background, and external factors including environmental stress, psychosocial stress, multiple fetuses, medical conditions (e.g., polycystic ovary syndrome), lifestyle choices (e.g., alcohol consumption, smoking), and assisted reproductive technologies. These same factors have similar consequences for placental growth and development, including vascular development. We and others have shown that placental vascular development begins very early in pregnancy and determines, to a large extent, placental function—that is, the magnitude of the increase in placental blood flow and thus nutrient transport to the fetus. During the peri-implantation period and also later in pregnancy, cloned (somatic cell nuclear transfer) embryos exhibit a variety of placental defects including reduced vascularization and altered expression of angiogenic factors. Although placental defects are less pronounced in pregnancies resulting from the transfer of in vitro fertilized embryos, we and others have recently demonstrated that vascularization, expression of angiogenic factors, sex steroid receptors, several epigenetic markers, and growth of utero-placental tissues all were altered during

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L. Zhang and C.A. Ducsay (eds.), *Advances in Fetal and Neonatal Physiology*, Advances in Experimental Medicine and Biology 814, DOI 10.1007/978-1-4939-1031-1_17,

early pregnancy after transfer of embryos obtained through natural mating, in vitro fertilization, or other assisted reproductive techniques. These observations are in agreement with the recent reports that in humans even singleton pregnancies established with assisted reproductive techniques are at increased risk of preterm delivery and low birth weight, and seem especially relevant considering the rapidly expanding use of these techniques in humans and animals.

Keywords
Placenta • Vascular defects • Angiogenesis • Vascular function • Pregnancy • Intrauterine growth restriction • Maternal stressors • Assisted reproductive technologies • Embryo transfer • In vitro fertilization • Cloning • Clones

1 Introduction

Compromised pregnancy encompasses any pregnancy in which fetal growth (and, by inference fetal development) are abnormal [1–3]. In addition, compromised fetal growth/development are normally accompanied by altered placental growth and development [1, 2, 4]. Practically, most compromised pregnancies involve poor fetal growth/development, accompanied by low birth weight.

In humans, the incidence of low birth weight, which is universally defined as <2.5 kg or 5.5 lb, is 15 % worldwide, or 20 million births, per year (WHO, 2008). In the U.S., low birth weight occurs in more than 8 % of pregnancies, and amounts to >350,000 births per year [5]. Low birth weight in turn is a major factor contributing to infant (birth to 1 year of age) mortality, which represents 0.7 % of infants in the U.S. (the highest rate in the developed world) and as high as 16.5 % of infants in some developing countries [5, 6]. Moreover, as mentioned low birth weight reflects poor fetal growth and development, which are major causes of 'developmental programming' in mammals, including humans and livestock [3, 7, 8]. Developmental programming, which refers to a long-term change in phenotype due to a developmental insult, leads ultimately to a two- to tenfold increase in the risk of developing so-called 'noncommunicable' diseases such as cardiovascular disease, diabetes, obesity, cognitive dysfunction, pervasive developmental disorders (e.g., autism, Asperger's and Rett's syndromes), etc. [3, 7–10]. In addition, the effects of developmental programming can also be transmitted across generations [2, 8, 9, 11, 12].

On the opposite end of the spectrum, infertility, or the inability to conceive and maintain a pregnancy, is a major socioeconomic issue [8]. Infertility results primarily from spontaneous abortion, or miscarriage, which amounts to 30–70 % of fertilized oocytes in humans, and 20–30 % in other mammals [13–16]. In addition, most embryonic loss occurs during the first third of pregnancy in all mammalian species studied, including humans and livestock [13].

The factors that negatively affect pregnancy establishment and subsequent fetal growth/development include maternal factors, such as nutritional stress, age, body mass index, and genetic background, as well as external factors including environmental stress (e.g., high altitude, heat stress), psychosocial stress, multiple fetuses, medical conditions (e.g., polycystic ovary syndrome), lifestyle choices (e.g., alcohol consumption, smoking), and assisted reproductive technologies [2, 8, 17–19].

2 Placental Vascular and Other Defects in Compromised Pregnancies

Placental vascular development begins very early in pregnancy and determines, to a large degree, placental function—that is, the magnitude of the increase in placental blood flow and thus nutrient transport to the fetus [17, 20–22]. Many of the same factors that negatively impact fertility and fetal growth and development also have similar consequences for placental growth and development. For example, in numerous models of compromised pregnancy in sheep reduced fetal weight near term is associated not only with reduced placental size but also reduced gravid uterine (maternal placental) and umbilical (fetal placental) blood flows as well as altered placental vascular development and function (Table 17.1) [8]. In addition, in one of these, namely nutrient-restricted ewes, the decrease in uterine arterial resistance that normally accompanies pregnancy is lost and umbilical vascular resistance is increased (Fig. 17.1) [23], indicating that placental vascular function also is compromised.

In humans, reduced placental vascular development and increased vascular resistance during early pregnancy have been associated with early embryonic mortality [24, 25]. Similarly, in human intrauterine growth restricted (IUGR) fetuses, although gravid uterine blood flow

Table 17.1 Changes in fetal and placental weights, uterine and umbilical blood flows, and placental (*CAR* caruncular or maternal placental; and *COT* cotyledonary or fetal placental) vascularity in various models of compromised pregnancy in sheep[a]

Model	Day of gestation[b]	Fetal weight	Placental weight[c]	Uterine blood flow	Umbilical blood flow	Vascularity
Overfed adolescent	130–134	↓20–28 %	↓45 %	↓36 %	↓37 %	↓59 % (total capillary vol., COT)[d]
Underfed adolescent	130	↓17 %	NSE	–	–	↓20 % (cap. area density, CAR)
Underfed adult	130–144	↓12 %	–	↓17–32 %	NSE	↓14 % (cap. area density, CAR)
Adolescent versus adult	135	↓11 %	↓29 %	–	–	–
Genotype[e]	130	↓43 %	↓47 %	–	–	↑28–43 % (cap. area density, CAR and COT, respectively)
Heat-stressed adult	133–135	↓42 %	↓51 %	↓26 %	↓60 %	–
Multiple pregnancy[f]	140	↓30 %	↓37 %	↓23 %	–	↓30 % (total cap. vol., COT)
High dietary Se	135	NSE	↓24 %	–	–	↑20 % (cap. number density, COT)
Hypoxic (hypobaric) stress[g]	140	NSE	–	↓35 %	–	↑19 % (cap. area density, CAR)

[a]Table adapted from Reynolds et al., 2010a; *cap.* capillary, *CAR* caruncular (maternal placenta), *COT* cotyledonary (fetal placenta/villous), and *NSE* no significant effect

[b]Length of gestation = approximately 145 days; thus, all measurements taken from late (0.9–0.99) of pregnancy

[c]Placental weight = total placentome weight

[d]Day 50 only, which was well before decreased fetal or placental weight, which was observed on day 130 but not on days 50 or 90 of gestation

[e]Genotype represents highly prolific (approx. 4 offspring per pregnancy) versus relative low prolificacy (approx. 1.2 offspring per pregnancy) breeds of sheep—the high prolificacy is associated with relatively small individual fetal and placental weights but a compensatory increase in placental vascularity to support the much large total fetal and placental mass

[f]Singletons versus triplets

[g]The increased placental vascularity is reflected by normal fetal weight despite a 35 % decrease in uterine blood flow

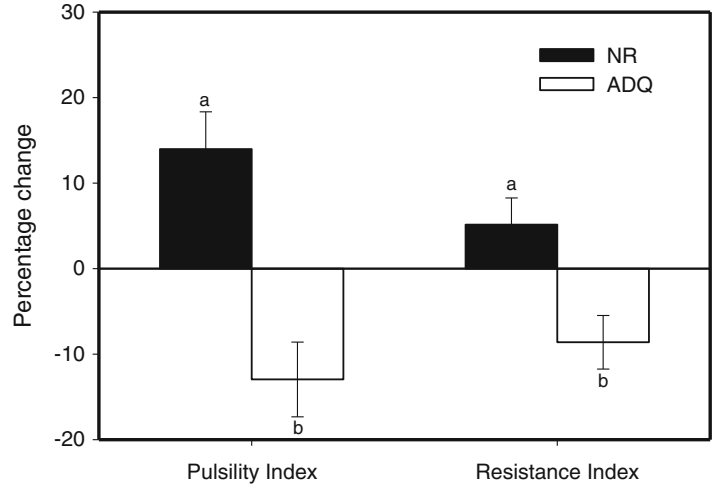

Fig. 17.1 Change in umbilical artery pulsatility and resistance indices from day 40 to 105 (0.28–0.72) of gestation in ewes that were nutrient-restricted (NR, *black bars*) or adequately fed (ADQ, *White bars*). [ab]Least-squares means ± SEM with different superscripts are different ($P < 0.01$). Taken from Reynolds et al., 2013

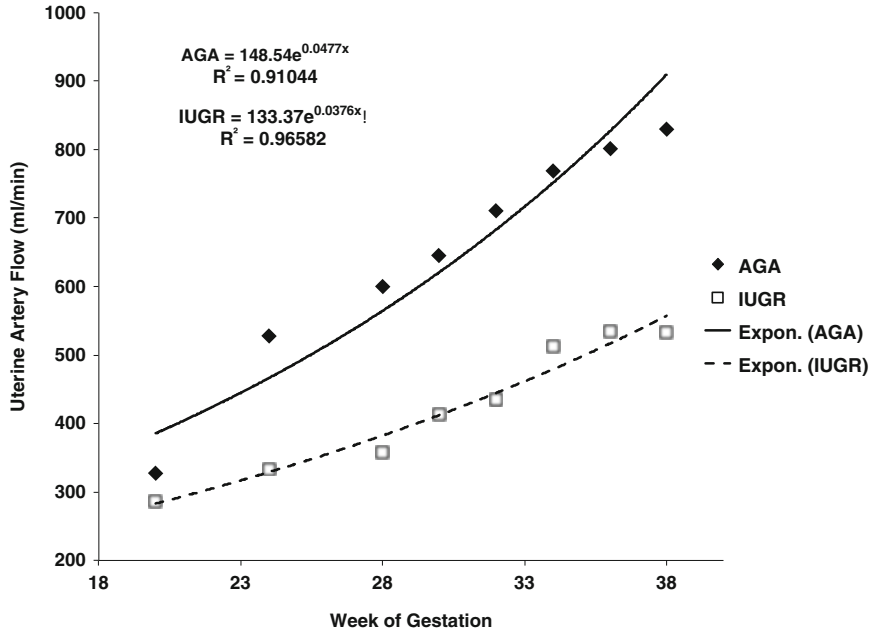

Fig. 17.2 Change in uterine artery flow (gravid uterine, or maternal placental, flow) throughout pregnancy for fetuses that were average size for gestational age (AGA; *solid square symbols* and *solid line*) or intrauterine growth restricted (IUGR, *open square symbols* and *dashed line*). The lines represent exponential changes across week of gestation, and the regression equations are shown. Data taken from Konje at al., 2003

increases exponentially as it does in average size for gestational age (AGA) fetuses, the magnitude of increase is dramatically reduced (Fig. 17.2) [26]. Additionally, in humans IUGR is reflected by dramatically reduced placental (villous) development including that of the capillary beds, but this does not occur in pre-eclamptic pregnancies without IUGR (Table 17.2) [27]. Altered angiogenesis or expression of selected angiogenic factors or reduced blood flow were also observed in the placenta in several pregnancy disorders including early intrauterine embryonic death,

Table 17.2 Placental development and angiogenesis in late pregnancy in control, preeclamptic (PE), and intrauterine growth restricted (IUGR) pregnancies in humans[a,b,c]

Variable[d]	Controls	PE	IUGR	PE + IUGR
Villous volume	219 ± 19	179 ± 171	130 ± 25	105 ± 20
Villous surface	11.1 ± 0.7	9.4 ± 0.2	6.6 ± 1.5	5.2 ± 1.1
Villous length	60.6 ± 6.8	45.3 ± 3.6	30.9 ± 7.0	26.7 ± 4.9
Capillary volume	65.5 ± 8.7	46.9 ± 3.5	35.3 ± 8.6	28.0 ± 6.9
Capillary surface	10.8 ± 1.4	8.2 ± 0.8	5.4 ± 1.4	3.8 ± 0.8
Capillary length	233 ± 33	172 ± 9	110 ± 28	90 ± 22

[a]Taken from Mayhew et al., Placenta 2004, 25:829–833
[b]Values are means ± SEM. Volumes in cm^3, surface areas in m^2, and lengths in km
[c]All placentas from term (in weeks of gestation): Controls, 39 ± 0.5; PE, 36 ± 1.5; IUGR, 37 ± 0.8; PE + IUGR, 33 ± 1.7
[d]Significant effects ($P < 0.05$) of IUGR but not PE

pre-eclampsia, placenta accretia, intrauterine growth restriction (IUGR) or diabetes in several species [1, 28–35]. Thus, the data available from sheep models of compromised pregnancy are in agreement with those from other mammalian species.

In addition to defects in placental size and placental vascular development and function, other placental defects include thickening of the subepithelial and capillary basement membranes; altered turnover of the trophoblastic epithelium; changes in the amount, type, and/or activity of glucose and amino acid transporters; altered lipid metabolism; altered steroid metabolism; and altered cytokine and growth factors expression, all of which affect placental transport capacity [2, 36–41]. Moreover, maternal stressors may epigenetically alter gene expression in the hypothalamic-pituitary-adrenal axis, which is a key regulator of development of fetal organs [42–45].

3 Placental Vascular and Other Defects in Pregnancies from Assisted Reproduction

Early pregnancy is a critical period because of the major developmental events that take place, including embryonic organogenesis as well as formation of the placenta, a process known as placentation and manifested by enhanced cell proliferation and vascular development (Fig. 17.3) [1, 2, 8, 46].

We have evaluated the pattern of placental vascular growth and development during early pregnancy after natural breeding in sheep and shown that the major initial changes appear in maternal placenta as early as day 16 and continue throughout pregnancy [21, 22, 47–49]. In addition, changes in vascularization measured by area, surface, number and density of capillaries were highly correlated with changes in expression of several angiogenic factors in maternal placenta [21, 22]. For several other species (e.g., humans, marmoset, rats) intensive vascular development in placenta was also observed during the first 3–4 weeks of pregnancy, and was associated with enhanced demand to support dramatic fetal growth [21, 22].

Comparison of placental development in natural pregnancies and pregnancies achieved by various ART, such as after transfer of embryos created through cloning or even in vitro fertilization (IVF), has demonstrated numerous significant effects of ART on placental and fetal growth and development, as well as offspring outcome in several species [50–60].

For animal models, including mice, cattle or sheep, impaired placental steroid metabolism, abnormal offspring syndrome, increased duration of gestation, altered placental vascularization, fetal weight/size and/or placental/fetus ratio have been reported after using ART [50, 53, 56, 57, 61, 62]. For early pregnancy in cows, both greater and less crown-rump length of fetuses created in vitro and then transferred compared to fetuses created in vivo has been reported [62, 63].

As we have discussed in our previous studies using different embryo origin and also ART [53], the embryo affects uterine function and has an active role in initiation of pregnancy, and in turn

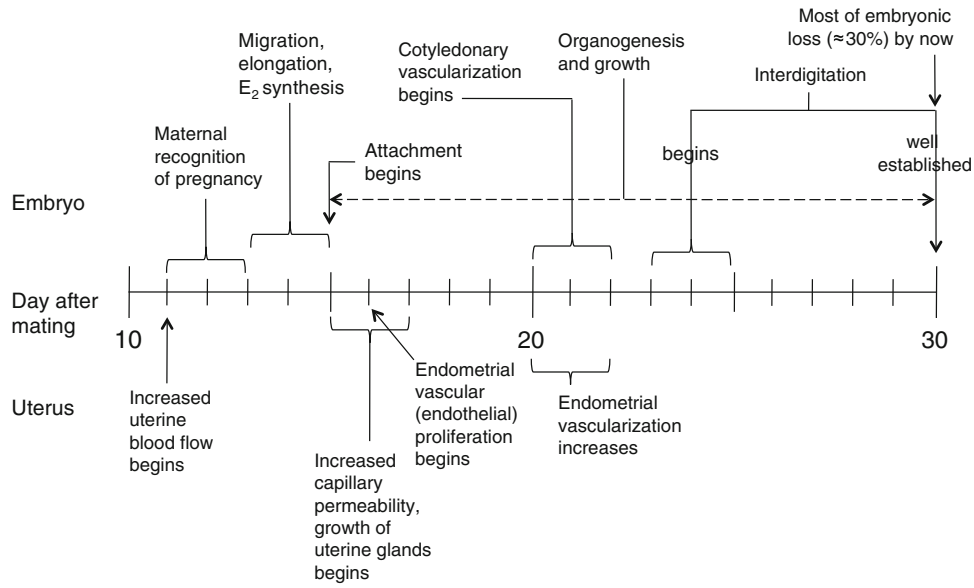

Fig. 17.3 Timeline of placental and embryonic/fetal development during early pregnancy in sheep. Taken from Reynolds et al., 2013. E2 = estradiol-17beta; 'Interdigitation' refers to interdigitation of maternal caruncular with fetal cotyledonary tissues in the placentomes (the sites of most intimate contact between maternal and fetal placental tissues)

the uterus affects fetal growth and development [14, 17, 53]. In fact, abnormal embryo/fetal-maternal communication, endometrial remodeling, reduced vascularization, altered expression of angiogenic factors and other problems (e.g., abnormal trophoblast differentiation, immunologic rejection) during the peri-implantation period and later in pregnancy have been observed for pregnancies achieved after transfer of embryos created through somatic cell nuclear transfer (SCNT) in animal models [64–73]. However, in pregnancies resulting from the transfer of embryos created through IVF, changes in endometrial vascularization, remodeling, and function were less pronounced than after transfer of embryos from SCNT in several species [64, 67, 68, 70–72, 74, 75]. In fact, endometrial (maternal placental) tissues possess mechanisms to adapt to embryos of different origin, which may serve as a biological sensor to meet embryonic demands or adaptation to environmental conditions [4, 76, 77]. This idea is in line with our hypothesis that during pregnancy some compensatory mechanisms may exist to reverse the negative effects of embryo manipulations on placental and fetal growth [2, 4, 20].

In human medicine ART is well established, broadly accepted and increasingly utilized [78, 79]. However, multiple pregnancies remain the principal cause of adverse outcomes after ART. On the other hand, singleton pregnancies also have an increased risk of preterm delivery and low birth weight at term [55, 80]. In addition, placental previa and other placental defects are increased following ART [54, 55, 66]. With the advancing age of women at the time of ART procedures, increased risks of higher rates of cesarean section and placenta accreta, preterm births and infants of lower than average birth weight have been demonstrated [51, 59, 60].

For sheep, we and others have recently demonstrated that vascularization, expression of several angiogenic factors, receptors for P4 and estrogens, and DNA methyl transferases (DNMT), as well as global DNA methylation and markers of growth of utero-placental tissues all were altered during early pregnancy after transfer of embryos obtained through natural mating, IVF, or in vitro activation (IVA, parthenotes, which have only maternal genome; Table 17.3) (Grazul-Bilska and Reynolds, unpublished) [53, 81]. These observations suggest to us that changes in

Table 17.3 Comparison of several factors in maternal and fetal placenta in early pregnancies after application of ART[a] with pregnancy achieved by natural breeding (control) in sheep[b]

		NAT-ET group		IVF group		IVA group	
Process	Factor	Maternal placenta	Fetal placenta	Maternal placenta	Fetal placenta	Maternal Placenta	Fetal placenta
Angiogenic factors mRNA expression	VEGF[c]	↓[d]	↓	–	↓	↓	↓
	FLT1	↓	↓	↓	↓	↓	↓
	KDR	–	–	–	–	–	–
	PGF	↓	↓	–	↓	–	–
	NP1	–	↓	–	↓	–	↓
	NP2	–	–	–	↓	–	↓
	ANGPT1	↓	↓	–	↓	–	↓
	ANGPT2	–	↓	–	↓	–	↓
	TEK	↓	–	↓	–	↓	–
	NOS3	–	↓	–	–	–	↓
	GUCY1B3	–	–	–	–	–	–
	HIF1A	–	↓	–	–	–	↓
	FGF2	–	↓	–	↓	–	↓
	FGFR	–	↓	–	↓	–	–
Global DNA methylation	DNMT1 mRNA	–	–	–	–	–	↑
	DNMT3a mRNA	↓	–	↓	–	↓	–
	DNMT3b mRNA	–	–	–	–	–	–
	5mC[e]	NP	–	NP	↑	NP	↑
Steroid receptor mRNA expression	Nuclear P4	↓	–	↓	–	↓	–
	Membrane P4 alpha	–	–	–	–	–	–
	Membrane P4 beta	–	–	–	–	–	–
	Membrane P4 Gamma	–	–	–	–	–	–
	Nuclear E alpha	↓	–	↓	↑	↓	↑
	Nuclear E beta	–	–	–	–	–	–
	membrane E (GPR30)	–	↑	–	–	↑	–
mRNA expression of diapauses markers	BTG1	–	↑	↑	↑	↑	↑
	IGF2R	–	↑	–	↑	↑	↑
Vascularization[f]	Blood vessel number	↓	–	↓	↓	↓	↓
	Average size of capillary	–	NP	↓	NP	↓	NP
Tissue growth[g]	Length of fetus	NA	↓	NA	↓	NA	↓
	Labeling index	↓	↓	↓	↓	↓	↓
Gap junctional connexins mRNA	C×26	–	–	–	–	–	↑
	C×32	↓	–	↓	–	↓	–
	C×37	–	–	–	–	–	–
	C×43	–	–	–	–	–	–

[a]ART = Assisted Reproductive Technologies

[b]Adapted from Reynolds et al., 2013. Three ART methods were used to establish pregnancies as follows: (1) superovulation induced by multiple injections of follicle stimulating hormone (FSH) combined with natural breeding, embryo flushing from donors and transfer to recipients (NAT-ET group), (2) transfer of embryos obtained through in vitro fertilization (IVF group) of oocytes collected after induction of multiple follicular development using FSH, and (3) transfer of embryos obtained through in vitro activation (IVA group; i.e., parthenotes, which are clones containing maternal genes only) of oocytes collected from FSH-treated donors. Analyses were performed for placental tissues collected on day 22 after breeding, or in vitro fertilization or activation

(continued)

Table 17.3 (continued)

[c]*VEGF* vascular endothelial growth factor, *PGF* placental growth factor, VEGF and PGF receptors FLT1 and KDR, *NP* neuropilin, *FGFR* fibroblast growth factor (FGF) 2 and receptor 2IIIc, *ANGPT* angiopoietin, ANGPT receptors TEK, endothelial NO synthase (NOS3) and receptor soluble guanylate cyclase (GUCY1B3), *HIF1A* hypoxia inducing factor 1 alpha, *DNMT* DNA methyltransferase, *5mC* 5 methylcytosine, *P4* progesterone, *E* estrogen, *GPC30* G protein-coupled receptor 30, *BTG1* B-cell translocation gene 1, *IGF2R* insulin-like growth factor 2 receptor, *Cx* connexin

[d]Compared to natural (control) pregnancy: ↓, downregulated; ↑ upregulated, – not different from control; *NA* data not available, *NP* not performed

[e]Expression of 5mC was determined based on immunohistochemistry and image analysis

[f]Blood vessel number was determined based on immunohistochemical staining of smooth muscle cell (SMC) actin (marker of SM cells and pericytes) and image analysis, and expressed as the number of blood vessels per tissue area; average size of capillary was based on histological staining by hematoxylin and periodic acid Shiff (marker of basement membranes) and image analysis

[g]Length of fetus was determined based on crown-rump distance; labeling index (proportion of proliferating cells) was determined based on immunohistochemical staining of Ki67 (marker of proliferating cells) and image analysis, and expressed as the proportion of proliferating cells out of total cells per tissue area

DNA methylation or histone modifications observed in embryos of several species (cattle, mice, sheep) after ART [82–86] continue during the critical period of placentation during early pregnancy, and probably contribute to poor placental vascularization and function later in pregnancy, ultimately leading to altered fetal growth/development and poor pregnancy outcomes associated with ART. Such epigenetic defects leading to altered placental gene expression and development have been observed in IVF and especially in SCNT clones not only in sheep but in cattle and mice as well [72]. These defects include altered expression of imprinted genes, which are thought to be critical for normal placental development [81, 87, 88].

4 Summary and Future Directions

As mentioned, early pregnancy is a critical period because of the major developmental events that take place, including embryonic organogenesis as well as formation of the placenta. As we have shown [21, 22, 47, 48], these processes include very early (days 16–18 after mating) increases in placental growth, vascular development, and angiogenic factor expression. Further, we have suggested that such dramatic changes are critical for pregnancy establishment as well as continued normal growth and development of the fetus [2, 20]. That is, we are convinced that altered placental growth and vascular development contribute to lower fertility, as reflected by spontaneous abortion, as well as poor pregnancy outcomes, reflected by low birth weights. Moreover, pregnancies established after ART seem to be the most highly compromised of all, which makes them a good model for examining the mechanisms of altered placental vascular development.

We and others also have suggested management and therapeutic strategies to improve placental vascular development and function, and thus pregnancy outcomes [2, 20, 89–95]. These suggested strategies have involved mostly targeted supplementation of maternal diet, either globally or with specific nutrients including dietary protein, specific amino acids, or micronutrients, and several of these strategies have shown promising results. However, because of the profound effects of other maternal factors, including for example use of alcohol, tobacco and other drugs, relational stress, and exercise, it seems likely that an integrated approach which takes maternal nutritional status as well as these other factors into account will be necessary to improve fertility and pregnancy outcomes [2, 20].

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How to Build a Healthy Heart from Scratch

18

Kent L.R. Thornburg and John R.G. Challis

Abstract

By any of several measures, the health of the American population has been worsening over the last two decades. Obesity, type 2 diabetes and heart failure have risen dramatically. All the while, the average birthweight at all gestational ages has declined. The relationship between robust growth in the womb and lifelong health is now well established. Likewise, babies born at the low end of the birthweight scale are known to have highly elevated risks for ischemic heart disease, hypertension, stroke and metabolic disease. The biological mechanisms by which developmental plasticity becomes a risk for cardiovascular disease are only now being understood. Translating from animal and human studies, low birthweight babies are likely to have endothelial dysfunction, fewer nephrons, fewer pancreatic beta cells, less vascular elastin, fewer cardiomyocytes, increased sympathetic tone and liver-derived dyslipidemias. Only in the past few years, however, has it become known that maternal and placenta phenotypes are associated with adult onset cardiovascular disease. Helsinki Birth Cohort studies have been especially important in the discovery of these relationships. Sudden cardiac death is associated with a thin placenta and heart failure is associated with a small placenta in short mothers. Coronary heart disease is associated with three combinations of maternal-placental phenotypes. Because the diet is important in providing nutrients for the development of the female body before pregnancy and for providing nutrients during pregnancy, there is increasing evidence that the

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L. Zhang and C.A. Ducsay (eds.), *Advances in Fetal and Neonatal Physiology*,
Advances in Experimental Medicine and Biology 814, DOI 10.1007/978-1-4939-1031-1_18,

western diet is an underlying cause for the increase in metabolic disease in the American population. A large segment of the American population suffers from high calorie malnutrition. Scientists in this field now have a responsibility to educate the public on the topic of nutrition and health. This chapter honors Lawrence Longo for decades of work in bringing health to pregnant women and their babies.

Keywords
Cardiovascular disease • Cardiac myocytes • Fetal programming • Placental phenotype • High calorie malnutrition

1 Introduction

This chapter is dedicated to Professor Lawrence Longo who has inspired an entire generation of young scientists in the field of fetal biology. He has continually encouraged them to persevere amidst the difficulties of modern science and indeed, many under his spell have become productive scientists well into their senior years. Over the duration of Professor Longo's scientific life, understanding of the physiology of pregnancy and the development of the fetus has grown exponentially. Professor Longo has seen a revolution in the field to which he has dedicated himself and in the midst of the revolution, he has left his mark on our understanding of the pathophysiology of pregnancy, placental function, cardiovascular development, neurodevelopment and many other aspects of reproductive biology.

Most of what we know today about pregnancy and fetal biology was discovered over the last half of the previous century. Looking back 25 years, it is clear that most of the basic aspects of fetal growth had already been cataloged through the enormous efforts of many fetal groups around the world who took advantage of the chronic sheep model. While fetal scientists were bathing in the joy of new knowledge there was full recognition that the descriptive era of fetal biology was coming to an end and new more translational avenues of investigation were needed. Within that same frame of time, the field gained further momentum as it gained new purpose. The new energy arose from the timely discovery that environmental conditions experienced by a fetus establish its risk for health and disease that will endure for the remainder of its life [1, 2]. Once it became clear that population health is largely driven by the prenatal development of the individuals within it, the urgency of further study was evident. The late Professor David Barker (1938–2013), the discoverer of the birthweight-disease relationship, carried the programming message directly to the fetal physiology community and urged them to determine how "normal" variations in prenatal growth could lead to disease risk in later life. This challenge became the mission of the fetal scientific community as they joined forces with many new partners, including epidemiologists, to understand the links between adult human disease and the intricacies of fetal growth and development.

1.1 The Health of the U.S. American Population Is Worsening

Figure 18.1 shows the increase in the prevalence of diabetes mellitus in the USA beginning in 1958 (http://www.cdc.gov/diabetes/statistics/diabetes_slides.htm). From 1958 to the mid-1990s there was a gradual increase in the portion of the population that acquired the disease. In 1958 less than 1 % of the population suffered from diabetes. Now, some 45 years later, the prevalence of diabetes has increased by some 7 times and even more in some states. In the mid-1990s the rate of rise increased to its present astonishing value. Diabetes prevalence now averages nearly 11 % in people older than 20 years with even higher rates found

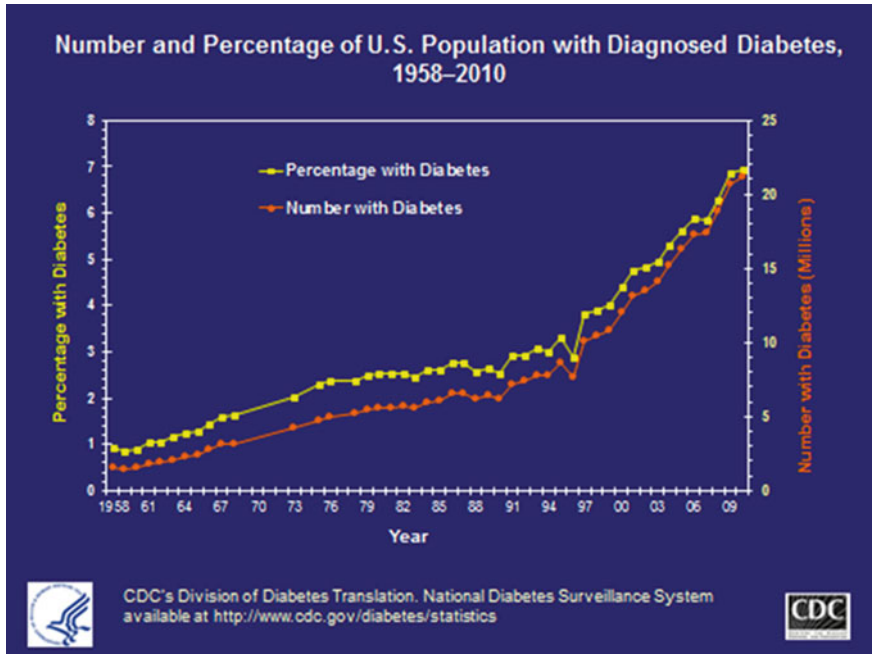

Fig. 18.1 Increase in the prevalence of people diagnosed with diabetes in the USA. (Data from Center for Disease Control)

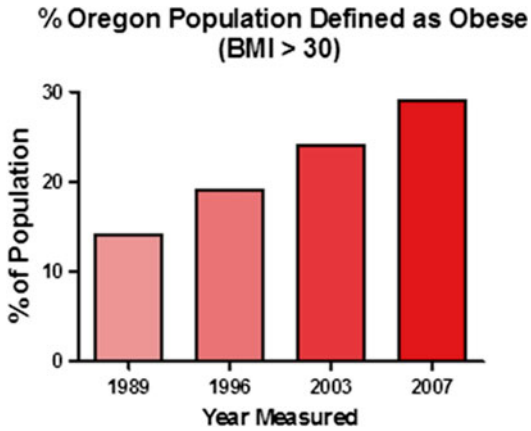

Fig. 18.2 Obesity rates in Oregon have doubled over the last 20 years as they have nationally. (Data from Center for Disease Control)

in the Deep South. The Centers for Disease Control and Prevention predicts that at the present rate of increase, some one in three members of the U.S. population will be diabetic by the year 2050. This prediction is a wakeup call for people living in the USA (http://www.cdc.gov/media/pressrel/2010/r101022.html).

Figure 18.2 shows changes in the prevalence of obesity, defined as a body mass index (BMI) exceeding 30 kg/m^2, in the State of Oregon, USA over the last 20 years. Oregon is in the middle of the pack with regard to obesity rates among the other 49 states. Over the period of time beginning 20 years ago when diabetes rates tripled in the USA, obesity rates in Oregon doubled. The obesity-diabetes link is well established and thus, it appears that the two will continue to rise in tandem.

Another interesting statistic that applies to the declining health of U.S. Americans is the fall in birthweight over that same recent period of time [3]. Figure 18.3 shows data from the U.S. National Center for Health Statistics for 36,827,828 singleton neonates born over 37–41 weeks of gestation during a 15 year period, 1990–2005. The data reveal decreases in national average birthweight at all gestational ages over that period of time. These data are especially interesting as they demonstrate a reversal of the trend in increasing birthweight that characterized the previous century [3]. While some hospitals are seeing increases in birthweight as increasing numbers of babies are born to mothers who are obese or who had poor glucose control during their pregnancy, the national trend is, nevertheless, downward. The implications of decreasing

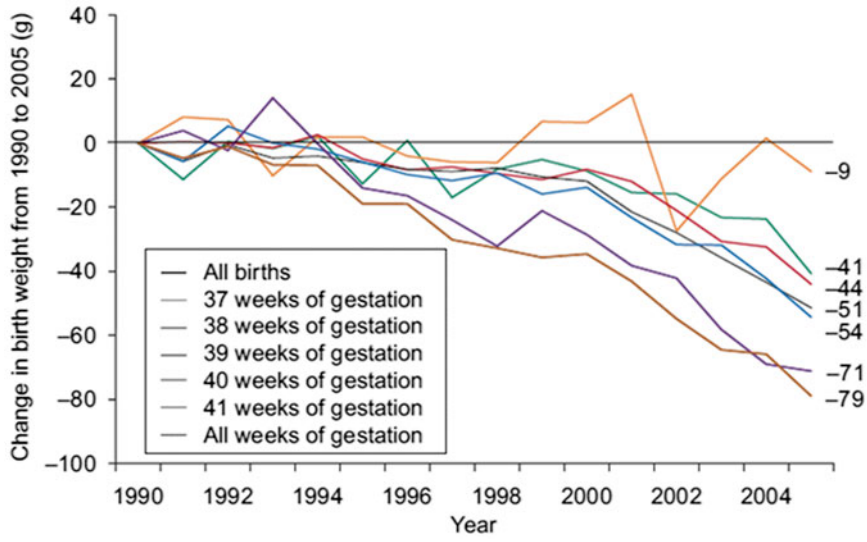

Fig. 18.3 Change in birthweight in grams from 1990 to 2005 across a wide range of gestation ages. (From Donahue SM, Kleinman KP, Gillman MW, Oken E. Trends in birth weight and gestational length among singleton term births in the United States: 1990–2005. Obstet Gynecol 2010 Feb;115(2 Pt 1):357–64)

birthweight are a reminder of the now well-known relationship between low weight at birth and health outcomes.

1.2 The Links Between Fetal Growth and Cardiovascular Disease

Barker's 1989 report [2] showing that ischemic heart disease risk was inversely related to birthweight among the inhabitants of Hertfordshire, UK (Fig. 18.4) was based on brilliant associations with infant mortality rates. Thus, the idea to study birthweight as an index of disease risk was actually derived from an earlier epidemiological observation [4]. What Barker and Osmond had observed was that the industrial regions of the UK with their high infant mortality rates also had high cardiovascular death rates among adults. Barker and his team could supposedly have spent the remainder of their lives looking for an unknown pollutant or infectious agent that caused both newborn babies and adults to die prematurely. Rather, they deduced that while many babies died as neonates, many more lived on as vulnerable adults. Among those that survived infancy were those that carried higher risks for death than those born in more affluent areas of the UK. Barker reasoned that both offspring who died early in life and those who died prematurely as adults lived shorter lives as the result of their developmental setbacks. Hundreds of studies since 1989 support the Barker team's intuition and the developmental roots of ischemic heart disease are no longer controversial. The team's new insights about chronic disease became known just at the time when chronic disease rates around the globe were rising as the western lifestyle became prevalent.

Since Barker's early revelations, scientists around the world have been hard at work to discover mechanisms underlying the now well-documented vulnerability for adult onset chronic disease that arises from environmental conditions during prenatal life. From these studies, it is becoming increasingly clear that inadequate fetal nutrition, fetal hypoxia, or maternal stress during pregnancy either independently or together, "program" the fetus in ways that make it vulnerable

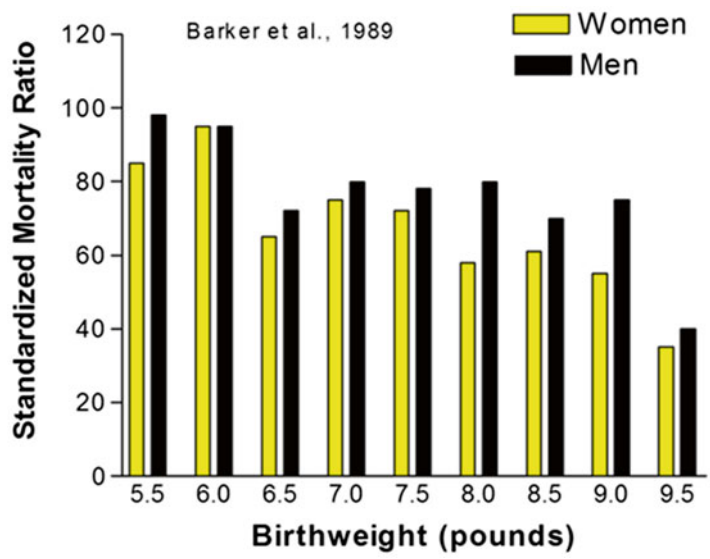

Fig. 18.4 Mortality ratio from ischemic heart disease in ~15,000 men and women in Hertforshire, UK. (Data from Barker DJ, Winter PD, Osmond C, Margetts B, Simmonds SJ). Weight in infancy and death from ischaemic heart disease. Lancet 1989 Sep 9;2(8663):577–580. & Osmond C, Barker DJ, Winter PD, Fall CH, Simmonds SJ. Early growth and death from cardiovascular disease in women. BMJ 1993 Dec 11;307(6918):1519–1524)

for later disease. "Programming" refers to the changes in fetal organ structure and function that predispose the fetus for disease over its lifetime.

Recent epidemiological data show that birthweight is but a crude indicator of disease risk. It is now clear that maternal phenotype, manifest as maternal stature, weight and bone structure, in combination with placental size and shape are more precise indicators of disease than has been previously appreciated [5]. Heart disease comes in many forms but the three categories of disease that are responsible for cardiac-related deaths include: (1) coronary heart disease, (2) heart failure and (3) ventricular fibrillation. Data from the Helsinki Birth Cohort are the first to show the strong relationships between maternal body composition and placental size and shape as they powerfully predict disease outcomes in all three of the aforementioned causes of cardiac death [6–8].

In the Helsinki studies, heart failure rates among adults were inversely related to the area of the surface of the delivered term placenta but only in placentas from mothers whose heights were below the median of the population [6]. There was no relationship between placental surface area and heart failure risk among the offspring of mothers who were taller than the median height. The maternal/placental origins of coronary heart disease in men are more complicated but three relationships between maternal phenotype and placental size and shape have been found thus far [8]. In primiparous (but not multiparous) short mothers, coronary heart disease was related to increases in the difference between the length and width of the placental surface. The more oval the placental shape, the higher the risk for disease in the individual who was once attached to the placenta. This is shown in Fig. 18.5 where the effect is striking in placentas that are much longer than wide and came from primiparous women who were short. In tall mothers whose body mass index was above the median, the disease risk was related to the decreasing surface area of the placenta. In tall mothers who were thin, coronary disease was related to the placental weight/birthweight ratio that was previously described [8].

Men and women of Helsinki who died suddenly out of the hospital had thin placentas [7]. The association was significant for men. This is the first example of a disease association with a thin placenta and points to the need to understand the determinants of placental size and shape in the human population. In addition to the thin

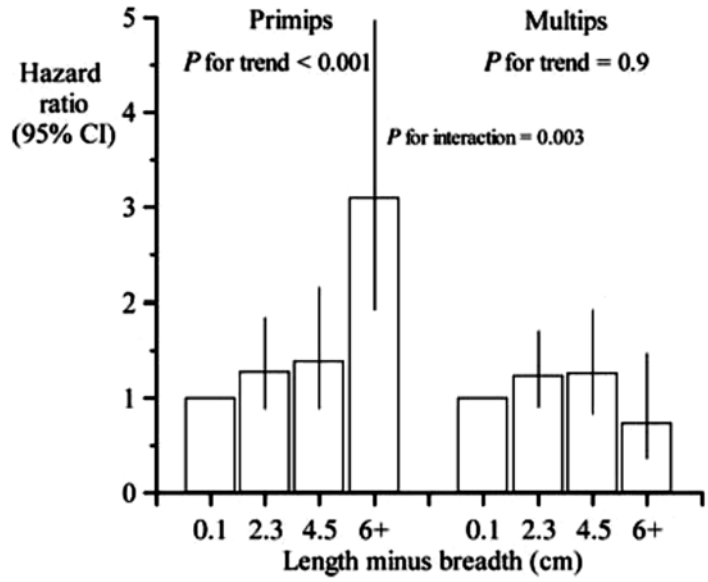

Fig. 18.5 The difference in the magnitude of the two placental axes is a powerful risk factor for coronary artery disease but only in people in the Helsinki Birth Cohort who were born to short women in their first pregnancy. (From Helsinki Birth Cohort and Eriksson JG, Kajantie E, Thornburg KL, Osmond C, Barker DJ. Mother's body size and placental size predict coronary heart disease in men. Eur Heart J 2011 Sep;32(18):2297–2303, with permission)

placenta, the men and women who died suddenly had low educational attainment. Because sudden cardiac death may be related to abnormal autonomic tone, these data hint that brain development and autonomic function may be linked in people with inadequate placentation.

1.3 How Does the Heart Become Programmed?

The inevitable question arises, how does a maternal-placental phenotype serve as an indicator for heart disease vulnerability in the offspring? The answer is complex and is undoubtedly related in part to the many cardiovascular processes that are already known to be programmed during fetal life. These include nutrition of the preimplantation embryo, hemodynamic forces in the embryonic circulation, endothelial dysfunction, architecture of the coronary tree, numbers of cardiomyocytes and epigenetic modification of expression patterns of genes encoding proteins needed for function and protection against stressful environments. Several of the aforementioned processes have been investigated at Oregon Health & Science University.

1.3.1 Hemodynamics in the Embryo

It has long been known that hemodynamic forces are sensed by cardiovascular tissues even during embryonic development. Abnormal forces lead to heart defects. One aspect of dynamic force generated by the beating heart is determined by the vascular architecture of the placenta as it develops. Inadequate vascular development in the placenta requires more effort by the developing heart than normal to overcome the resistance. Even though the chicken embryo does not have a full-fledged chorioallantoic placenta because it lacks a maternal interface, it does however have a chorioallantoic membrane that adheres to the shell. This fetal side of the "placenta" is available for study in the avian embryo. The role of changes in hemodynamic forces during the looping stage of embryonic heart development can be determined in this model. The outflow tract (OTB) of the embryonic 3 day heart is about 200 μm in diameter. It connects the primitive single ventricle to the vascular tree and will later become the pulmonary artery and the aorta of the four chambered heart. The outflow tract can be constricted by a fine suture, to mimic a high impedance placenta (outflow tract banding). In response the embryonic heart alters its developmental pattern

through the expression of a host of force-responsive genes. Blood pressure upstream to the band will increase as will surface shear forces across the constriction and beyond [9]. It appears that the outcome of this perturbation is a change in the mechanical properties of the embryonic vascular tree. For example, increases in vascular stiffness following 24 h of OTB are suggested by increased pulse transit times keyed to the QRS complex of the electrocardiogram [9]. In addition, a number of proteins including atrial natriuretic peptide in the ventricle and collagens in the outflow tract (unpublished) are increased. The increase in pulse wave velocity even downstream from the constriction suggests that the whole embryonic arterial tree is modified in response to banding. Thus it is evident that the arterial vascular elements within the embryo are exquisitely sensitive to changes in mechanical forces that accompany changes in hemodynamic state. This suggests that these forces will influence the development of the heart during the period of time that the chorioallantoic placenta is becoming established.

1.3.2 Regulation of Cardiomyocyte Number

The regulation of organ size and composite cell number is a fundamental unknown in biology [10]. There are increasing data showing that the regulation of cardiomyocyte number in the developing heart is a complex process that includes signals from hemodynamic sources, oxygen, nutrients, growth factors and hormones all superimposed on a developmental cascade directed by sequential gene expression programs. Data from several sources suggest that under usual circumstances, heart cell numbers are set around the time of birth and that the ensuing endowment is maintained until aging or some pathological process sets in [11, 12]. During late gestation in the ovine fetus, cardiomyocytes go through a maturation phase whereby dividing mononucleated cells become binucleated and stop dividing [13, 14]. Some 60–80 % of the cardiomyocyte population is binucleated at birth and that number rapidly increases to 95+% within 2 weeks after birth (unpublished). The terminal differentiation process is not well understood but it is clear that factors that accelerate it or depress it will influence the total cardiomyocytes endowment for life.

It is increasingly evident that two sets of antagonistic growth-altering biological agents are key factors in regulating cell numbers in the ovine heart [15]. Pro-proliferation hormone/growth factors include insulin like growth factor-1 (IGF-1), cortisol and angiotensin II (Ang II). Interestingly in adult life, these hormones are not growth-promoting agents in the heart. Two hormones that act in opposition to the aforementioned pro-proliferation hormones have been identified as thyroid hormone and atrial natriuretic peptide (ANP). It appears that the balance of the hormones is not based exclusively on their relative concentrations. Receptor affinity and "gain" of the signaling process are also important.

The thyroid hormone story is especially interesting. As term approaches and cortisol levels in the fetal blood rise, the protein levels and activities of deiodinases in the liver and other tissues increase [16]. The activity of these enzymes converts thyroxine (T_4) to its more potent form tri-iodo-L-thyronine (T_3). T_3 has at least three powerful effects on the developing myocardium [17, 18]: (1) it suppresses proliferation of cardiomyocytes and (2) it "forces" the maturation of cardiomyocytes including their binucleation and the maturation of their organelles and their physiological function (Fig. 18.6) and (3) it stimulates cellular hypertrophy through cardiomyocyte widening. T_3 also stimulates maturation of intracellular systems including expression of sarco/endoplasmic reticulum Ca^{2+}-ATPase ($SERCA_{2a}$), and ANP (Fig. 18.7). T_3 is a very powerful suppressant of any pro-proliferation stimulant, even in mid gestation [18]. Professor Longo showed nearly 30 years ago that increasing thyroid hormone levels by 10 times in the ovine fetus increases heart rate and fetal oxygen consumption by about 30 %, which adds a significant work load burden to the developing heart [19]. Thus, excessive thyroid hormone levels are likely to alter myocardial growth indirectly via increased heart rate and directly by modifying growth and maturation gene expression patterns. Fetal hearts exposed to excessive plasma T_3 levels have fewer cardiomyocytes at birth.

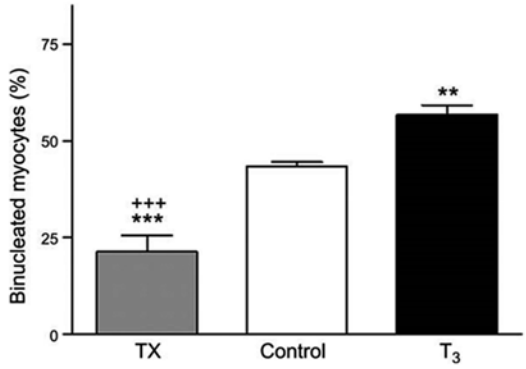

Fig. 18.6 Increased terminal differentiation of fetal sheep cardiomyocytes (binucleated) with T_3 infusion with reduced maturation in hearts from fetuses that were thyroidectomized. Data are means ± SE; $n = 8$/group. $^{**}P < 0.01$, $^{***}P < 0.001$ versus control; $^{+++}P < 0.001$ versus T_3. (From Chattergoon NN, Giraud GD, Louey S, Stork P, Fowden AL, Thornburg KL. Thyroid hormone drives fetal cardiomyocyte maturation. FASEB J 2012 Jan;26(1):397–408, with permission)

A different effect is found in hearts that are exposed to inadequate levels of T_3. In thyroidectomized ovine fetuses, cellular proliferation is also depressed because at least minimal levels of T_4 are required to maintain mitotic potency. Fetuses with low levels of thyroid hormone have low rates of cardiomyocyte binucleation [18]. Thus, hearts born to hypothyroid mothers are likely to be relatively immature. This finding is important because low birthweight is associated with hypothyroidism in adult life—thus in hypothyroid women who become mothers, the negative cycle continues, if not treated.

Contrary to previous reports, ANP suppresses the proliferative activity of the immature cardiomyocytes in culture following stimulation with Ang II or serum [20]. The signaling cascade includes augmentation of the GTPase activity of the bound receptor. In right and left ventricular myocytes a 10-7 M dose of exogenous ANP inhibited Ang II-stimulated BrdU uptake by 60+% as did exogenous 8-bromo-cGMP. Intracellular cGMP was increased by exogenous ANP exposure [20]. The phosphorylation of extracellular signal regulated kinases (ERK I, II) and Akt were inhibited by exogenous ANP in the media. Stimulation with 8-bromo-cGMP had effects on the cardiomyocytes that were similar to ANP. Figure 18.8 shows that both ANP and cGMP prevent the augmentation of cell division by Ang II. Thus, both thyroid hormone and ANP have similar suppressive effects on immature cardiomyocytes and each signals through different intracellular cascades; both could be important in regulating the number of cardiomyocytes at birth.

Hypoxemia during gestation is known to alter the vulnerability of the myocardium in adult life and render it more likely to suffer ischemic reperfusion injury in adulthood. Zhang and team have demonstrated that this is because of epigenetically driven suppression of the epsilon isoform of phosphokinase C in rat myocardium [21]. In addition, there is ample evidence that anemia before birth leads to permanently altered development of the fetal coronary tree and vascular physiology in sheep such that conductance is elevated enormously in the womb [22–25]. The conductance is defined as the slope of the relationship between blood flow and driving pressure; the latter is usually the difference between arterial and venous pressures. What is surprising about this finding is that the augmented conductance in the near term fetus in response to 4 or 5 days of anemia persists into adulthood (Fig. 18.9). The conductance is determined physiologically by the overall resistance of the coronary bed. The findings of this study are an amazing example of how the physiological conditions of the fetus may lead to structural changes that persist beyond birth. In fact, one would surmise that the augmented conductance was beneficial to the adult since low levels of blood flow are so often the cause of myocardial dysfunction or death. However, while the hearts of sheep with high conductance functioned better under conditions of mild acute hypoxemia, they were much more vulnerable to ischemic damage and infarcts when coronary flow was severely reduced by coronary occlusion.

It is now clear that building a healthy heart requires nature's blueprint encoded in the genome. However, if building materials, amino acids in particular, are in short supply or if metabolic fuel is in short supply, a heart will be built nonetheless. It will not be a perfect heart,

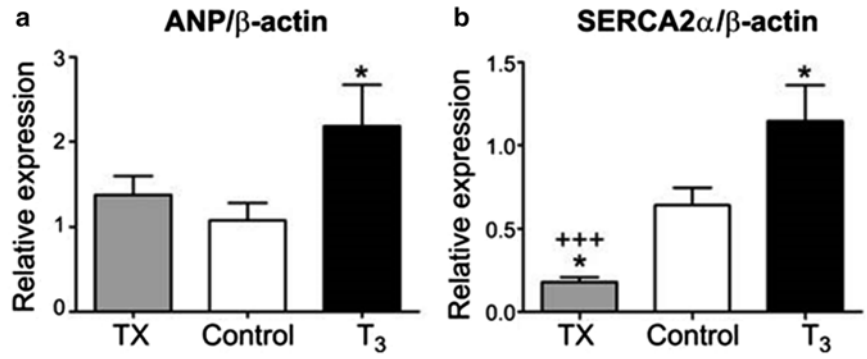

Fig. 18.7 T_3 promotes cardiomyocyte maturation. (**a**) ANP expression levels were increased in the *left ventricles* of fetuses receiving exogenous T_3. (**b**) SERCA2a expression levels were suppressed in TX fetal hearts and stimulated in T_3-infused hearts compared to controls. Data are means ± SE; $n = 8$/group. $^*P < 0.05$ versus control; $^{+++}P < 0.001$ versus T_3 (From Chattergoon NN, Giraud GD, Louey S, Stork P, Fowden AL, Thornburg KL. Thyroid hormone drives fetal cardiomyocyte maturation. FASEB J 2012 Jan;26(1): 397–408, with permission)

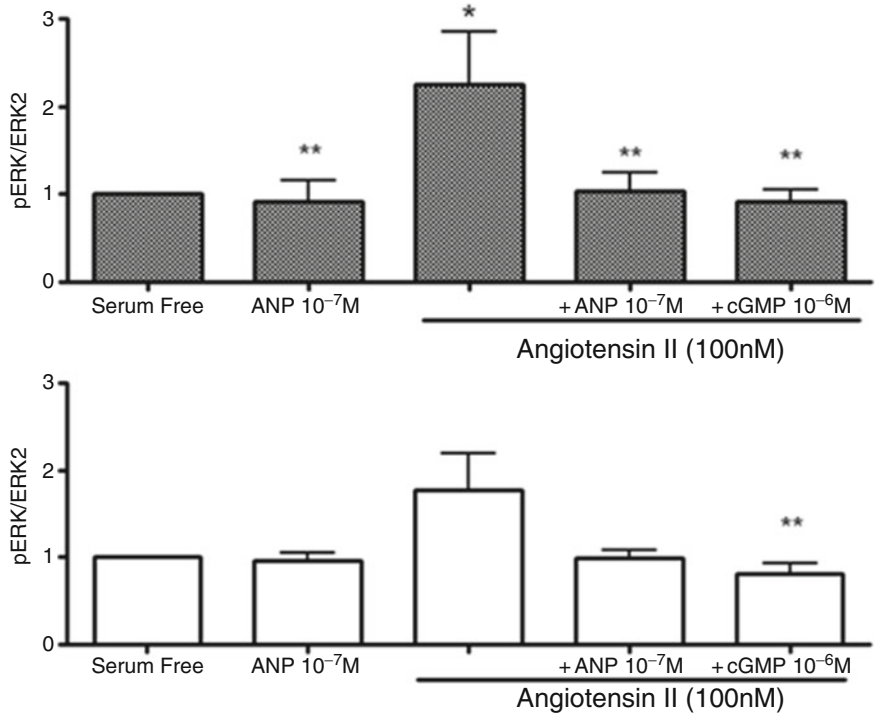

Fig. 18.8 Ang II-stimulated BrdU uptake (an index of cell proliferation) in cardiomyocytes isolated from *right* (*filled bars*) and *left* (*open bars*) near-term ovine fetal ventricles was inhibited by 48 h of treatment with the cGMP analogue, 8-bromo-cGMP. Control cells were cultured in serum-free conditions. Data are means ± S.E.M. $N = 5$ or 6 fetuses per group. $^*P < 0.05$ versus control; $^{**}P < 0.05$ versus Ang II alone. (From O'Tierney PF, Chattergoon NN, Louey S, Giraud GD, Thornburg KL. Atrial natriuretic peptide inhibits angiotensin II-stimulated proliferation in fetal cardiomyocytes. J Physiol 2010 Aug 1;588 (Pt 15):2879–2889 with permission)

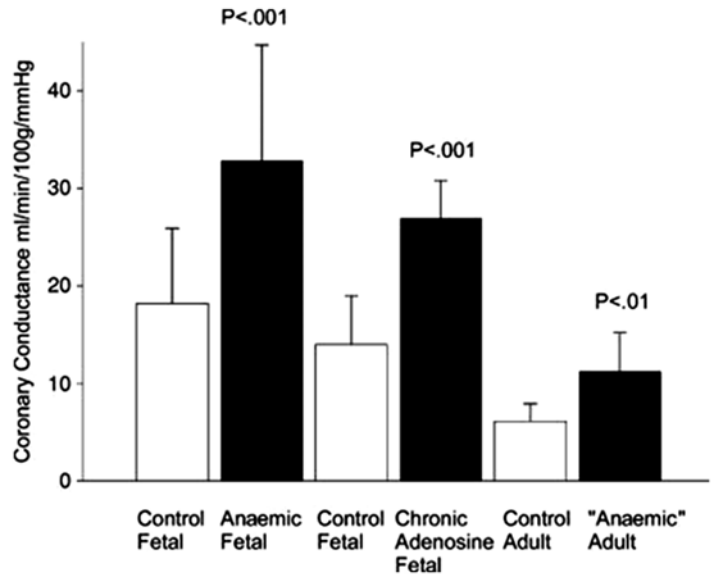

Fig. 18.9 Shows that conductance of the coronary circulation is increased in fetuses that were made anemic or that were chronically infused with adenosine in the circumflex artery. The elevated conductance remained into adult life. (From Davis L, Thornburg KL, Giraud GD. The effects of anaemia as a programming agent in the fetal heart. J Physiol 2005 May 15;565(Pt 1):35–41, with permission)

however. It is possible for the fetus to cut corners and construct a body that is less sturdy than optimal for a long life of use. A compromised fetus may have an altered coronary architecture and it may have fewer cardiomyocytes than it needs. It may also lack the biochemical backup systems required to protect it under episodes of stress over the life course. The owner of the heart will be vulnerable for one or more of the three ways that people die: a heart attack, a heart that fails or a heart that stops because it cannot maintain synchronized electrical impulses.

Thus, the fetal environment provides the underpinnings of chronic disease in adults. While we know that many other modifications of fetal organ structure and epigenetic alterations of gene expression occur, the field is ripe for young investigators to see this hot area of research in their future. The question now is how to link the increases in chronic disease with the biological underpinnings of programming. Barker showed an eightfold increase in the risk for type 2 diabetes across the birthweight spectrum and it is well known that some 65 % of people with type 2 diabetes suffer coronary heart disease and/or heart failure (http://www.heart.org/HEARTORG/Conditions/Diabetes/WhyDiabetesMatters/Cardiovascular-Disease-Diabetes_UCM_313865_Article.jsp). Thus the link between fetal nutrition and later disease is certain. Perhaps it is time to be courageous and state that the nutritional environment in which westerners live is driving dramatic and unprecedented increases in disease among humans.

1.4 The Link Between Poor Diet and the Current Chronic Disease Epidemic

The twenty-first century will be known as a time when high death rates from communicable diseases were replaced by those from chronic diseases. For example, now a dozen years into the century, cardiovascular disease dominates mortality worldwide. This powerful trend is found even in developing countries that were once dominated by mortality from infectious diseases. A worrisome corollary of the global picture is the disease trend in the USA, a country which has recently become the world's leader in declining health.

Why are westerners suffering from worsening chronic disease at this time in human history?

Chronic disease progression around the world is especially noteworthy in populations that have adopted the "western diet," rich in processed foods that are energy dense but lacking nutrients [26]. For example, Americans have relinquished their control over their intake of nutritious foods by purchasing time-saving processed or fast foods that have poor nutrient content or contain outright harmful substances rather than eating foods grown in nutritious soil. Over the past three generations, the quality of the American diet has deteriorated. Women bearing children have become increasingly less able to afford wholesome foods and are less capable of preparing and cooking wholesome foods for their families. As an ever larger portion of families are slipping across the poverty line, the diets of people without means are likely to become even more important in determining population health. Nearly half of all children in Oregon are born to mothers that are enrolled in the WIC program for low income families. WIC provides but meager support for the purchase of nutritious food during pregnancy and child rearing for women from low income families. The shift away from eating and storing traditional garden-grown foods over the past 50 years is easily explained by the fact that the food industry provides tasty but non-nutritious calories at a very low price. Fetal physiologists have a role to play in this important health issue. The magnitude of nutrient flow during gestation directly affects compromises that the fetus must make to construct a healthy body. Only the science of fetal biology can bring clarity to this issue.

Experts in programming hold the knowledge base that will provide a solid way forward in driving the case for changing America's food culture. Rarely has any scientific group has such an awesome responsibility to the public.

2 Conclusions

Over the past 20 years, the field of fetal biology has transformed from one driven to understand the basic function of fetal organs to one focused on understanding how disordered prenatal growth provides substrate for disease in the next generation. This shift in emphasis follows the discovery that chronic diseases have their roots in the quality of embryonic and fetal growth. Among important discoveries in this field are the findings that even the embryo heart developmental pattern can be detrimentally modified by the shear and wall forces forced upon it by poor placental vascularization. Likewise developmental processes during the late fetal period determine the degree to which the adult myocardium is endowed with cardiomyocytes. Insulin like growth hormone-1, thyroid hormone and atrial naturietic peptide are important determinants of cardiomyocyte numbers at the end of gestation. These biological findings translate into public health issues as well as economic issues with great social ramifications. Thus, fetal biologists are now faced with opportunities to show the public how an understanding of fetal growth can benefit public health policy.

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Estrogen in the Fetus

19

Charles E. Wood

Abstract

Estradiol and other estrogens are important modulators of fetal and maternal physiology in pregnancy. Much is known about the biosynthesis of estrogens in fetus and mother, and much is known about the role that estrogen plays in labor and delivery. However, much less is known about the regulation of estrogen biosynthesis throughout the latter half of gestation, and the role that estrogen plays in homeostatic and neuroendocrine control in the fetus. This review focuses on the biosynthesis and actions of estrogen in the fetal circulation, the role that it plays in the development of the fetus in the latter half of gestation, and the role that is played by the estrogen milieu in the control of the timing of birth. Estrogen circulates in fetal blood in both unconjugated and conjugated molecular forms, with the conjugated steroids far more abundant than the unconjugated steroids. This review therefore also addresses the biological significance of the variety of molecular forms of estrogen circulating in fetal and maternal blood.

Keywords

Estradiol • Pregnancy • Estrogens • Fetal blood • Steroids

Estradiol and other estrogens are important modulators of fetal and maternal physiology in pregnancy. Actions of estrogen in the maternal circulation include the modulation of uterine and systemic vascular tone [1–4], uterine growth and differentiation of the uterine glands [5], growth and terminal differentiation of mammary ducts and lactation [6, 7]. Much is known about the ontogeny of estrogen biosynthesis and secretion into the maternal circulation, and much is known about the biochemical mechanism underlying the function of the feto-placental unit in the human being and nonhuman primate. The feto-placental unit, which typifies primate placental estrogen biosynthesis, represents a de facto collaboration between mother and baby [8]. While control of estrogen production

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L. Zhang and C.A. Ducsay (eds.), *Advances in Fetal and Neonatal Physiology*, Advances in Experimental Medicine and Biology 814, DOI 10.1007/978-1-4939-1031-1_19,

in the human is dependent upon fetal hypothalamus-pituitary-adrenal axis integrity and activity, more attention has been given to the role of estrogens in the maternal, rather than the fetal, circulation. In this review, I will focus on the biosynthesis and actions of estrogen in the fetal circulation, the role that it plays in the development of the fetus in the latter half of gestation, and the role that is played by the estrogen milieu in the control of the timing of birth. This review also focuses on the biological significance of the variety of molecular forms of estrogen circulating in fetal and maternal blood.

Much of our understanding of the role of estrogens with regard to fetal physiology has derived from work in the chronically catheterized fetal sheep model. Biosynthesis of estrogen in the sheep differs from that in the human or nonhuman primate because of the ability of the sheep placenta to induce 17α hydroxylase activity, encoded by the CYP17 gene, in late gestation [9, 10]. In the human and nonhuman primate (Fig. 19.1), an increase in the secretion of the estrogen precursor, dehydroepiandrosterone sulfate (DHAS), by the fetal adrenal increases placental synthesis of estradiol [8, 11]. In the sheep (Fig. 19.1), the placenta can synthesize estradiol directly from maternally-derived cholesterol after induction of CYP17 but may also be in part dependent on supply of precursors from the fetal adrenal cortex [12–15]. In both cases, the increase in estrogen secreted into the maternal circulation is an important step in the chain of events that culminates in parturition. Also in both cases, the ultimate stimulation of estrogen production is the adrenocorticotropin (ACTH) secretion by the fetal pituitary [16]. Prior to the preparturient induction of CYP17, however, the sheep expresses little or no CYP17 [17, 18]; nevertheless, the ovine fetus—like the human fetus—has estrogen circulating in its blood. The mystery of the origin of these fetal estrogens is perhaps at least partly explained by secretion of estrogen precursors from the adrenal cortex [15].

Viewed through the lens of late gestation and parturition, the role of estrogen in the fetus is at least in part characterized by the action of estradiol to stimulate the fetal HPA axis. We have previously hypothesized [19] that increases in plasma concentrations of estrogens [20] or increases in the abundance or balance of estrogen receptors in target tissues [21] participate in a progressive stimulation of the fetal HPA axis by estradiol and other estrogens. Chronic estradiol treatment of fetal sheep increases basal and stress-induced fetal HPA axis activity [22–25]. Partial blockade of aromatase by infusion of inhibitor into the fetal circulation decreased circulating fetal ACTH, although with little effect on cortisol [26]. In late gestation baboons, blockade of aromatase decreased plasma cortisol concentration in umbilical cord blood [27].

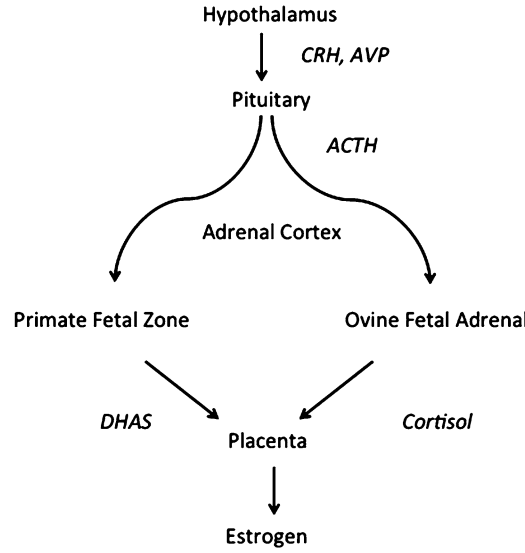

Fig. 19.1 Estrogen production in primate and sheep with respect to control by the fetal hypothalamus-pituitary-adrenal axis. In the human and nonhuman primate, there is a functional feto-placental unit for biosynthesis of estrogen. Estrogen precursors, mainly dehydroepiandrosterone sulfate (DHAS), are supplied to the placenta as substrate for synthesis of estrogens. The placenta of the primate requires DHAS as substrate because of a lack of CYP17 (which is responsible for 17α-hydroxylase activity). The placenta of the sheep lacks CYP17 before it is induced by the preparturient increase in fetal plasma cortisol concentration that heralds the onset of labor in this species. Prior to the induction of CYP17, the sheep placenta synthesizes estrogens from precursors that are secreted by the fetal adrenal cortex

Estradiol appears to have a stimulatory effect on the fetal HPA axis via an action on the fetal central nervous system. Evidence of this is the increasing abundance of arginine vasopressin in the ovine fetal hypothalamus with estradiol treatment [28]. Evidence of estradiol action in the fetal brain can also be seen as increased Fos immunostaining in paraventricular nucleus of the hypothalamus and other regions important for control of ACTH secretion [23]. The mechanism of neuronal stimulation by estradiol is not known, but it is likely that at least one component of the mechanism involves brain prostaglandin biosynthesis.

There is a longstanding recognition of the effect of prostaglandin E2 (PGE2) on fetal ACTH and cortisol secretion [29, 30]. Parturition is triggered after prolonged administration of PGE2 [31]. There is a dramatic increase in circulating concentrations of PGE2 in the plasma of fetal sheep that originate in the placenta and peaks at the time of spontaneous parturition [30, 32]. Intravenous infusion of high doses of PGE2 into the fetus increase fetal HPA axis activity [33]. Whole body blockade of prostaglandin biosynthesis with nimesulide (a cyclooxygenase-2, COX2, inhibitor) inhibits HPA axis activity in fetuses of laboring sheep [34]. In a similar experiment, McKeown and colleagues demonstrated that, after initiation of labor with RU-486, treatment of fetal sheep with meloxicam (a COX2 inhibitor) decreased plasma PGE2 and fetal ACTH concentrations [35]. Inhibition of prostaglandin synthesis has been shown to prolong gestation in several species, although this is the direct result of reduced prostaglandin stimulation of the myometrium. The link between COX2, ACTH, and parturition in sheep is clear, although the critical site of COX2-mediated prostaglandin biosynthesis is the fetal brain. Infusion of PGE2 into the carotid arterial blood of fetal sheep at rates that modestly exaggerate the spontaneous increase in plasma PGE2 from the placenta are ineffective in stimulating fetal pituitary ACTH secretion [36]. Intravenous infusion of PGE2 is more effective at releasing immunoreactive ACTH from lung than from pituitary [36]. Blockade of COX2 specifically in the fetal brain

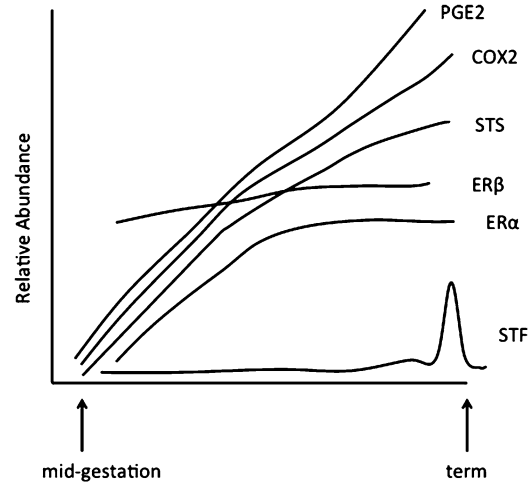

Fig. 19.2 Schematic representation of tissue content of prostaglandin E2 (PGE2), mRNA abundance of cyclooxygenase 2 (COX2), steroid sulfatase (STS), estrogen sulfotransferase (STF), and estrogen receptors alpha (ERα) and beta (ERβ) in the cerebral cortex of fetal sheep throughout the latter half of gestation [21, 41, 92]. The relative abundance of these enzymes is consistent with the net deconjugation of sulfoconjugated estrogen, increasing transcriptional activity of the estrogen receptor, increasing transcription of COX2, and increasing local biosynthesis of PGE2

(by infusion of small amounts of nimesulide into the lateral cerebral ventricle of the fetal sheep) decreases fetal ACTH responses to stress (cerebral hypoperfusion) and prolongs gestation [37, 38]. NMDA glutamatergic neurotransmission, which mediates the fetal ACTH response to cerebral hypoperfusion [39], stimulates the HPA axis in part by increasing brain (COX2-dependent) prostaglandin biosynthesis [40]. The concentration of PGE2 in cerebral cortex and hippocampus greatly exceeds that in plasma, suggesting local biosynthesis, and the concentrations increase in these brain regions prior to parturition [41]. The fetal brain and pituitary express the enzymatic machinery needed for prostaglandin biosynthesis (Fig. 19.2) [41–43]. Activation of neuronal pathways subserving fetal responsiveness to stress increase the expression of these enzymes [44].

It is possible that there exists a positive feedback relationship between placenta and the fetal HPA axis. This idea was first proposed as a link between placental PGE2 production and

fetal ACTH secretion [32]. However, even with the understanding that the site of prostaglandin production relevant to fetal ACTH secretion is in the fetal brain and/or pituitary, it is still logical to posit a functional communication between placenta and fetal brain. There is evidence to support the notion that estrogen secreted by the placenta augments fetal HPA activity, and that at least a part of that mechanism of the estrogen effect on ACTH is via stimulation of COX2 expression in the fetal brain. Estradiol treatment increases COX2 abundance in fetal brain and increases the magnitude of the increase in COX2 abudance after cerebral hypoperfusion [24]. Infusion of ICI 182,780, an estrogen receptor antagonist intracerebroventricularly into fetal sheep decreases COX2 mRNA abundance [45]. The mechanism of the estrogen effect on COX2 expression in the brain is unknown.

There is evidence that estrogen alters COX2 expression in endothelial cells via an effect on membrane-bound ER, transduced by the activity of phosphatidylinositol 3-phosphate/Akt [46]. Similarly, upregulation of COX2 expression in breast cancer cells by the xenoestrogen o′p′-DDT is dependent on CRE activation, and PKA and Akt/PI3 kinase activities [47]. As might be expected, several endocrine stimuli to COX2 generation utilize phosphorylation of CREB as the mechanism. For example, in adult fibroblasts, cortisol upregulates COX2 via phosphorylation of CREB [48].

In cardiac myocytes, corticosterone increases COX2 transcription via binding of the GR and C/EBP to the COX2 promoter. In the placenta of sheep, the mechanism of upregulation of COX2 at the end of gestation might also be glucocorticoid, not estrogen, dependent. Whittle and colleagues reported that cortisol increases COX2 mRNA in placenta, and the effect is not blocked by concomitant blockade of aromatase [49]. It is possible that, in differing cell types within placenta, both glucocorticoid and estrogen receptor upregulated the PTGS2 (COX2) gene expression using the same molecular mechanism, CREB phosphorylation. However, in many tissues, the predominant effect of glucocorticoid is inhibition of the PTGS2 gene, secondary to a GR-NFkB interaction that blocks the upregulation of gene expression by NFkB [50].

1 Cellular Responses to Estrogen in the Fetus

Numerous studies have demonstrated that estrogen receptors are expressed in various tissues in the late-gestation fetus [51, 52]. ERα functions as a ligand-gated transcription factor (Fig. 19.3) [53, 54], or as an estrogen binding site tethered to the plasma membrane [55–58]. The role of ERβ is somewhat less clear. ERβ could act as a functional inhibitor of ERα action [59, 60]. ERβ is also known to play an important role in mitochondrial function [61–63]. Estrogen receptors are found in the fetal brain

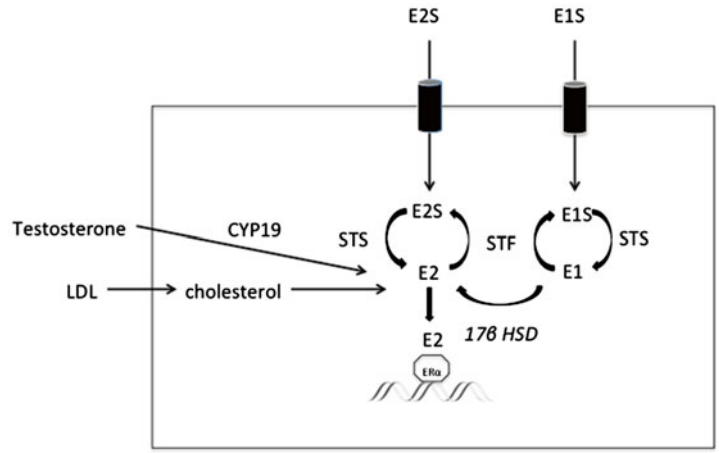

Fig. 19.3 Schematic representing the local synthesis of estradiol (E2) from testosterone and de novo from cholesterol, the interconversion of E2 and estrone (E1), the sulfonation/desulfonation cycle catalyzed by steroid sulfatase (STS) and sulfotransferase (STF), and entry of E2S and E1S into the cell by carrier-mediated transport

of sheep [64–66], and mouse [67, 68]. In the cerebral cortex of the bovine fetus, ERα, ERβ, and aromatase are expressed throughout gestation, with increases in both ERα and ERβ at the end of gestation [69].

Changes in the abundance of estrogen receptors might predict developmental changes in sensitivity to estrogen. In a recent study, we reported that there is an increase in ERα and a decrease in ERβ in the ovine fetal pituitary in late gestation [21], suggesting a possible increase in pituitary sensitivity to estradiol in the peripartum period. Interestingly, the ratio of ERα/ERβ (at the level of mRNA abundance) favors ERα in the pituitary, but ERβ throughout the fetal brain. In hypothalamus, ERα and ERβ abundances are highest at 120 days gestation (~80 % gestation), with decreases at term. The decreases might be caused by receptor downregulation secondary to rising plasma concentrations of estradiol. In hippocampus, the brain region in which there is increasing biosynthesis of PGE2 in late gestation, there is an increase in both ERα and ERβ from 120 days through the end of gestation, with further increases postnatally. These changes in ER abundance in pituitary and in various brain regions suggests an increasing pattern of estrogen signaling in the brain as the fetus matures and approaches the normal time of spontaneous parturition. The molecular development of the brain could therefore account for changes in estrogen responsiveness prior to the ontogenetic increase in plasma concentrations of estradiol at term.

2 17β-Hydroxysteroid Dehydrogenase

In addition to developmental changes in estrogen receptor abundance, local synthesis and enzymatic biotransformation of steroids within target tissues can alter estrogen signaling independent of changes in plasma concentrations of estradiol (Figs. 19.3 and 19.4). Estradiol and estrone are interconverted by the action of 17β-hydroxysteroid dehydrogenase (17β-HSD) activity. Resko and Stadelman reported that this activity, converting E2 to E1, was higher in the fetal pituitary of the rhesus monkey at 80 (~48 % gestation) days gestation compared to 120 (~73 % gestation) and 150 (~90 % gestation) days gestation [70]. In that study, the investigators reported lower levels of

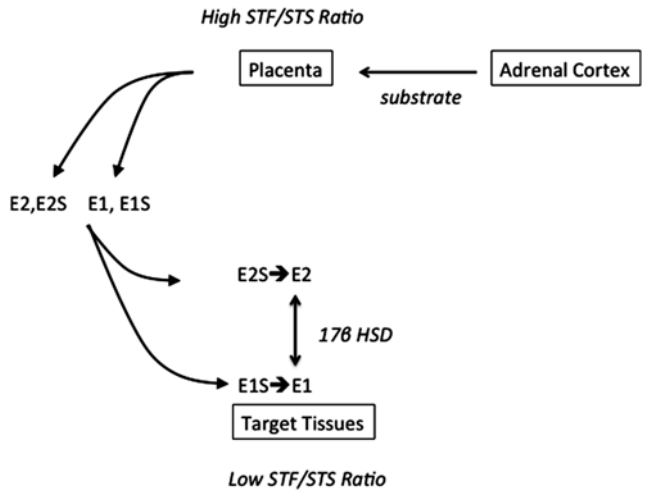

Fig. 19.4 Schematic representation of the targeting of estrogen from its source (placenta) to tissues that deconjugate E2S and E1S and therefore enhance local estrogen signaling. The placenta expresses sulfotransferase but very little sulfatase, resulting in predominant secretion of sulfoconjugated estrogen. Target tissues that express sulfatase but little sulfotransferase would be expected to have higher cellular content of unconjugated estrogen than tissues that cannot locally deconjugate the high concentrations of E2S and E1S that are in fetal blood

17β-HSD activity in the brain of the fetus than in pituitary and other peripheral tissues. In first and second trimester human fetuses, Millewich, MacDonald, and Carr reported higher oxidative activity (E2 to E1) in liver, intestine, stomach, kidney, brain, and heart, and relatively higher reductive activity (E1 to E2) in placenta and in the fetal zone of the fetal adrenal cortex [71]. In a later study, Takeyama and colleagues reported that in human fetuses 11–20 weeks gestation, 17β-HSD activity could be measured in various tissues, but that both the oxidative and reductive activities were far lower in fetal brain than in placenta and liver, with intermediate activity in regions of the gastrointestinal tract and in the kidney [72]. Using Northern blot, these investigators reported that type 1 enzyme (HSD17B1) was found in placenta, while the type 2 enzyme (HSD17B2) was found in liver, kidney, and gastrointestinal tract [72]. Recent molecular studies have revealed that there are multiple enzymes with 17β-HSD activity. Currently, there are 14 known enzymes that have been identified as members of the family of enzymes that have similarities in enzyme activity and gene structure. These are named HSD17Bx (HSD17B1, HSD17B2, etc.) [73]. Our understanding of the distribution of these enzymes in the developing fetus is incomplete, and an improvement in this regard can improve our understanding of molecular targets that can be exploited for the purpose of altering the course of fetal development or timing of parturition.

3 Aromatase

Aromatase (CYP19), is present in brain [74, 75] and is usually understood as a marker of local estrogen synthesis (Fig. 19.3) [76]. In the developing rat brain, aromatase activity increases between day 16 of gestation (~75 % gestation) and day 20 of gestation [77]. Highest activities were found in the preoptic area. However, because the altricial nature of the in utero development of the rat brain, these results cannot easily be extrapolated to species that are more mature at birth. Aromatase activity has been found in the term and newborn rhesus monkey brain, with highest levels in the POA and hypothalamus [78, 79], and has been found in the fetal sheep brain, again with high levels of activity in the fetal hypothalamus [80, 81]. Similarly, CYP19 mRNA is found in human fetal (15–19 weeks gestation) brain [82]. Tissue-specific expression of the CYP19 gene involves multiple alternative splicing schema, although the alternative splicing is not in the coding region [83].

The origin of steroid in the fetal brain is generally thought to be placental or maternal in origin. In the case of glucocorticoids and mineralocorticoids, the fetal and maternal adrenal cortices are the most likely source. However, there is increasing evidence of the possibility that steroids are synthesized from cholesterol in the brain. Adult human brain expresses low levels of steroidogenic enzymes that could subserve local synthesis of mineralocorticoid and glucocorticoid [84]. Brain steroid concentrations in adrenalectomized rats reveals functional local steroidogenesis in varous brain regions [85]. Pezzi and coworkers, using PCR, have demonstrated the presence in the fetal brain of StAR protein and all the enzymes needed for synthesis of estradiol from cholesterol [82].

4 Sulfoconjugation of Estrogens

The most abundant molecular forms of estrogen in fetal and maternal blood are the conjugated estrogens. In the sheep, E1 sulfate (E1S) is more abundant than E1, and E2 sulfate (E2S) is more abundant than E2 [86, 87]. The placenta of the sheep has abundant activity of estrogen sulfotransferase [88, 89] but very little estrogen sulfatase activity [18]. In the maternal circulation, E1 and E1S increase progressively through the latter half of gestation from approximately 10 to approximately 50 pg/mL and from approximately 50–100 to approximately 2,000–5,000 pg/mL, respectively [20]. The pattern of E1 and E1S in the fetus is similar, although plasma concentrations of E1S are higher than on the maternal side of the placenta [20, 86]. Maternal plasma E2, like

E1, increases throughout the latter half of gestation, increasing from <10 pg/mL at mid-gestation to approximately 50 pg/mL at term [86]. Maternal E2S displays the same pattern but at higher levels, increasing from <100 pg/mL to approximately 500 pg/mL [86]. Arteriovenous differences across the uterine vascular bed support the conclusion that estrogen synthesis is dominated by the placenta, but it is clear that the pattern of change in the plasma concentrations for E1, E1S, E2, and E2S are not identical in fetal and maternal blood, suggesting either directional secretion of steroid or an independent source of estrogen secretion in the fetus. In any event, the high concentration of E2S in uterine venous blood compared to E2S in jugular venous blood strongly suggests that E2S is secreted from the placenta.

What is the endocrine function of the sulfoconjugated E1 and E2 in fetal and maternal blood? Is it simply a metabolite or is it an active hormone? We have proposed that required steps involved in cellular action of sulfoconjugated estrogens would include carrier-mediated transport [90] and deconjugation [91, 92]. Sulfoconjugation blocks binding of the steroid to the estrogen receptor [93, 94]. Removal of the sulfate group, liberating E1 or E2, would be required to convert the sulfoconjugated steroid to a steroid that can act at the ER. It is possible that secretion of sulfoconjugated steroid allows targeting to tissues that express the deconjugating enzyme [19]. Local deconjugation has been proposed as one cellular mechanism by which breast cancer increases local estrogen action [95]. However, a similar mechanism could work in both mother and fetus. Fetal brain takes up sulfoconjugated estrogen from fetal plasma [96], and fetal brain contains both conjugating and deconjugating enzymes [91, 97]. The ratios of mRNA abundance for the two enzymes [92] suggest that the predominant reaction is deconjugation (i.e., liberation of E2 from E2S and E1 from E1S). Quantification of the ratio of mRNA for the deconjugating to the conjugating enzymes reveals that, at term, this ratio ranges from approximately 10 to 100, depending on brain region [92]. The general pattern of expression in most brain regions (brainstem, cerebellum, and cerebral cortex) is an increasing expression for steroid sulfatase (the deconjugating enzyme) and in several of the brain regions (cerebellum, hippocampus, and hypothalamus) an increasing expression of the sulfotransferase (conjugating) enzyme [92]. It is possible that, as the fetus matures and the HPA axis is activated, the increasing plasma cortisol concentration induces SULT1E1, the gene that encodes the sulfotransferase enzyme [98]. If so, estrogen action in the fetal brain could be modified by cortisol. Along with the dynamics of deconjugation and conjugation, the dynamics of E1 and E2 interconversion in the fetal brain, local synthesis of estrogen from cholesterol or from aromatization of testosterone and androstenedione should be considered as a possible factor in the action of estrogen on the fetal brain. Tissue content of E1, E2, E1S, and E2S are higher in the cerebral cortex than in fetal plasma (Wood, Chang, Keller-Wood, submitted manuscript), suggesting either that these estrogens are concentrated in the brain or that they are synthesized locally.

Contrary to the hypothesis that E1S and E2S actions require deconjugation and subsequent binding to the ER, we have found that the physiological and molecular responses of the fetus to E2S appear to have overlapping, yet distinct, actions compared to the responses to E2 [92, 96, 99]. This suggests that E2S acts, at least in part, through mechanisms not subserved by deconjugation and binding to the ER. One possibility is that E2S has a neurosteroid action on $GABA_A$ or on NMDA receptors, analogous to the action of pregnenolone sulfate [100–103]. E2S stimulates genomic responses in the fetal hypothalamus that suggest that it is orexigenic, perhaps encouraging feeding behavior in early neonatal life. E2S also stimulates a subset of genes that are hypoxia-sensitive, including genes important for neovascularization [99].

It is likely that estradiol sulfate acts, in part, via cellular mechanisms that do not involve classical estrogen receptor binding. However, little is known about the binding of E2S or E1S to cell surface receptors. To date, there have been no reports of studies reporting the binding affinity of E2S or E1S with the palmitoylated and membrane bound ERα or the putative GPCR estrogen receptor GP30 (GPER). However, there is

reported evidence of sulfoconjugated estrogen interacting with ion channels [104], analogous to the neurosteroid action of pregnenolone sulfate [102]. For example, microinjection of estradiol sulfate into the striatum of rats impaired response learning [105]. In the striatum, membrane-bound ER activates metabotropic mGluR3 and mGluR5 glutamate receptors, which in turn alter MAP kinase-dependent CREB phosphorylation [106]. Perhaps using the same mechanism, DHEAS is known to be neuroprotective, working by activation of CREB and NFkB [107].

5 A Model of Fetal Estrogens in Late Gestation

Estrogens in plasma and target tissues are important components of the endocrine milieu that modulate fetal physiology and—in late gestation—are involved as a component of the mechanism by which parturition is triggered. It is perhaps most likely that estrogen acts via several mechanisms. The large supply of sulfoconjugated estrogen in fetal blood, the ready availability of the deconjugating enzyme (STS) in the fetal brain, the abundant uptake of sulfoconjugated estrogen into the fetal brain from blood, and the expression of estrogen receptors in the brain are all consistent with local deconjugation and action of the sulfoconjugated estrogen (Fig. 19.4). In this model, the majority of estrogen secretion in the fetus is in the sulfoconjugated form, affording a large supply of water-soluble precursor hormone that can be locally deconjugated in and targeted to the brain and other tissues that express steroid sulfatase. However, the unique endocrine and molecular responses to E2S as compared to E2 suggests that there are mechanisms of action unique to sulfoconjugated estrogens, perhaps as neurosteroids interacting with glutamate or GABA receptors.

The importance of estrogen supporting the physiological changes associated with a healthy pregnancy is well-known, as is the importance of estrogen in normal, spontaneous parturition. The role of sex steroids in patterning of early brain development is also well-known. However, the view of biologically active fetal estrogen as E2 and/or E1, and the view of the nuclear ERα as the unitary signal transduction mechanism for estrogen are overly simplistic. The secretion of both conjugated and unconjugated forms of E1 and E2, the tissue-specific and gestation-specific expression of sulfatase and sulfotransferase, the known activities of ERα and ERβ as both ligand-gated transcription factors and membrane receptors, and the possibility of estrogen signaling mediated by GPR30, suggests a far more complex endocrine axis. The complexity of estrogen signaling in the fetus strongly argues for a better understanding of the role of the sulfoconjugated estrogens in fetal development. Concentrations of sulfoconjugated steroid, long assumed to be inactive, should be routinely included in the assessment of steroid concentrations in the fetus and neonate.

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Calcitonin Gene Related Family Peptides: Importance in Normal Placental and Fetal Development

20

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Abstract

Synchronized molecular and cellular events occur between the uterus and the implanting embryo to facilitate successful pregnancy outcome. Nevertheless, the molecular signaling network that coordinates strategies for successful decidualization, placentation and fetal growth are not well understood. The discovery of calcitonin/calcitonin gene-related peptides (CT/CGRP) highlighted new signaling mediators in various physiological processes, including reproduction. It is known that CGRP family peptides including CGRP, adrenomedulin and intermedin play regulatory functions during implantation, trophoblast proliferation and invasion, and fetal organogenesis. In addition, all the CGRP family peptides and their receptor components are found to be expressed in decidual, placental and fetal tissues. Additionally, plasma levels of peptides of the CGRP family were found to fluctuate during normal gestation and to induce placental cellular differentiation, proliferation, and critical hormone signaling. Moreover, aberrant signaling of these CGRP family peptides during gestation has been associated with pregnancy disorders. It indicates the existence of a possible regulatory role for these molecules during decidualization and placentation processes, which are known to be particularly vulnerable. In this review, the influence of the CGRP family peptides in these critical processes is explored and discussed.

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L. Zhang and C.A. Ducsay (eds.), *Advances in Fetal and Neonatal Physiology*,
Advances in Experimental Medicine and Biology 814, DOI 10.1007/978-1-4939-1031-1_20,

Keywords
CGRP • Adrenomedullin • Intermedin • RAMP • Implantation • Decidua • Trophoblast • Placentation • Organogenesis • Pregnancy disorders

1 Introduction

Mammalian reproduction is a complex but well-coordinated process designed to produce successful pregnancy by safeguarding critical steps of the regulatory systems. Normal preimplantation embryo development, timely journey of embryos from the oviduct into the uterine lumen, differentiation of the uterus to the receptive state, trophoblast cell fusion, trophoblast invasion and hormone production are all prerequisites for successful pregnancy; dysregulation of these processes leads to pregnancy complications, including fetal growth restriction, preeclampsia, preterm birth, gestational diabetes, etc. In recent years, calcitonin/calcitonin gene-related peptide (CT/CGRP) family peptides, a group of endogenously produced peptides, have emerged as major players in reproduction. Supported by mechanistic studies, the importance of CT/CGRP family peptides during pregnancy is growing through their involvement in vascular adaptations, utero placental functions and fetal growth.

The CT/CGRP family consists of five peptides: calcitonin (CT), amylin (AMY), CGRP, adrenomedullin (AM), and AM2/intermedin (IMD). These peptides share similar molecular structure and the majority of their functions overlap. Although these peptides have little sequence homology, they share similar secondary structure consisting of an amino acid ring structure formed by a single disulfide bond and an amidated carboxyl terminus [1, 2]. Moreover, the receptors for these peptides consist of components that are common to the majority of these peptides further adding to the overlapping functionality. Recent studies implicate CT/CGRP family peptides in multiple essential roles in a variety of functions including vascular adaptations, uteroplacental functions and fetal growth for a successful healthy pregnancy. For detailed up to date information on the role of CGRP family peptides in vascular adaption please refer to our recent review [3]. In this review, we now provide a comprehensive account of functions, shared receptors and mechanisms of action for CGRP, AM and IMD with respect to their role in regulating various functions during pregnancy especially placental development fetal growth and their implications for pregnancy complications.

2 CGRP Family Peptides and Their Receptor Components

2.1 Peptides

The CT, AMY, CGRP, AM, and IMD originated phylogenetically from CALC1 gene (CALCA) [4] and share structural similarities. C-cells in the thyroid gland were found to be the source of CT which was presented as an endocrine hormone based on its potent hypocalcemic activity [5].

The *CALCA* gene produces CT mRNA after alternative splicing when exons 1, 2, 3, and 4 are spliced together, and αCGRP mRNA when exons 5 and 6 are included instead of exon 4 [6]. A second form of CGRP, βCGRP, which differs from αCGRP by 1 amino acid in the rat and 3 amino acids in the human, is produced by a separate gene. AMY was isolated from amyloid deposits in pancreatic islets of patients with type II diabetes, is produced mainly by β-islet cells, and is co-secreted with insulin [7, 8]. AMY shares only 40 % amino acid identity with CGRP. AM was originally isolated from human pheochromocytoma but is also expressed in various other tissues [9].

The preproAM is processed to form a 164-amino acid peptide proAM, which is then cleaved to give rise to biologically active AM [9]. Rat AM is 50 amino acids long and differs from human AM in only 6 positions [10]. There is a 27 % homology between the peptide sequence of AM and CGRP. The human IMD gene encodes a prepro-protein of 148 amino acids and a predicted 47-amino-acid mature peptide. IMD has ~28 % sequence identity with AM and <20 % with CGRP, and is expressed primarily in the intermediate lobe of the pituitary and gastrointestinal tract, and appears to have distinct physiological effects [2].

All these peptides share several common features of secondary structure which includes a ring structure formed by an intramolecular disulfide linkage and a C-terminal amide structure that are essential structural features of the CT/CGRP family. The N-terminus of these peptides has di-sulfide bonded residues important for their biological activity and approximately 30 amino acids required for binding [1]. Distinct biological activity is exhibited by these peptides despite their sequence homology due to the interaction with their receptors.

2.2 Receptors

Seven-transmembrane (TM) domain G-protein-coupled receptors, CT receptor (CTR) and calcitonin receptor-like receptor (CRLR) are the two G-protein coupled receptors assigned to this peptide family that interact with their respective ligands. The CTR is for CT and AMY, whereas CGRP, AM and IMD exhibit their function through CRLR. The ligand binding specificity of these receptors is conferred by 1 of the 3 receptor activity modifying proteins (RAMPs). CTR and CRLR share >50 % amino acid identity, and CTR by itself acts as a specific receptor for CT, whereas transport of CRLR to the plasma membrane and its ability to bind ligands are dependent on its heterodimerization with one of the RAMPs [11–13]. The RAMPs are 150 amino acids long single TM proteins. RAMPs share a similar basic structure—a large extracellular domain of 120 amino acids containing 4 conserved cysteine residues, a single TM domain of 20 amino acids, and a short intracellular domain of 10 amino acids. $RAMP_1$ was discovered while screening an expression cDNA library from the human neuroblastoma cell line SK-N-MC in *Xenopus* oocytes while aiming to clone a specific CGRP receptor [13]. A sequence database search for expressed sequences similar to $RAMP_1$ identified $RAMP_2$ and $RAMP_3$. The 3 RAMPs show only about 30 % amino acid sequence identity. The type of RAMP protein associated with CTR or CRLR would dictate the specificity of binding to each of the CT/CGRP family peptides.

$RAMP_1$ co-expressed with CRLR constitutes CGRP receptor, which is antagonized by truncated $CGRP_{8–37}$ [14]. Co-expression of $RAMP_2$ or $RAMP_3$ with CRLR constitutes specific AM receptors. CRLR forms a heterodimer with either $RAMP_2$ to form AM_1 receptor or with $RAMP_3$ to form AM_2 receptors. Studies of the reconstituted CGRP and AM receptors in yeast suggest that $CGRP_{8–37}$ and $AM_{22–52}$ are selective for the CRLR receptor/$RAMP_1$ and CRLR receptor/RAMP2 combinations, respectively [15]. IMD actions are mediated via formation of a heterodimeric complex of CRLR with any 1 of the 3 RAMPs. However, as demonstrated by Roh et al. actions of IMD are more potent in the presence of CRLR/$RAMP_1$ or CRLR/$RAMP_3$ compared to CRLR/$RAMP_2$ [2]. A truncated form of IMD, $IMD_{17–47}$, is the suggested antagonist of IMD [2]. Since IMD functions through CGRP and AM receptors, some of the effects of IMD are antagonized by both CGRP and AM antagonists. It appears that CT/CGRP peptides can bind to CRLR, but the presence of RAMPs is critical for signaling.

3 Expression and Localization of CGRP Family Peptides and Their Receptor Components

CGRP, AM and IMD and their receptors have been shown to be expressed in placenta and implantation sites [16–19]. CRLR and RAMPs are localized in numerous reproductive tissues

including the uterine endometrium [20], fetal membranes [21, 22], placenta [23], and trophoblast cells [16, 21, 24–27], suggesting an important role for CGRP, AM and IMD in feto-placental development. mRNA for receptor components CRLR, $RAMP_1$, $RAMP_2$ and $RAMP_3$ are expressed in implantation sites, inter-implantation sites, fetus and placenta in rat gestation. These receptor components are differentially regulated during pregnancy in a spatio-temporal manner [17]. CRLR and $RAMP_1$ immunoreactivity was specifically shown to be concentrated in the cytotrophoblast and syncytium in labyrinth, trophoblastic giant cells and basophilic cells in trophospongial cell layer, and endothelium and smooth muscle cells in fetal vessels [28]. Transcripts of CRLR were detected by RT-PCR in both decidual and extravillous trophoblast cells, whereas transcripts of $RAMP_1$ were detected in decidual cells only [16]. No $RAMP_1$ immunostaining was observed in either EVCTs or immune cells [16]. Thus, a functional CGRP receptor appears to be present in decidual cells but not in extravillous trophoblast cells.

AM localizes to the human epithelium and endothelium of the endometrium [20] and in stromal macrophages [29]. In the rat AM was localized in the endometrial stroma with increased immunoreactivity from nonpregnancy to pregnancy [17]. Robust levels of *AM* gene expression were found in the mural trophectoderm cells of a preimplanting blastocyst at E3.5. The relative level of fluorescence in the trophectoderm lineage was slightly higher than the levels observed in the inner cell mass [30]. AM along with CRLR and $RAMP_2$ mRNAs was reported in fetal membranes and umbilical vein endothelial cells (HUVECs) [31–33]. $RAMP_3$ mRNA is also expressed in HUVECs.

Expression of IMD is reported in placenta throughout gestation [19, 34]. Immunoreactive IMD was reported in cytotrophoblast cells, syncytio-trophoblast, placental vascular endothelial cells, decidual trophoblast cells and natural killer (NK) cells that are infiltrated into deciduas [35]. Expression of CGRP, AM and IMD and their receptor components are also demonstrated in the immortalized first trimester trophoblast derived normal extravillous cytotrophoblast cell and trophoblast cells derived from choriocarcinoma such as JEG-3, HTR, JAr and TEV-1 cells [34, 36]. Availability of these cell lines has greatly facilitated many in vitro studies to explore functional roles of these peptides in pregnancy [36].

Expression of these peptides and their receptor components appear to be regulated by sex steroid hormones. During pregnancy, 17beta-estradiol inhibits, while progesterone stimulates, placental mRNA and proteins for CRLR and $RAMP_1$. Antiestrogen, ICI 182780, increased, whereas antiprogesterone, RU 486, inhibited expression of CRLR and $RAMP_1$ [28, 37].

4 Physiological Roles of CGRP Family Peptides During Pregnancy

4.1 Implantation

After fertilization, the morula becomes a blastocyst as fluid accumulates and polarization of cells occurs. The blastocyst has an outer layer of cells (trophoblast) that will form the placenta and fetal membranes, an inner cell mass at one pole that will form the embryo, and a fluid filled cavity. The inner and outer cell masses multiply and the fluid cavity enlarges until the expanded blastocyst hatches out of the zona shell. Initially it is bathed in uterine secretions that provide oxygen and metabolic substrates; however, these secretions soon become inadequate for support of further development. Therefore, within 24 h of hatching (about day 6 after fertilization), the blastocyst implants in the uterine lining, which provides access to substrates (glycogen filled stromal cells) necessary for continued growth. Implantation involves movement of the blastocyst to an optimal location (typically the mid to upper anterior or posterior wall of the human uterus), adhesion, and invasion.

CGRP is found to be produced by decidual cells (but not by extravillous trophoblast cells) at the implantation site, where it is suggested to be involved in important events, such as the complex immunomodulation that abrogates rejection

of trophoblast cells by decidual cells and immunocompetent cells present in the decidua [16]. CGRP stimulates cAMP production in cultured decidual cells while it also acts on the nearby extravillous trophoblasts to increases NO release [16]. Thus, CGRP has paracrine and autocrine effect on decidual and extravillous trophoblast cells, two major players in implantation [16].

Fetal trophoblast cells and the maternal uterine wall have coordinated and localized increases in *AM* gene expression at the time of implantation. AM peptide is abundantly expressed by both the maternal uterine luminal epithelium and the fetal trophectoderm at the time and site of implantation [30]. Northern blot and in situ hybridization analyses showed that the AM mRNA is detected just after implantation and its level peaks at 9.5 days of post conception and decreases coincidentally with the completion of the mature chorioallantoic placenta. Decidual *AM* expression is strictly localized and concentrated around the implanting and developing embryo. Specifically, the luminal epithelium and several surrounding subepithelial cell layers of the stroma express high levels of AM, which rapidly dissipate away laterally from the implanted embryo. The robust expression of *AM* in the maternal decidua, compared with weak expression in the fetal placenta, persists throughout development. In contrast, an AM receptor was not detected in either embryo or trophoblast giant cells at 7 days, suggesting that the AM produced and secreted from the embryo's trophoblast giant cells acts on the maternal tissues rather than on the embryonic tissues [27]. Vasodilation being the hallmark function of AM [38] suggests that the most likely role for AM in the maternal uterine tissue is to maintain uterine quiescence in pregnancy and promote blood flow to the implantation site. In addition, AM is also an angiogenic factor [32, 39, 40] that has been shown to regulate vascular permeability [41] and trophoblast invasion [42, 43] that may support maternal vascular remodeling and permeability that occur during implantation. This notion is supported by maternal expression of AM in both the epithelium and endothelium of receptive uterine tissue [20] as well as stromal macrophages [29]. Inhibition of endogenous AM action via the AM receptor for just 3 days from post-copulation to preimplantation caused deleterious effects, including irregular implantation spacing at mid-pregnancy, and this effect is shown to be mediated by the AM receptor and not by the CGRP receptor [44]. $AM^{+/-}$females are shown to display abnormal spacing of and overcrowded conceptuses within the uterine horns [30]. These results demonstrate that maternal *AM* expression is tightly coordinated, localized to implantation sites, and persistently robust throughout development [30, 35].

Expression of IMD is reported in implantation sites at decidual trophoblast cells and infiltrated decidual NK cells. Recent report shows secretion of IMD by human blastocysts on day 5 [35] and its mRNA expression in day 9 rat implantation sites. Further, infusion of IMD antagonist from day 3 causes a significant decline in weights of implantation sites on day 9 [19]. Therefore, IMD may have a potential role in arterial remodeling and thus contribute to efficient implantation to ensure a healthy pregnancy.

4.2 Syncytialization and Hormone Production

After implantation, cytotrophoblasts differentiate into the villous cytotrophoblast and the extravillous cytotrophoblast (EVCT). The former fuse to form the multinucleated syncytiotrophoblasts responsible for fetomaternal exchange and production of hormones. The latter form migratory cell columns that invade the endometrium [45]. Placental hormones such as human chorion gonadotrophin (hCG) are crucial for maintenance of gestation and successful pregnancy outcome. In placental tissue, the major source of hCG is the multinucleated syncytiotrophoblast layer. Transcription of hCG subunit, mRNA expression and secretion strongly increase during in vitro cell fusion of primary trophoblasts [46–48]. Since production of hCG could be stimulated by placental as well as decidual growth factors, several investigators attempted to study the involvement of CGRP family peptides in syncytialization and placental hormone production.

The first evidence for a critical role of CGRP peptides in hormone production was obtained from studies in choriocarcinoma cell lines. Studies have shown that CGRP stimulates human villous cytotrophoblasts to aggregate and fuse to form multinucleated syncytiotrophoblasts [49]. CGRP also increases hCG, 17β-estradiol and progesterone secretion from human term trophoblasts. This CGRP-induced increase in trophoblast hormone secretion is time- and dose-dependent, and is blocked by CGRP antagonist, $CGRP_{8–37}$ [49]. Although AM and IMD immunoreactivity has been reported in syncytiotrophoblast cells of placental villi, their role in syncytialization and hormone production is yet to be established [25, 34].

4.3 Trophoblast Proliferation and Invasion

The progenitor cytotrophoblast cells are the stem cells of the placenta. These cells proliferate throughout gestation, differentiating along two pathways to form either villous cytotrophoblast, which ultimately can become syncytiotrophoblasts (outer cellular layer) or EVCTs (inner cellular layer). Syncytiotrophoblast is a specialized epithelium that has several functions, including transport of gases, nutrients, and waste products and synthesis of peptide and steroid hormones that regulate placental, fetal, and maternal systems. Extravillous trophoblasts (EVT) have a proliferative component and an invasive component. There is also a migratory EVT, which is neither invasive nor proliferative. These cells populate the cell islands, septum, chorionic plate and chorion laeve.

Invasion by trophoblast cells involves cellular proliferation, attachment of cells to and degradation of extracellular matrix, and migration through connective tissue [50]. It is well established that signaling through the RAMPs in general promotes cellular division and differentiation. Specifically, in human or rodent cells, reducing CGRP family peptides signaling by infusion with antagonists directly correlates with increased apoptosis or programmed cell death both in vitro and even more so in vivo [51, 52]. In preimplantation rats, antagonist infusion induces apoptosis, which manifests as increased resorption rates. In vivo, AM and IMD contribute for protection against apoptosis especially in trophoblast cells in the labyrinth zone of placenta and uterine decidua in rats [51, 52]. AM increases the invasive capacity and migration of trophoblast cells [34–36, 53].

Fetal *AM* gene expression is upregulated in invasive trophoblast giant cells [30]. AM is shown to enhance the invasive capabilities of JAr cells and HTR-8/SV neo cells through increasing the gelatinolytic activity of MMP-2 [43], increased expression/activity of uPA [36] and reduction in plasminogen activator inhibitor-1 expression [43]. These actions of AM were completely blocked by administration of human $ADM_{22–52}$ [36]. It is likely that AM secreted either from maternal or fetal tissues act as a migratory factor for these cells during trophoblast invasion. In support of this possibility, AM is shown to be an effective chemoattractant and migratory factor for a variety of cell types [54, 55], including cultured choriocarcinoma JAr cells and first-trimester cytotrophoblast cells [43].

Similar to AM, IMD also stimulates an increase in trophoblast invasion and migration [34, 35]. Recent report shows that IMD regulates the invasive capacity of first trimester EVCTs via suppressing decidual expression of tumor/metastasis suppressor KAI-1(Kangai-1) in human pregnancy [35]. In addition, based on the in-vivo studies IMD may involve NO and MMP/uPA system to facilitate trophoblast invasion [19].

4.4 Fetal Growth and Developmental Consequences of Direct Perturbations

Infusion of CGRP antagonist, $CGRP_{8–37}$, caused significant fetal growth restriction and pup mortality [56]. Rats infused with AM antagonist, AM_{22-52} during pre- (i.e., from gestational day 1–4) or post-implantation period (i.e., from gestational day 8–15 or 14–22) is shown to induce dose-dependent decrease in both placental and

fetal weights along with increase in fetal resorption sites [44, 52]. The AM antagonist induced placental and fetal growth restrictions appeared more pronounced when infused during late gestation (day 14–22). Fetal as well as placental growth restriction with impaired placental vasculature was also reported in pregnant rats infused with IMD antagonist in mid gestation [51]. Reduced weights of implantation sites were observed when the infusion of IMD antagonist was done during peri-implantation period [19]. Thus, IMD has a potential role in mediating early placentation and fetal-placental growth.

Genetically modified mouse models have been developed for most of the CGRP family peptides and their receptor components. CRLR, $RAMP_2$, and AM—but not CGRP, $RAMP_1$, and $RAMP_3$—null mice are embryonically lethal [3]. A modest 50 % reduction in uterine AM in $AM^{+/-}$ female mice caused significant reductions in fertility and fetal growth restriction even in wild-type and $AM^{+/-}$embryos, demonstrating a critical role for maternal AM on fetal growth. Genotype analysis from $AM^{+/-}$ intercrosses and results from reciprocal crosses using wild-type females mated to $AM^{+/-}$males did not reveal a significant dose effect of heterozygous loss of fetal AM on fertility or fetal growth. However, the incidence of fetal growth restriction was significantly exacerbated when the implanting blastocyst was null for AM. These data suggest that fetal expression of AM also contributes significantly to the early stages of embryonic development. Taken together, these data implicates that both maternal and, to a lesser extent, fetal sources of AM peptide are involved in early fetal growth [30].

5 CGRP Family Peptides and Pregnancy Diseases

5.1 Immunological Effects

Uterine NK cells constitute the largest proportion of immune cells in the decidua. The uterine NK cells have an important role in spiral artery remodeling and cooperation between decidual NK cells and the EVT are considered primary events in this vascular remodeling event [57, 58]. Recently, a direct link between AM and uterine NK immune cell function was identified [59]. Dynamic differences were reported in uterine NK cell recruitment between AM null and AM wild type placentas in a mouse model of gestation, reflecting concomitant changes in the expression of numerous chemokines and cytokines [59]. Uterine NK cells expressed high levels of CRLR, and treatment with AM responded with increases in MMP9 but not MMP2 consistent with the previously described functions of uterine NK-derived MMP9 in SA remodeling [59]. Thus, fetal AM may greatly influence the immune milieu of the placenta by recruiting and activating uterine NK cells to secrete chemokines, cytokines, and MMPs to facilitate SA remodeling. Understanding regulation of uterine NK cell effector molecule expression is important, as in addition to MMPs and cytokines such as IFN- , uterine NK cells also secrete angiogenic factors vascular endothelial growth factor and angiopoietin II [60]. In support of Li and colleagues [59], we observed that NK cells in human decidua express abundant AM receptor components (CRLR, RAMP2, RAMP3) in early gestation (Yallampalli and coworkers, unpublished observations). In ongoing studies in a rat pregnancy model [52], in vivo antagonism of AM results in decreased uterine NK numbers, and reduced IFN- expression by uterine NK cells, at implantation sites at day 8 of gestation (Yallampalli and coworkers, unpublished observations). Uterine NK cells are the predominant source of IFN- in the decidua microenvironment while exogenously administered IFN- is sufficient for SA remodeling in NK cell deficient mice [61]. Inhibition of trophoblast invasion during SA remodeling, however, is linked to induction of EVT apoptosis by uterine NK cell-derived IFN- [62, 63]. These opposing effects suggest the IFN- levels may be tightly regulated to optimize vasculogenesis while controlling EVT invasion. Understanding how neuroendocrine peptide mediators, such as CGRP and AM, regulate recruitment and activation of uterine NK cells at the fetal/maternal interface is

an important avenue of investigation relevant to diseases associated with vascular dysfunction (e.g., preeclampsia).

5.2 Spontaneous Abortion

The causes of recurrent pregnancy loss are classified as genetic, anatomic, hormonal, metabolic, immunologic, microbiologic, and environmental [64, 65]. Plasma AM concentrations were similar in women who are spontaneously aborting and their controls. However another recent study reported that the plasma AM levels in women with recurrent pregnancy loss (5.6 ± 1.9, mean ± standard deviation) were significantly higher ($P > 0.001$) than that in control women (3.6 ± 1.7) [65]. In the placenta, AM was localized at the fetomaternal interface, and the prevalence of positive cells, particularly of trophoblast cells, stained for AM was significantly lower in spontaneous abortions than in controls [66]. Our preliminary studies [67] and that of Urban et al. [68] showed that ir-AM is reduced at the fetomaternal interface in women with spontaneous abortion compared to controls. In spontaneous abortion before 10 gestational weeks, AM immunopositive cells are reduced by more than 50 % in the decidua and up to 30 % in the extravillous trophoblast cells [59]. Recent study shows that lower serum as well as placental IMD levels are associated with spontaneous abortion compared to the age matched controls [35]. IMD mRNA expression in the first trimester villous tissue from spontaneously aborted placenta were 100-fold lower at all weeks of gestation compared with elective abortion [35]. This suggests that either pathological pregnancy decreases IMD levels or the effect of lower IMD levels in these spontaneous abortions could possibly be a cause for spontaneous abortion. However, the limitations of the studies involving tissues from spontaneous abortions in human pregnancy cannot be ignored. Future studies, perhaps in a nonhuman primate model, may help to address the relationship of lower IMD levels in spontaneous abortion with the occurrence of pathology.

5.3 Preeclampsia and Intrauterine Growth Restriction

Transcripts of CRLR and $RAMP_1$ are substantially reduced in fetoplacental vessels from preeclamptic women [42]. In addition, trophoblast cells also showed decreased expressions for CRLR and $RAMP_1$ proteins and CGRP binding sites were lower in preeclamptic placentas. In addition, relaxation of umbilical and chorionic arteries to CGRP in preeclamptic women is significantly attenuated compared to their age-matched controls. Therefore, it is likely that the fetoplacental vascular resistance in normal pregnancies is regulated by CGRP, which appears to be compromised in preeclamptic pregnancies [42, 69].

Maternal circulating AM has been reported as either increased [70], decreased [71] or unchanged [23, 59, 72–74], whereas in umbilical plasma and amniotic fluid, its concentrations are higher than in normotensive pregnancies [72]. Conflicting results have been reported also in the expression of AM in fetoplacental tissues in preeclampsia. Ir-AM in placentas of preeclamptic women was found to be decreased [75] or unchanged [72], and AM mRNA expression has been shown to be either decreased [18] or unchanged in the placenta and uterine muscle [76], decreased in fetal membranes and increased in umbilical artery [76]. Similarly, receptor component for AM ($RAMP_2$) has been shown to be unchanged in the placenta [77], decreased in cord and uterus and increased in fetal membranes [77] although no correlation was found between mRNA level and blood pressure. Li et al. [78] reported a ninefold decrease in AM output from cultured preeclamptic placentae. Differences in the criteria for diagnosis of preeclampsia or in the uteroplacental or fetal hemodynamic condition between studies may account for the controversial results found. However, recent study in AM knockout mice showed characteristics of preeclamptic placenta such as failed SA remodeling and reendothelialization with retained smooth muscle actin layer as they approached the maternal fetal interface and reduced number of uNK cells in AM-null placentas [59].

Di Iorio and colleagues found that fetoplacental levels of AM peptide were increased in human patients with intrauterine growth restriction [79], while another group found no significant differences in fetal or maternal AM levels between normal pregnancies and pregnancies with fetal growth restriction [80].

Since expression of receptor components for CGRP peptide family are altered in preeclampsia, involvement of IMD in the pathophysiology of this pregnancy complication cannot be ruled out. Our unpublished data shows that expression of IMD transcript are significantly lower in preeclamptic villi compared to the age matched controls and this effect appeared to be more pronounced in pregnancies with male fetus compared to the female. Clearly, more studies are needed to understand the mechanisms of IMD action and the cause and consequences of altered IMD levels in pathological pregnancies such as miscarriages and preeclampsia.

6 Summary

It is evident from in-vivo and ex vivo studies that, the CGRP family peptides involving CGRP, AM, and IMD in endometrium, decidua and placental tissue could be an important system for various adaptive changes that occur during pregnancy. CGRP family peptides are found to have pleiotrophic effects on placenta and endometrium. In the uterus, these peptides may facilitate decidualization, favor implantation, angiogenesis and proliferation of endometriotic tissue and amplify the uNK cell responses. In the placenta, CGRP family peptides may facilitate trophoblast cell proliferation and fusion and estradiol and progesterone production.

Despite our increasing knowledge on the diverse functions CGRP family peptides in normal and pathological reproduction, much remains to be learned about CGRP family peptides-dependent signaling cascades in the diverse gestational tissues and its interactions with other molecules. Although CGRP, AM and IMD appears to play an important role in regulating feto-placental growth and development, most of the functions of these peptides appear redundant, yet knock out or inhibition of one peptide or its receptor component cause deleterious effects on fetal growth and development. Whether function of each of these peptides are complimentary, additive or synergistic; and if the signaling of these peptides integrates at some point downstream is not known. Conspicuous similarities in the structure and function and yet distinct physiological roles of these peptides provoke future studies to identify their relative roles in placental functions and if these peptides compete for their shared receptor components to create a favorable physiological milieu in pregnancy. Future explicit mechanistic studies may identify these peptides and or their receptor components as a new class of clinically useful tools in pregnancy related disorders such as recurrent miscarriages, preeclampsia and intrauterine growth restriction. Therefore, continuous research and improvement of model systems are required to gain more insights into the complex functions of CGRP family peptides in physiological and pathological placenta and endometrium.

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