Microvascular Changes in the Diabetic Foot

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Abstract

Microvascular changes in diabetes are related to the presence of neuropathy and highlighted by increased vascular permeability and impaired autoregulation of blood flow and vascular tone. The functional impairment of the microcirculation has been attributed to deficiencies at the level of the nerve-axon reflex and endothelial cell dysfunction, resulting in diminished expression of endothelial nitric oxide synthetase and poly polymerase. Consequently, there is a diminished hyperemic response, resulting in failure to achieve maximal blood flow following injury. This observed functional ischemia may be a possible mechanism for the poor wound healing in diabetic foot ulcers.

Keywords

Microcirculation • Endothelial dysfunction • Hyperspectral imaging • Near-infrared spectroscopy • Nerve-axon reflex

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Abbreviations

PAS	Periodic acid Schiff
ACh	Acetylcholine
SNP	Sodium nitroprusside
EDRF	Endothelium-derived relaxing factor
EDNO	Endothelial-derived nitric oxide
PKC	Protein kinase C
PARP	Poly ADP-ribose polymerase
AGEs	Advanced glycosylated end products
VPF	Vascular permeability factor
TXA_2	Thromboxane
PGH_2	Prostaglandin
vWF	von Willebrand factor
CAM	Cellular adhesion molecule

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sICAM	Soluble intercellular adhesion molecule
sVCAM	Soluble vascular cell adhesion molecule

Introduction

The notion of "small vessel disease" in diabetic peripheral arterial disease has successfully been dispelled in the last decade through studies demonstrating similar patterns of occlusive disease in both diabetic and nondiabetic limbs at the arteriole level [1, 2]. Since then, numerous studies have refuted the notion of "small vessel disease" and, more importantly, shown that diabetic peripheral arterial disease can be successfully treated with endovascular or surgical bypass techniques [1–4]. Furthermore, vascular reactivity in the vessels of diabetic patients has been shown to be comparable to those of nondiabetic patients based on physiologic studies involving the administration of the vasodilator papaverine into femoro-popliteal bypass grafts [3]. This data, coupled with a vast clinical experience of nearly 3 decades of successful arterial reconstruction in patients with diabetes, has revolutionized the notion of diabetic "small vessel disease" and led researchers to investigate the fundamental changes in diabetic microcirculation [4].

Recent work suggests that while an occlusive disease of the microcirculation does not exist, the microcirculation (predominantly capillaries and arterioles) is impaired in the patient with diabetes. In simplest terms, microvascular dysfunction in diabetes may be described by an increased vascular permeability and impaired autoregulation of blood flow and vascular tone. It is postulated that metabolic derangements as a result of hyperglycemia and insulin resistance work synergistically to create the basis of microvascular dysfunction. Consequently, these metabolic alterations produce functional and structural changes at multiple levels within the arteriolar and capillary levels.

Histology of Skin

Skin receives a rich blood supply from penetrating vessels located in the skeletal muscles and in the connective tissue of the subcutaneous fat septa. These vessels give origin to two distinct microvasculature plexuses, a ramifying arteriole and a venule network. The former, the superficial or subpapillary plexus, lies between the papillary and the reticular dermis, delimitating their boundaries. The latter, the subcutaneous plexus, is instead located between the dermis and the subcutaneous fat. These two parallel oriented plexuses are connected by a rich network of vertical reticular dermal vessels. The small capillary loops extend from the superficial plexus more superficially into the dermal papillae that are closer to the epidermis. The return loop of these small vessels is the so-called postcapillary venule. Other blood vessel plexuses can be also found both in the surrounding dermal tissue in the periphery of the cutaneous appendages and in the hair follicles during the anagen growth phase.

Lymph vessels are distributed around the subpapillary layer. They arise as a blind-ending initial lymphatic vessel (lymphatic capillaries) and extend through the postcapillary lymph vessels to the dermal and subcutaneous lymph vessels. The lymph vessels during their route follow the course of the main blood vessels, veins, and arteries.

Capillaries consist of a single layer of endothelial cells (ECs) and a basement membrane with the adjunction of ascent pericytes. The basement membrane is significantly different in arterial and venous capillaries, since in the former it is solitary and homogenous, and multilayered in the venous system. Arterioles contain, from the lumen outwards, a thin intima, the internal elastic lamina (IEL), the media consisting of one or two layers of smooth muscles, and finally the adventitia composed of loose connective tissue. The ECs in the venules are surrounded by the basement membrane and pericytes, and the ECs in the lymph vessels by elastic fibers and a lax basement membrane.

Skin Innervation

The skin is innervated by efferent nonmyelinated system responsible for the function of cutaneous vasculature and skin appendages and an afferent myelinated and nonmyelinated system responsible for the detection of cutaneous sensation.

The microanatomy of nerve fibers in the skin is similar to that of the vascular plexus. The nerves of the skin derive from musculocutaneous nerves that arise from spinal nerves and follow the main routes of the vascular plexuses.

The autonomic nerves are subdivided according to their function in adrenergic and cholinergic systems. The adrenergic sympathetic nerves are distributed in the arrector pili muscles, blood vessels, and glomus apparatus. The cholinergic nonmyelinated sympathetic nerves innervate the eccrine and apocrine sweat glands. Initially, it was believed that sebaceous glands were not innervated and that the peripheral nervous system had no effect on sebaceous gland activity in normal skin. However, it has been demonstrated that small fibers can be detected around sebaceous glands indicating that sebaceous glands in the skin are controlled through neuronal activity.

The sensory innervation protects the skin from thermal and noxious injuries. These fibers are of type Aδ and C, and their free nerve endings are distributed subepidermally in the papillary dermis and into the epidermis as a 3D network of unmyelinated nerve fibers. These free endings are then connected with special neurogenic structures and individual cells.

The neurogenic specialized structures in the skin include the Merkel cells and the Meissner corpuscles responsible for the detection of light touch. The Pacini corpuscles are found deep in the dermis and the subcutaneous tissue, and are specialized in detecting pressure. The Krause bulbs and the Raffini corpuscles are activated, respectively, by cold and heat. Naked nerve endings in the basal layer of the epidermis are responsible for transmission of pain. Collectively, these structures work as a unique system in a suitable hormonal milieu, where neurotransmitters and various inflammatory factors play an important role to retrieve external stimuli.

Structural Changes in the Microcirculation

Structurally, the most notable changes affecting the microcirculation in diabetes involves thickening of the basement membrane and an observed reduction in the capillary size [5, 6]. However, the density of the skin capillaries does not differ from healthy subjects [7]. These structural changes are more pronounced in the legs, likely being the result of increased hydrostatic pressures [8]. The extent of basement membrane thickening has also been observed to be related to glycemic control, with increased basement thickening in poorly controlled diabetic patients [9].

In the diabetic foot, basement membrane thickening has been demonstrated in the muscle capillaries [10]. The sequence of events leading to basement membrane thickening begins with the increased hydrostatic pressure and shear force in the microcirculation. This is thought to evoke an injury response on the part of the microvascular endothelium with subsequent release of extravascular matrix proteins. Subsequently, thickening of the basement membrane with arteriolar hyalinosis occurs [11].

Because changes in the basement membrane can affect numerous cellular functions, such as vascular permeability, cellular adhesion, proliferation, differentiation, and gene expression, alterations in its components may cause vascular dysfunctions. Thickening of the basement membrane impairs the normal exchange of nutrients and activates leukocyte migration between the capillary and interstitium. Furthermore, the elastic properties of the capillary vessel walls are diminished, limiting their ability to vasodilate [12]. As a result, the normal hyperemic response to injury is impaired, limiting the compensatory arteriolar dilatation in response to local injury, resulting in a reduced hyperemic response [13]. It is important to note that basement membrane thickening does not appear to lead to narrowing of the capillary lumen; instead, arteriolar blood flow is observed at normal levels or even increased despite these changes [14].

Functional Changes in the Microcirculation

The observed failure of the microcirculation to vasodilate in response to injury has been described as a functional ischemia and has been demonstrated to be a result of a number of factors at play in the diabetic patient's

microcirculation. Alteration in the microcirculation of the foot has been postulated to be an important factor in the poor wound healing associated with chronic diabetic foot ulcerations. Recent work has investigated these changes, with specific emphasis placed on the changes in the diabetic foot microcirculation, nerve function, and muscle metabolism.

Functional changes in the microcirculation include reduced elasticity of capillaries (and thereby vasodilating capacity) and impaired cellular migration and nutrient exchanges. These abnormalities are thought to be due to endothelial dysfunction, smooth muscle cell dysfunction, and impairment of the nerve-axon reflex. While the exact mechanisms of endothelial dysfunction and smooth muscle cell dysfunction remain unknown, measurements in the diabetic neuropathic foot have shown that the neurovascular response is impaired, leading to a significant reduction in blood flow under conditions of stress.

The functional impairment of the microcirculation has been attributed to reduced expression of endothelial nitric oxide (NO) synthetase and poly polymerase [15, 16]. Furthermore, expression of endothelial nitric oxide synthetase is reduced in peripheral neuropathy, suggesting a relationship between neuropathy and endothelial dysfunction. Under conditions of stress such as pain and trauma, the C fibers secrete peptides, such as Substance P, Neuropeptide Y, Neurotensin, and others, that exert vasodilation and increase vessel permeability. This represents a protective mechanism, and it has been shown to be impaired in the diabetic patients with and without neuropathy [17–19], with the largest reduction observed in neuropathic feet (Fig 10.1). This characteristic impairment at the foot level can also be considered as a functional ischemia and it may be another possible mechanism that explains poor wound healing in DFU.

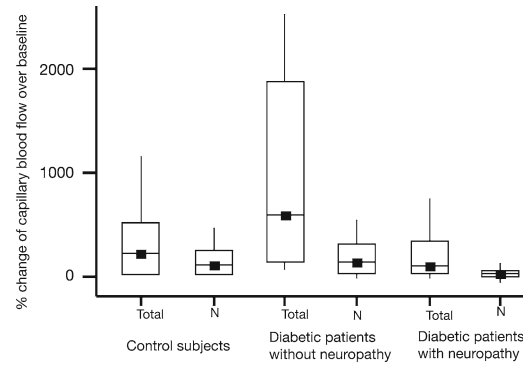

Fig. 10.1 Total and neurovascular (N) change in skin blood flow in response to acetylcholine at the foot level. The median, first quartile, and third quartile and the range are shown. The total response is significantly lower in neuropathic diabetic patients than it is in control subjects and diabetic patients without neuropathy ($p<0.01$). The percentage contribution of neurovascular response to the total response is also significantly lower in neuropathic diabetic patients than in control subjects and diabetic patients without neuropathy ($p<0.01$) (reprinted with permission from Parkhouse and Le Quesne [19])

Functional Changes in the Diabetic Foot

The resting total skin microcirculation in the diabetic foot is comparable to that of the nondiabetic foot, when peripheral neuropathy is absent. However, when neuropathy is present, the capillary blood flow has been shown to be reduced [16, 20]. This may indicate a maldistribution of blood flow to the skin, with a resultant functional ischemia. As previously mentioned, the hyperemic response is impaired in the diabetic microcirculation, thereby failing to achieve maximal blood flow following injury.

Functional changes in the microcirculation appear to impact the ability of precapillary arterioles and capillaries to vasodilate in periods of stress or injury. Clinical examination of the neuropathic diabetic foot with an ulcer may demonstrate a warm foot with palpable pulses and distended veins; paradoxically, however, this foot may be functionally ischemic. In fact, diabetic autonomic neuropathy with sympathetic denervation may lead to the opening of subpapillary arteriovenous shunts with a resultant augmentation of blood flow maldistribution between the nutritional capillaries and the subpapillary vessels [7, 21]. Therefore, although there appears to be no reduction in foot vascularization, the skin microcirculation will be dramatically reduced [22–24]. Ultimately, arteriovenous shunting further aggravates the functionally ischemic foot, as evidenced by studies using venous occlusion

plethysmography, Doppler sonography, and venous oxygen tension measurements [25].

Vasodilation

The endothelium is a diaphanous cellular monolayer that forms the inner layer lining of blood vessels. By virtue of its direct contact with circulating blood, endothelial cells serve as important autocrine and paracrine regulators of vascular function, and provide a critical interface between the elements of blood and tissues. Vascular smooth muscle cells (VSMCs) are small, spindle-shaped mononucleated cells that surround the endothelial monolayer with a variable number of layers depending on vessel location and size: large (elastic) arteries contain many layers of VSMCs alternated with sheets of elastic laminae; precapillary (resistance) arteries may instead have only a single VSMC layer [26]. The IEL lies in between the smooth muscle cell and endothelium layers [27]. Direct communication among cells of the artery wall is enabled by gap junctions. These contacts allow the passage of small molecules and electrical current, and are present both among same cell types (EC–EC and VSMC–VSMC), called homo gap junctions, and in between different cell types (EC–VSMC), known as hetero or myoendothelial gap junctions (MEGJs). MEGJs comprise an EC projection that reaches a VSMC through an IEL perforation [27]. Transmission of membrane hyperpolarization through gap junctions is the key factor of the phenomenon called "spreading vasodilation" that allows diffusion of vasodilation from stimulated to adjacent vessel segments.

In 1980, Furchgott and Zawadzki discovered that arterial vasodilation was dependent on an intact endothelium and its release of a substance they called endothelium-derived relaxing factor (EDRF), which causes arterial smooth muscle relaxation in response to ACh and other vasodilators [28]. Later, this molecule was identified as endothelial-derived nitric oxide (EDNO), a gas that is synthesized from the precursor L-arginine in a reaction catalyzed by nitric oxide synthase. After its secretion from the endothelium, NO diffuses to the adjacent smooth muscle cells and induces VSMC relaxation through the activation of the cyclic GMP and the hyperpolarization of the muscle cell membrane. The activation of cyclic GMP in fact induces the activation of VSMC guanylate cyclase, with subsequent elevation of cGMP levels which in turn leads to a reduction in intracellular Ca^{++} resulting in smooth muscle relaxation and thereby vasodilation.

As previously stated, ECs synthesize and release both relaxing factors, such as NO and prostacyclin (PGI2), and contracting factors, such as endothelin-1 (ET-1), prostaglandins, and angiotensin II (ANG-II). NO is the main vasodilatatory mediator; however, other vasodilating agents exist, such as PGI2, a cyclooxygenase-dependent metabolite of arachidonic acid [29]. Among these, increased evidence for endothelium-derived hyperpolarizing factor (EDHF) has accumulated in the last years. The name originates by the observation that this vasodilating mechanism is abolished by potassium channel (K^+-channel) blockers or by depolarizing concentration of K^+. Direct hyperpolarization of the VSMC membrane is instead achieved through nitrosylation of the K-ATP channel and, therefore, an increase of the pump's activity [30]; this mechanism is extremely important since it counteracts the effects of vasoconstrictive agents.

Under physiological circumstances, mechanisms leading to vasodilatation and vasoconstriction are balanced, so vascular tone and permeability and balance between coagulation and fibrinolysis are finely regulated. Meanwhile, in case of endothelial dysfunction, this balance is altered predisposing the onset and progression of atherosclerosis. Endothelial dysfunction is associated with decreased NO availability, either through loss of NO production or loss of NO biological activity [31]. The significance of endothelial dysfunction on the micro- and macrocirculation and the variety of proposed mechanisms affecting normal function are discussed in further detail.

Endothelium-Dependent Vasodilation

The majority of studies agree that the endothelium-dependent vasodilation in the large vessels

is impaired in diabetes, irrespective of the presence or absence of long-term complications [32–36]. Initial studies of endothelium-dependent vasodilation used venous occlusion plethysmography, while subsequent studies employed flow-mediated vasodilation, a noninvasive technique. Through these techniques, endothelium-dependent vasodilation has been shown to be impaired in adolescents with type 1 diabetes, a population that is generally spared from the micro- and macrovascular complications of diabetes [37]. This finding suggests that endothelial dysfunction is present before the development of these vascular complications and may play an important role in their development. Finally, endothelial function in type 1 diabetes has been shown to be associated with total cholesterol, red cell folate, blood glucose levels, and duration of diabetes [38–40].

In the last decade, extensive research effort has focused on the relationship of type 2 diabetes and vascular disease. Thus, it is currently well established that endothelium-dependent vasodilation is impaired in both the micro- and macrocirculation in type 2 diabetes. Furthermore, there is almost universal agreement that changes in the endothelial function precede the development of diabetes and are already present in the prediabetic stage. It is also of interest that endothelial dysfunction is associated with insulin resistance in nondiabetic subjects, suggesting a cause–effect in the relationship of these two conditions [36].

A study conducted in our unit found that the vasodilatatory response to ACh was reduced in patients with diabetes complicated by neuropathy alone, neuropathy and vascular disease, and patients with Charcot neuroarthropathy, meanwhile no difference was found between patients with diabetes not complicated by neuropathy and the healthy controls (Fig. 10.2). We also found that the vasodilatory response was not diminished in subjects with neuropathy and vascular disease compared to subjects with neuropathy alone. All together, this data suggested for the first time the fundamental role played by the peripheral nerval system in regulating microcirculation and in the pathogenesis of diabetic foot ulceration [16].

Impairment in the microcirculation was also found to be present in the absence of large vessel disease. These findings implied that the main reason for reduced microvascular reactivity was the presence of neuropathy, as indicated by the fact that no other abnormalities were found in the non-neuropathic diabetic patients. Further support for this claim is provided by the findings that the coexistence of neuropathy and vascular disease did not result in a greater decrease in endothelium-dependent vasodilation than that due to neuropathy alone.

Endothelium-Independent Vasodilation

VSMCs are to be considered "the muscle behind vascular biology," since they represent the final effectors of the vasodilating process. Vasodilation is determined by relaxation of VSMCs, mainly achieved through the NO activation of soluble guanylate cyclase with subsequent formation of cyclic GMP that in turn activates protein kinase G, causing phosphorylation of myosin light-chain phosphatase and, therefore, inactivation of myosin light-chain kinase that ultimately leads to the dephosphorylation of the myosin light chain. EDHF determines vasodilation by transmitting hyperpolarization from endothelial cell to VSMCs through gap junctions and/or the release of diffusible factors. Hyperpolarization of the endothelium depends on the activation of the intermediate- and small-conductance calcium-activated potassium channels (IKCa and SKCa, respectively) present on the surface of the ECs.

Data on endothelium-independent vasodilation function in complicated and noncomplicated diabetes is controversial [41–45]. Recent investigation has suggested that endothelium-independent vasodilation is decreased in patients with diabetes [16] (Fig. 10.2). Using laser Doppler imaging, measurements of vasodilatory response to iontopheresis of sodium nitroprusside on VSMC function have been shown to be significantly reduced in diabetic patients with vascular disease, suggesting that the endothelium-independent response may be spared. Since ACh stimulates the production of nitric oxide, it was surmised that an impaired nitric oxide production was responsible for the impaired vasodilatory response observed.

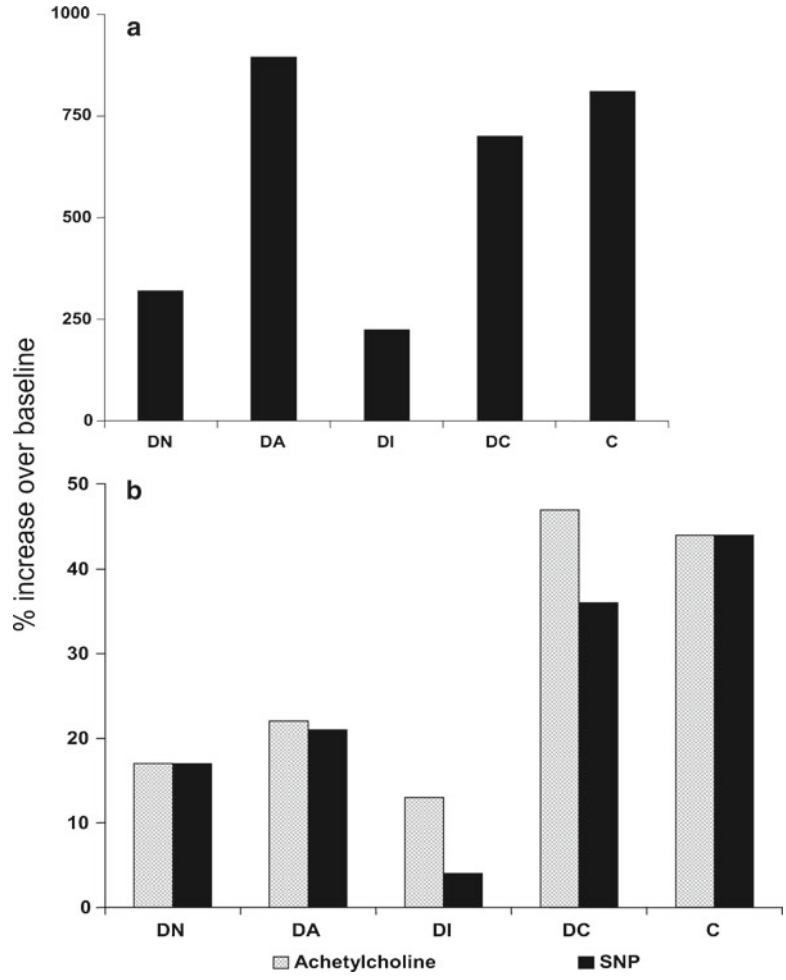

Fig. 10.2 (**a**) The maximal hyperemic response to heating of foot skin at 44°C for at least 20 min (expressed as the percentage of increase over baseline flow measured by a single-point laser probe) is reduced in the diabetic with neuropathy (DN) and in diabetic patients with neuropathy and peripheral vascular disease (DI) when compared with diabetic patients with Charcot arthropathy (DA), diabetic patients without complications (DC), and normal control subjects (C) ($p < 0.001$). (**b**) The response to iontophoresis of acetylcholine and sodium nitroprusside (SNP) (expressed as the percentage of increase over baseline flow measured by laser scanner imager). The response to acetylcholine is equally reduced in the DN, DI, and DA groups when compared with the DC and C groups ($p < 0.001$). The response to SNP was more pronounced in the DI group and also reduced in the DN and DA groups compared with the DC and C groups ($p < 0.001$) [26]

Nerve-Axon Reflex

Nerve dysfunction contributes to the diminished vasodilatatory response observed in diabetes. Under normal conditions, the ability to increase blood flow to the skin depends on the existence of an intact neurogenic vascular response. This protective hyperemic response, also known as Lewis' triple flare response or the nerve-axon reflex vasodilation (NARVV), begins with the stimulation of C-nociceptive nerve fibers, leading to antidromic stimulation of the adjacent C fibers (Fig. 10.3). The activated C fibers then secrete neuropepetides, such as substance P,

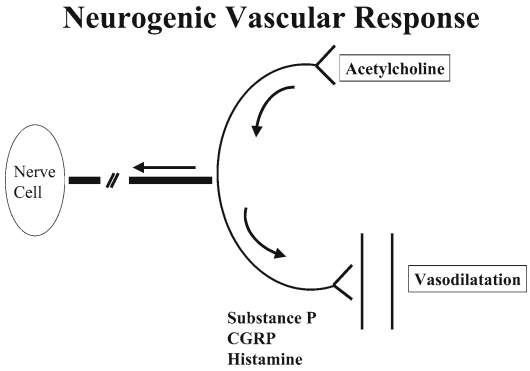

Fig. 10.3 Stimulation of the C-nociceptive nerve fibers leads to antidromic stimulation of the adjacent C fibers, which secrete substance P, calcitonin gene-related peptide (CGRP), and histamine that cause vasodilatation and increased blood flow

calcitonin gene-related peptide, and histamine, causing vasodilation and increased blood flow to the injured tissues. Typically, this response is equal to one-third of the maximal vasodilatory capacity and depends on the existence of an intact neurogenic vascular response.

Measurements in the diabetic neuropathic foot have shown that this neurovascular response is impaired, leading to a significant reduction in blood flow under conditions of stress. It has been postulated that the observed reduction in the NARV in diabetic neuropathy is related to both impaired C-nociceptive fiber function and impaired ability of the microvasculature to respond to vasomodulators secreted by these fibers [46]. Evidence for this vasodilatatory impairment related to the presence of diabetic neuropathy is provided by studies in our lab that used the previously described single-point laser probe technique to evaluate the NARV response. In patients with neuropathy alone, neuropathy and peripheral vascular disease, and Charcot arthropathy the iontophoretic response to ACh was significantly reduced when compared to patients with not complicated diabetes and to healthy subjects. This phenomenon was apparent in the skin adjacent to ACh, but not in areas in direct contact with it [47].

The impairment in axon-related vascular reactivity is believed to further aggravate the diabetic microcirculatory abnormalities, determining a vicious cycle [16]. Thus, in the diabetic neuropathic foot, the involvement of the C-nociceptive fibers does not only lead to the well-known altered pain perception, but also to impaired vasodilation under stresses, such as infection and injury (i.e., functional ischemia).

Mechanisms of Endothelial Dysfunction

Endothelial dysfunction is expressed in increased interactions with leukocytes, smooth muscle growth, vasoconstriction, impaired coagulation, vascular inflammation, thrombosis, and atherosclerosis. There is substantial evidence that endothelial function is abnormal in patients with both type 1 and type 2 diabetes mellitus [34, 35]. The causes of endothelial dysfunction have been postulated to include hyperglycemia, insulin resistance, and inflammation as possible mediators of abnormal endothelium-dependent responses.

Endothelial Dysfunction and Hyperglycemia

A variety of mechanisms have been proposed for endothelial dysfunction in hyperglycemia, mainly through the induction of oxidative stress. The main mechanisms involved in this process include the activation of protein kinase C (PKC), increased vasoconstrictor protanoids synthesis, reduction of the Na^+-K^+ ATPase activity, poly (ADP-ribose) polymerase (PARP) activation, production of oxygen-derived free radicals, increased synthesis of ET-1, induction of the polyol pathway, and generation of advanced glycosylated end products (AGEs).

1. PKC

   PKCs include a superfamily of cytoplasmic serine/threonine kinases isoenzymes. Increased activation of PKC, a key player in intercellular signal transduction for hormone and cytokines, may be the result of hyperglycemia and elevated fatty acids present in T2DM [48]. PKCs participate in vascular cell signal transduction and mediate diverse signaling, including oxidant, inflammatory, mitogenic, and angiogenic effects, in diabetic

vascular tissues that may promote atherosclerotic CVD [49, 50]. PKC activation leads to increased production of extracellular matrix and cytokines; it enhances contractility, permeability, and vascular cell proliferation; induces the activation of cytosolic phospholipase A2; inhibits the activity of Na^+-K^+-ATPase; and regulates neovascularization via the expression of growth factors, such as VEGF/vascular permeability factor (VPF) [51]. Ruboxistaurin (RBX) mesylate is a PKC inhibitor that specifically inhibits PKC-β overactivation. RBX has shown to improve neural function in diabetic animals [52]; but in a double-masked randomized clinical trial conducted in patients with DPN, RBX failed to achieve the primary end point, that is, the improvement of quantitative sensory testing for vibration detection threshold among all symptomatic patients. However, the study showed that treatment with RBX at the dosage of 64 mg, compared to placebo, determined a significant improvement in the neuropathy total symptoms score-6 (NTSS-6) at 6 and 12 months, in the group classified at baseline with clinically significant sensorial neuropathy and treated with RBX [53].

2. Vasoconstrictor protanoids synthesis
   Experimental studies in diabetic animals have also indicated that abnormal endothelial production of vasoconstrictor prostanoids may be a cause of endothelial cell dysfunction. Increased levels of thromboxane (TXA_2) and prostaglandin (PGH_2) have been isolated from segments of diabetic vascular tissue. In human studies, however, the role of vasoconstrictor prostanoids is less clear. Flow-dependent vasodilation in healthy subjects, which may be used as an index of endothelial function, is unaffected by aspirin, thus demonstrating that it is entirely mediated by EDNO and independent of vasoactive prostanoids [54].
3. PARP
   Recent work has also shed light on the role of PARP in endothelial function [15]. PARP is a nuclear enzyme that responds to oxidative DNA damage by activating an inefficient cellular metabolic cycle, often leading to cell necrosis. PARP's activation, besides being related to endothelial dysfunction in patients with diabetes, has been observed also in healthy patients at risk for developing diabetes [15]. It was observed that the activation of PARP was associated with changes in the vascular reactivity of the skin microcirculation, supporting the hypothesis that PARP activation contributes to changes in microvascular reactivity. These findings overall suggest that changes in the microcirculation due to PARP activation may begin in the prediabetic state.
4. Oxygen-derived free radicals
   It has recently been proposed that oxidative stress contributes to the development of diabetic vascular complications through an increased production of oxygen-derived free radicals. This increased production in diabetes directly inactivates endothelium-derived nitric oxide, thereby reducing the bioavailability of EDNO [55]. In animal models, endothelium-derived free radicals impaired EDNO-mediated vasodilation. In human studies, administration of vitamin E (400 IU/day), a potent free radical scavenger, had no apparent effect on cardiovascular outcomes in patients with diabetes with complications [56]. However, early studies showed that high-dose vitamin E (1,800 IU/day) normalized hemodynamic abnormalities, suggesting that administration of an antioxidant may reduce the risks of diabetic vascular complications [57]. Later studies involving long-term high-dose vitamin E found no beneficial effects on endothelial function or left ventricular function in type 1 and type 2 diabetic patients [58]. Furthermore, high-dosage vitamin E was also associated with worsening in some vascular reactivity measurements when compared with control subjects.
5. ET-1
   Hyperglycemia, oxidative stress, and AGEs induce the synthesis of ET-1, a potent vasoconstrictor, through the activation of NF-kB [59]. The upregulation of ET-1 seems to be a consequence of the NO–Ang II imbalance; however, the exact mechanism by which hyperglycemia induces its increase is still not

completely understood. Another possible pathway responsible for the increase of ET-1 is the PKC-mediated induction of the endothelin-converting enzyme (ECE)-1, which catalyzes the conversion of the inactive ET-1 to the active form [60].

6. Polyol pathway
   In the presence of hyperglycemia, glucose enters the polyol pathway. In this pathway, aldode-reductase (AR), using NADPH as a cofactor, catalyzes the reduction of glucose to sorbitol, an organic osmolyte. Subsequently, sorbitol dehydrogenase oxidizes sorbitol to fructose, with production of NADH from NAD^+. Since NADPH is fundamental for synthesizing nitric oxide and glutathione, the polyol pathway activation results in increased concentrations of sorbitol (with subsequent osmotic stress), fructose (which is ten times more potent glycation agent than glucose), and reactive oxygen species, and in decreased concentrations of NO and glutathione [61]. The role for AR in the pathogenesis of diabetic chronic complications has been extensively reviewed [62]. New evidence has recently emerged on the increased activity of AR during hyperglycemia- and diabetes-induced oxidative–nitrosative stress [61] and the downstream activation of mitogen-activated protein kinase (MAPK) [63], PARP [64], and NF-kB [63].

   The accumulation of sorbitol causes the depletion of other osmolytes, like myo-inositol and taurine [65]. The depletion of myo-inositol impairs the phosphoinositide metabolism and, through this, is in part responsible for the reduction in Na^+/K^+-ATPase activity. The proposed mechanism by which myo-inositol affects Na^+/K^+-ATPase function is through the impaired activation of neural PKC caused by diminished phosphoinositide-derived diacylglycerols [66]. The Na^+-K^+-ATPase is involved in the maintenance of cellular integrity and functions of contractility, growth, and differentiation. Therefore, impairment of this mechanism can lead to vascular dysfunction and the early reversible nerve conduction defect in experimental diabetes [67].

   In the last 30 years, over 32 randomized controlled trials have tested the efficacy of AR inhibitors (ARIs), such as ranirestat and epalrestat, in the treatment of diabetic neuropathy. A meta-analysis involving 879 ARI-treated and 909 control (placebo or no treatment) participants showed no overall significant difference in the treatment of diabetic polyneuropathy between the groups [68].

7. AGEs
   AGEs result from a nonenzymatic reaction when proteins are exposed to hyperglycemic environments. The resultant Schiff bases can be rearranged to form Amadori products, AGEs, and reactive oxygen species. Increased AGE levels have been found in patients with diabetes and may contribute to the increased vascular permeability of diabetes, since blockade of a specific receptor for AGE reverses diabetes-mediated vascular hyperpermeability [69]. Furthermore, the generated reactive oxygen species have been shown to cause severe disturbances in the regulation of coronary flow and cellular hemostasis, leading to the severe macrovascular lesions typically observed in diabetic patients after more than 10 years of disease [70]. Interestingly, inhibition of reactive oxygen species also prevents the generation of AGE products, suggesting that the autoxidative process plays an important role in the complex reaction cascade leading to AGE.

Endothelial Dysfunction and Insulin Resistance

Besides its anabolic action, insulin also exerts a hemodynamic action that results in peripheral vasodilation and capillary recruitment. Insulin mediates vasodilation through modulating the synthesis and release of NO [47, 71]. Insulin's stimulation of NO is mediated by the activation of signaling pathways involving the recruitment of phosphoinositide-3 (PI-3) kinase that leads to the phosphorylation of eNOS. Interestingly, it has been proposed that as much as 25% of insulin's stimulatory effect on muscle glucose uptake is related to its hemodynamic actions [72]. By contact with the endothelial barrier, insulin

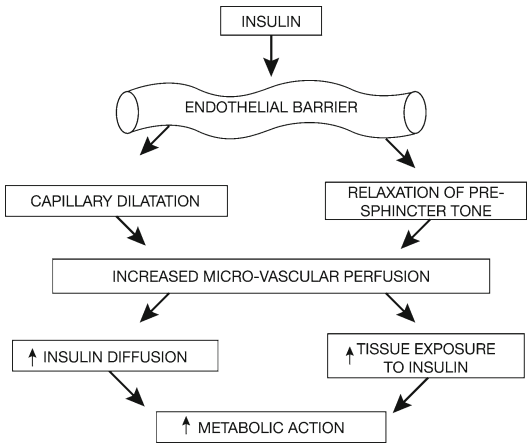

Fig. 10.4 Hemodynamic and metabolic actions of insulin. Insulin stimulates an increase in recruitment of microvessels, expansion of the capillary network, and perfusion of the microcirculation via relaxation of the precapillary sphincter tone and dilatation of the capillaries. Insulin, thereby, diffuses into the interstitium more readily and the target tissues are exposed to high insulin concentrations. This determines ultimately an increased insulin-mediated glucose metabolism (reproduced with permission from Cersosimo and DeFronzo [73])

determines dilation of the capillaries and relaxation of the presphincter. In this way, insulin's metabolic action is enhanced, both by recruiting new capillary beds and redirecting the capillary blood flow more toward insulin-sensitive tissues (muscle and adipocytes) and away from insulin-independent tissues (bone and skin) [73] (Fig 10.4).

The bidirectional link between hyperinsulinemia and endothelial dysfunction is well established. On one hand, the exposure of vascular endothelium to hypertriglyceridemia and elevated small dense LDL cholesterol particles, typical of insulin resistance states, is accompanied by reduced NO availability [74]. On the other hand, endothelial dysfunction contributes to impaired insulin action by altering the transcapillary passage of insulin to target tissues. Although the molecular mechanisms determining the metabolic and vascular abnormalities associated with the insulin resistance state have yet to be entirely elucidated, the impaired NO production clearly appears to play a pivotal role.

Endothelial Dysfunction and Inflammation

The origins of heightened inflammatory activity in type 1 and type 2 diabetes are very different. In type 1 diabetes, even though the inflammatory process plays a greater role in the long-term progression of disease, the onset is the consequence of an islet inflammation that is thought to be a local phenomenon triggered by a focal autoimmune activation. In T2DM, the activation of inflammation results from systemic etiologic factors, such as central obesity and insulin resistance. In both T1DM and T2DM, an important role in the maintenance and exacerbation of the inflammatory reaction is played by hypertension and dyslipidemia in concert with hyperglycemia.

An early feature of inflammation is the release of chemokines, such as C-reactive protein (CRP), fibrinogen, interleukin-6 (IL-6), interleukin-1 (IL-1), TNFα, and monocyte chemoattractant protein (MCP)-1. These factors promote the expression of interstitial and vascular cellular adhesion molecules, like ICAM-1, VCAM-1, and E-selectin, and attract monocytes and immunocytes [75]. Inflammatory cytokines increase the vascular permeability, change the vasoregulatory responses, and increase the adhesion of leukocyte to endothelium. Furthermore, inflammatory cytokines facilitate thrombus formation by inducing procoagulant activity, inhibiting anticoagulant pathways and impairing fibrinolysis by increasing the expression of plasminogen activator inhibitor (PAI)-1 and tissue factor, through platelet activation and acute-phase reactions that increase the circulating levels of coagulation factors, such as fibrinogen and factor VIII [75].

Biochemical Markers of Endothelial Dysfunction

When the endothelium has been injured, a number of vasoactive substances are produced in response. As a result, these biochemical markers, such as von Willebrand factor (vWF) and cellular

adhesion molecules (CAMs), have been employed to evaluate endothelial dysfunction. vWf, a multimeric glycoprotein mainly synthesized by endothelial cells, is involved in platelet adhesion and aggregation and acts as the carrier of coagulation factor VIII in plasma. Increased levels of vWf, reflecting activation of or damage to endothelial cells, have been described in association with atherosclerosis and diabetes. Initial studies in patients with diabetes have demonstrated increased plasma levels of vWF [76]. Furthermore, these elevations preceded the development of albuminuria and peripheral nerve dysfunction. Therefore, it has been suggested that vWF could be used as a predictive indicator of vascular complications.

CAMs are expressed on endothelial cells in response to inflammation and facilitate the adhesion of circulating leukocytes to their surface. Increased levels of soluble intercellular adhesion molecule (sICAM) in healthy individuals have been linked with a higher risk of future cardiovascular complications [77]. Furthermore, both sICAM and soluble vascular cell adhesion molecule (sVCAM) levels have been reported to be higher in patients with diabetes and, in some instances, individuals with impaired glucose tolerance (IGT) [78, 79].

In vitro studies of sICAM and sVCAM demonstrated that these biochemical markers were expressed by endothelial cells following a short period of incubation in high glucose conditions [80], lending support that hyperglycemia plays a role in activation of these molecules. Furthermore, a direct correlation has been detected between VCAM-1 and VEGF, suggesting that cellular adhesion and neovascularization may be linked processes [81].

Methods of Evaluating the Microcirculation of the Feet

Recent technological advances over the last decade have enabled us to evaluate the functional microcirculation of the feet. Methods, such as Laser Doppler flowmetry, flow-video microscopy, cannulation measurements of capillary pressure, and transcutaneous oxygen tension measurements, have all been used. The most commonly used technique, and the one used in our lab, for evaluating blood flow in the skin remains Laser Doppler flowmetry.

Laser Doppler Flowmetry

This method is considered the most widely accepted technique for evaluating capillary blood flow in the skin microcirculation based on its ease of use and reproducibility. This method uses a red laser light that is transmitted to the skin through a fiber-optic cable. The frequency shift of light backscattered from the moving red blood cells beneath the probe tip is used to give a measure of the superficial microvascular perfusion [16]. The method of laser scanning also uses the technique of iontophoresis to evaluate microvascular reactivity. More specifically, a device consisting of two chambers that accommodate two single-point laser probes is applied to the skin. A small quantity (<1 mL) of the vasoactive substance is placed in the chamber while a second nonactive electrode is placed 10–15 cm away from the chamber. A constant current of 200 μA is applied, creating movement of the solution toward the skin, causing vasodilation. Iontophoresis of ACh chloride measures the endothelium-dependent vasodilation, while sodium nitroprusside measures the endothelium-independent vasodilation.

There are two types of laser probes available for use with this method: a single-point laser probe or a real-time laser scanner. The single-point laser probe measures the microvascular blood flow at a single point in the skin, and has been used for evaluating the hyperemic response to a heat stimulus or for evaluating the NARV-related hyperemic response. In conjunction with the technique of iontophoresis and the addition of a second probe, the single-point laser probe method can be used to assess the integrity of the NARV. In the two single-point laser probe technique, the first probe is exposed to ACh in order to measure the blood flow to a specified area of skin. The second probe is situated in close proximity (5 mm) to the first probe, and consequently measures the indirect effect of the iontophoresed ACh. The indirect effect of the ACh results from stimula-

tion of the C-nociceptive nerve fibers and, therefore, the NARV hyperemic response [82, 83]. Following iontophoresis of the vasoactive substance, the adhesive device is removed and the area of skin is scanned with a laser Doppler scanner. Measurement of the hyperemic response to a heat stimulus is performed by first taking baseline blood flow measurements. Next, the skin is heated to 44°C for 20 min using a small brass heater or, in our experience, maintaining the ambient room temperature at this level. Following this, the maximum blood flow is determined by the magnitude of blood flow change in response to heat.

The laser Doppler perfusion imager uses 1-mW helium–neon laser beam of 633-nm wavelength to sequentially scan the area of skin. Increased blood flow at the skin level is recorded by the scanner and expressed in volts. This technique has been validated against direct measurements of the capillary flow velocity with consistent measurements achieved. In clinical settings, measurements of endothelial function with laser Doppler in conjunction with iontopheresis of ACh yielded consistent results in patients with diabetes and coronary heart disease.

Flow-Video Microscopy

Flow-video microscopy enables measurements of capillary blood flow along with such parameters as average flow velocity, peak postocclusive hyperemic flow velocity, and response to other physiologic maneuvers. With the use of an image-shearing monitor, the capillary red cell column width is calculated. This is performed by lighting mercury vapor onto skin that has previously been brushed with a thin film of oil or varnish in order to limit the scattering of light. The image of the moving blood elements can then be recorded with a low-light-sensitive video system [84].

More recently, a digitized system has been developed that is capable of recording continuous capillary blood flow. Measurements are then calculated through an integrated software program. However, this method may underestimate the true capillary lumen due to the unvisualized marginal plasma layer.

Capillary Pressure Measurements

The measurement of capillary pressure involves direct cannulation of a single vessel. Following cannulation of the vessel, the transmitted pressure can be measured manometrically or through use of an electronic device. This invasive technique is capable of detecting small changes in the capillary pressure as low as 1–2 mmHg. Additionally, it has the added benefit of being able to measure the capillary pressure continuously. However, the procedure can be complex and may require significant expertise.

Transcutaneous Oxygen Tension Measurements

The measurement of oxygen transcutaneously ($TcPO_2$) can be performed based on the fact that oxygen is capable of diffusing throughout the body tissue and skin. While the rate of diffusion is very low at normal surface body temperature, application of heat to a localized area can sufficiently enhance the flow of oxygen through the dermis to allow for noninvasive measurement of the capillary oxygen level. However, these measurements suffer from inconsistency as they can be inaccurate as they appear to fluctuate with the skin temperature and room temperature. As a result of this variability, the clinical use of $TcPO_2$ has been restricted [85, 86].

Latest Developments

Hyperspectral Imaging

Hyperspectral imaging (MHSI) emerged as a method of "imaging spectroscopy" that provides spatial measurements of the skin's oxy- and deoxyhemoglobin levels [87] and quantifies the skin oxygenation by combining the chemical specificity of spectroscopy with the spatial resolution of imaging. A spectrum of reflected light is acquired for each pixel in a region, and each spectrum can be subjected to standard analysis. This allows the creation of an image based on the chemistry of the region of interest (ROI).

MHSI has been used for decades in a wide variety of applications ranging from geological and agricultural to military and industrial, and recently

has begun to be applied also to biomedicine [19]. Using spatially resolved oxygen saturation maps, MHSI can predict tissue viability following plastic surgery [88], differentiate tumor from normal tissue, assess local tissue viability following partial thickness burns [85, 86], and determine the local skin manifestations of larger systemic problems, such as shock [87]. These capabilities are highly advantageous in the evaluation of small wound regions, where the detection of subtle microcirculatory differences is necessary.

In the research arena, MHSI has been used to identify differences of the skin oxygenation between healthy control subjects and diabetic patients with and without neuropathy. MHSI has shown that hemoglobin saturation is reduced in the skin of patients with diabetes and that this impairment is accentuated in the presence of neuropathy in the foot, suggesting that microcirculatory changes could play a major role in the development of neuropathy. Furthermore, these changes could underlie the development of foot ulceration and, more importantly, preclude the healing of existing ulcers [89, 90].

In a follow-up study, the ability of this technique to predict diabetic foot ulceration healing was examined and the progress of foot ulcers was tracked over a period of 6 months [90]. The results of this study provided the proof of concept that MHSI, evaluating wound oxygenation and its relationship to wound healing, could satisfactorily predict ulcer healing and therefore has the capability to assist in the management of DFU [90].

Near-Infrared Spectroscopy

Near-infrared spectroscopy (NIR) is a spectroscopic method which uses the near-infrared region of the electromagnetic spectrum. NIR can typically penetrate much farther into a sample than mid infrared radiation and can be used to monitor changes in vascularization, hemoglobin concentration, and collagen concentration, and can therefore be used to obtain a measure of the optical absorption and optical scattering properties of probed tissue. The optical properties of tissues at visible and near-infrared wavelengths are determined mostly by the levels of oxygenated and deoxygenated hemoglobin in the probes tissue. The sensibility and specificity of NIR in evaluating changes in muscle blood flow are currently under investigation. Changes in blood flow at resting conditions and during exercise may suggest a relationship between lower extremity dysfunction with changes in both the micro- and macrocirculation and the development of peripheral neuropathy.

Conclusion

In conclusion, while an occlusive disease of the microcirculation does not exist, functional impairment of the microcirculation in diabetes may contribute to secondary complications, such as foot infections and ulcerations. Microcirculation to the diabetic foot suffers both structural and functional derangements. Nerve-axon-related microvascular reactivity is clearly impaired in the diabetic population and there is a growing belief that both the failure of the vessels to dilate and the impairment of the nerve-axon reflex are major causes for impaired wound healing in diabetic patients. Further studies are necessary to clarify the precise etiology of observed endothelial dysfunction in diabetic and neuropathic patients and to identify the possible potential therapeutic interventions to prevent or retard its progression.

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