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Regulation of prostaglandin synthase-1 and prostaglandin synthase-2

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Abstract

It has been assumed that the rate-limiting step in the ligand-induced synthesis of prostaglandins is the release of arachidonic acid from membrane phospholipid stores as a result of the activation of phospholipase. The assumption has been that the arachidonic acid is converted to PGH_2 by the constitutive prostaglandin synthase/cyclooxygenase EC1.14.99.1 (PGS-1) enzyme present in cells. In this model, PGS-1 is proposed to be present in excess, and the production of arachidonic acid is thought to be rate limiting. However, a second prostaglandin synthase gene, PGS-2 has recently been described. The PGS-2 gene is induced by a variety of ligands, in cells as diverse as fibroblasts, monocytes, macrophages, smooth muscle cells, ovarian granulosa cells, epithelial cells, endothelial cells, and neurons. Moreover, PGS-2 induction is inhibited in nearly all contexts by glucocorticoids. It seems likely, therefore, that the regulation of PGS-2 expression plays a critical role in the production of prostanoids, both in normal physiological processes and in pathophysiological processes involving these paracrine mediators. In this review, we consider the regulation of the two genes, PGS-1 and PGS-2, that encode the isoforms of prostaglandin synthase.

Prostanoids are involved in a wide range of normal and pathophysiological responses

The prostanoids – prostaglandins, prostacyclins, and thromboxanes – play pivotal but complicated and often contradictory roles in a wide range of normal autocrine and paracrine cellular interactions. There is substantial experimental and clinical evidence demonstrating that the prostanoids play important roles in both activation and inhibition of the immune response, inflammatory responses, wound healing, female reproductive biology (where luteinization, ovulation and parturition are all modulated by alterations in prostaglandin production), bone development, glomerular filtration and water balance, and hemostasis. Aberrations of prostanoid production are implicated in a wide range of pathophysiological alterations in these various biological systems. Alterations in prostaglandin production have been associated with, and in some cases been demonstrated to be causal, in arthritis, asthma, bone resorption, cardiovascular disease, nephrotoxicity, atherosclerosis, hypotension and shock, acute inflammation, immunosuppression, gastric ulcers, and cancer. Because of the complicated biochemistry of the biosynthesis and degradation of the prostanoids, the often contradictory roles of closely related structures, and the different effects of the same prostanoid in different biological contexts, a clear picture of their roles and their importance in intercellular communication in normal and pathophysiological contexts has been difficult to achieve. However, interdiction of prostaglandin production by nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin is associated with reduction in pain, fever, inflammation, a lessening of risk for cardiovascular problems, and a lowered incidence of colon cancer. Steroid-based anti-in-

flammatory pharmaceuticals that block prostaglandin production also play a major role in reduction of inflammation. No more dramatic demonstration of the likely causal role of prostanoids in the biology of cancer can be made than the relatively recent observations, discussed extensively elsewhere in this collection of review articles, that inhibition of prostaglandin production by NSAIDs can substantially reduce the occurrence of colon cancer and its mortality [1–3].

Prostaglandin synthase/cyclooxygenase plays a key role in prostanoid synthesis

Although the biosynthesis of the arachidonic acid metabolites is discussed at length in other reviews in this collection, I will briefly review the salient points necessary for the remainder of this manuscript, for purposes of clarity and self-containment. Synthesis of the prostanoids begins with the release of arachidonic acid from membrane stores, as a result of its cleavage from phospholipids by cellular phospholipases. Free arachidonic acid is the substrate for lipoxygenases, leading to the biosynthesis of the leukotrienes, and for prostaglandin synthase, the enzyme that catalyzes the production of the common intermediate from which the various prostaglandins, prostacyclins, and thromboxanes are subsequently synthesized. Prostaglandin synthase carries out two sequential reactions. In the first, the cyclooxygenase activity of the enzyme catalyzes the conversion of arachidonic acid to PGG2. In a second catalytic reaction the peroxidase activity of the enzyme reduces PGG2 to PGH2 [4]. This enzyme is widely referred to as either prostaglandin synthase (prostaglandin endoperoxide synthase) or as cyclooxygenase. The PGH2 formed from arachidonic acid by prostaglandin synthase is then converted to the various prostaglandins, thromboxanes, or prostacyclins. The end products of this pathway produced by various cell types reflect the particular spectrum of enzymes they express that use PGH2 as substrate. However, production of prostaglandins, prostacyclins, and thromboxanes all depend on the presence and activity of prostaglandin synthase. The absence or inhibition of PGS will prevent the synthesis of all the prostanoids.

Prostaglandin synthase EC1.14.99.1 has been purified, cloned, and extensively studied biochemically

The anti-inflammatory and analgesic consequences of interfering with prostanoid production have made prostaglandin synthase the subject of a great deal of basic and applied research. Prostaglandin synthase was first purified from sheep seminal vesicles, a rich source of this enzyme [5–7]. An extensive amount of structural, biochemical, enzymatic, and pharmacologic research has been carried out on the ovine and human enzymes, because of the pivotal role of PGS in the production of prostanoids, and because of its role as a target for pharmacologic intervention in pain, fever, and inflammation by NSAIDs. The biochemical, structural, and pharmacologic studies of PGS EC1.14.99.1 have been reviewed previously [4]. Three groups cloned a cDNA for the ovine PGS EC1.14.99.1 enzyme, using structural information from the purified ovine enzyme [8–10]. The murine [11] and human [12] PGS EC1.14.99.1 cDNAs were cloned on the basis of sequence similarity and nucleic acid cross hybridization potential.

Exaggerated prostanoid production occurs in a wide range of cell types, in response to alterations in the extracellular environment

Essentially all cells have detectable levels of PGS activity, and can synthesize basal levels of one or more of the prostanoids. A wide variety of stimuli can elicit exaggerated production of prostanoid synthesis. To give the reader a sense of the variety of cell types and stimuli that can elicit enhanced prostanoid production, without however trying to be exhaustive in these citations, I will cite a number of such responses: mitogenic stimulation of fibroblasts, endotoxin stimulation of monocytes and macrophages, the response of ovarian granulosa cells to surges of luteinizing hormones, exposure of

vascular endothelial cells to interleukin-1, epithelial responses to ultraviolet irradiation, growth factor activation of smooth muscle cells, glomerular inflammation, oncogenic transformation by a subset of retroviruses, and inflammatory cytokine action on chondrocytes [13].

Regulatory, molecular, and biochemical studies suggest the existence of a second form of prostaglandin synthase

The classic model for the regulation of prostanoid synthesis has assumed the constitutive, nearly ubiquitous presence of a single PGS species in a wide range of distinct cell types. In this model, the critical step in the induced production of prostanoids in response to extracellular signals is the activation of phospholipase, to release arachidonic acid from membrane stores. Ligands of various types (e.g. growth factors, endotoxin, tumor promoters, hormones) are proposed to activate, via receptor-mediated mechanisms, the phospholipase activity of cells to release arachidonic acid. Excess PGS EC1.14.99.1 is assumed to be present, and available to convert the released arachidonic acid to PGH2. This formulation suggests (i) that phospholipase is the rate-limiting reaction in prostanoid biosynthesis, (ii) that arachidonic acid availability is rate-limiting in the production of prostanoids from substrate, and (iii) that PGS EC1.14.99.1 is present in excess, and converts into PGH2 the arachidonic acid released from membrane stores following phospholipase activation.

Following the purification of seminal vesicle PGS, antibody was prepared by several research groups. Studies with radioactive labelling followed by immunoprecipitation suggested that the synthesis of PGS was elevated following ligand stimulation, and accompanied the rise in prostaglandin biosynthesis, in both fibroblasts [14, 15] and monocytes and macrophages [16]. The assumption made at that time was that, in contrast to the previous model, that ligand treatment increased the production of PGSEC1.14.99.1 antigen. However, studies of glucocorticoid inhibition of enzyme production suggested that the basal and induced synthesis of PGS were differentially modulated by hormone [14–16]. Moreover, when northern analysis for the PGS EC1.14.99.1 mRNA was examined following ligand induction, the level of this message was often unchanged, despite alterations in prostaglandin levels, cyclooxygenase enzyme activity, and synthetic rates of PGS antigen. These observations led to the suggestion that mitogen-treated fibroblasts and activated monocytes induced to produce exaggerated levels of prostaglandins contain two pools of prostaglandin synthase, one that is constitutively expressed and insensitive to glucocorticoids, while the other is highly sensitive to extracellular ligands and is sensitive to glucocorticoid inhibition [17].

At about this time Rosen *et al.* noted that although the 2.8 kb message for PGS EC1.14.99.1 was not elevated in mitogen-treated sheep tracheal mucosal cells, a cross-hybridizing message at 4.0 kb was increased following stimulation. These authors suggested that this message might encode a second form of PGS. Immunochemical studies of the production of PGS in the rat ovary also suggested that there were two distinct forms of this enzyme. Using several different polyclonal antibodies to PGS EC1.14.99.1, Richards and her colleagues [19, 20] identified two distinct species of the enzyme. Moreover, one of the two immunodistinct forms accumulates rapidly, but transiently, in granulosa cells of preovulatory follicles following stimulation by luteinizing hormone, while the second form is constitutively expressed [19]. These studies also lead to the suggestion that a second, distinct PGS enzyme exists.

A second, rapidly inducible prostaglandin synthase gene was discovered by groups studying gene expression in response to oncogenes, tumor promoters, and growth factors

The suggestion of a second PGS molecule arose from studies in the areas of inflammation and reproductive biology. However, the identification of a second PGS gene occurred serendipitously in laboratories studying the regulation of gene expression by oncogenes and mitogenic stimuli.

Increased metabolism of arachidonic acid and

production of prostanoids as a result of expression of *v-src*, the oncogene of Rous sarcoma virus, are among the many consequences of transformation by this virus [21]. Han *et al.* [22], using giant two-dimensional gels, identified a 72/74 kilodalton doublet that is persistently increased in *v-src* transformed BALB/c 3T3 cells. Immunoprecipitation experiments with antiserum to sheep seminal vesicle PGS EC1.14.99.1, coupled with measurements of the cyclooxygenase activity, identified this protein as cyclooxygenase. *v-src* induction of the doublet was blocked by glucocorticoids. Platelet-derived growth factor (PDGF) and tetradecanoyl phorbol acetate (TPA) induced transient accumulation of the 72/74 kd doublet. Although the authors of this article did not propose that the elevated PGS activity they observed might be a second form of the enzyme, it seems clear in retrospect that this is the case. Xie *et al.* [23] used a differential cDNA screen of a library prepared from chick embryo fibroblasts carrying a temperature-sensitive *v-src* gene to identify cDNAs for a number of messages that are persistently elevated in *v-src* transformed cells. Sequencing of one of the mRNAs, originally called CEF-147, demonstrated 'significant sequence identity' with ovine PGS [23]. The CEF-147 message is also elevated in response to treatment of chick embryo ifbroblasts with serum or TPA. These authors speculated that the CEF-147 message might be the product of a second PGS gene, related to the message seen by Rosen *et al.* [18] in ovine tracheal mucosal cells. However, they could not be certain of this conclusion as the avian homologue of sheep seminal vesicle PGS EC1.14.99.1 had not been characterized.

Our laboratory has been studying the G0 to G1 transition in reponse to mitogens. One of our experimental approaches has been to use differential screening of cDNA libraries prepared from TPA-treated Swiss 3T3 cells to identify genes whose message levels are elevated in response to mitogen stimulation [24]. We refer to these cDNAs as 'TPA induced sequences', or TIS genes., The TIS10 cDNA sequence has substantial amino acid identity (60%) to the previously cloned murine PGS EC1.14.99.1 cDNA [25]. However, the differences between the two in amino acid sequence demonstrate that these messages are products of distinct genes. From this point on I will refer to the TIS10 gene and its product as PGS-2, and term the originally described ovine gene and enzyme PGS-1. The PGS-1 protein contains a hydrophobic sequence in the amino-terminal end of the molecule that is not present in PGS-2. In contrast, the PGS-2 molecule contains an 18 amino acid sequence in the carboxy-terminal region of the protein that is absent from PGS-1 [25]. Cyclooxygenase and hydroperoxidase assays of cells transfected with expression vectors encoding PGS-2 demonstrate that this gene does, indeed, encode a protein with both enzyme activities [26–28]. The PGS-1 and PGS-2 genes map to distinct murine chromosomes [29].

In our initial report we demonstrated that the TIS10 message is absent in untreated cells, and is rapidly and transiently inducible by epidermal growth factor (EGF), forskolin, TPA, and serum in Swiss 3T3 cells. The PGS-2 gene can, therefore, be induced through tyrosine kinase, protein kinase A and protein kinase C mediated signal transduction pathways. In contrast, the PGS-1 message was present in uninduced 3T3 cells, and was essentially unchanged by any of these treatments [25]. Although the TIS10 gene had previously been shown to be rapidly induced by expression of *v-src* [30], its identity as a second PGS was not known at the time of those experiments. PGS-2 protein is rapidly destroyed after it is synthesized; its half-life is only four hours [31]. The short message and protein half lives of PGS-2, and the transient induction, result in a bolus of enzyme appearing in the cell following stimulation, resulting in a wave of ligand-induced prostanoid synthesis.

O'Banion *et al.* [32] initially reported a partial sequence of a cDNA for a serum-induced message and suggested it encoded a PGS molecule from a gene distinct for PGS-1. They later sequenced the complete open reading frame, and confirmed the mitogen induction of PGS-2 in murine fibroblasts [27]. PGS-2 was also subsequently identified as a serum-inducible gene in 3T3 cells by differential screening of a cDNA library from serum-stimulated cells [32a]. Using sequence data from the murine gene, c-DNAs for the rat [33] and human [34, 35] PGS-2 genes have also been cloned and sequenced.

The structures of the PGS-1 and PGS-2 genes are quite similar

Using the cDNA probes, the murine [36] and human [37] PGS EC1.14.99.1 genes have been cloned and characterized. Both the human and murine genes are composed of eleven exons and ten introns. The borders between the exons and introns are completely conserved between the murine and human genes. Intron sizes also appear to be similar between the two genes. In both cases the genomic region encoding the transcription unit covers approximately 22 kilobases of DNA.

The gene for the murine TIS10/PGS-2 gene has also been cloned and characterized [26]. The PGS-2 gene contains only ten exons and nine introns. The intron that encodes the hydrophobic region in the amino-terminal region of the PGS-1 protein is absent from the PGS-2 gene. All the other exons that encode the PGS-1 and PGS-2 transcripts are homologous for PGS-1 and PGS-2, and show the same sites of splicing, with the exception of the most 3′ exon. In the PGS-2 gene this exon includes the 18 amino acid insert in the carboxy-terminal region of the PGS-2 protein, as well as a longer 3′ untranslated region that includes several copies of the AUUUA sequence that confers message instability to many rapidly degraded messages. The major difference in the structures of the PGS-2 and PGS-1 genes is in their sizes; the 11 exons and 10 introns of the human and mouse PGS-1 gene are about 22 kilobases in length, while the 10 exons and nine introns of the murine PGS-2 gene are only 8 kilobases long. Like the murine gene, the chicken PGS-2 gene contains 10 exons, and is only approximately 9 kilobases long [38]. The rapidly inducible primary response genes are, in general, quite small when compared to constitutively expressed genes [39]. The regulatory regions of the PGS-1 and PGS-2 genes will be discussed below.

Glucocorticoid hormones block PGS-2 induction and prostaglandin production in murine fibroblast cell lines

In their initial experiments demonstrating that *v-src* and PDGF could induce the 72/74 kd PGS doublet, Han *et al.* [22] demonstrated that glucocorticoids could block the appearance of this labelled species. In retrospect, it is clear that this doublet is encoded by the PGS-2 gene but cross-reacts with antibody to the PGS-1 product. The level of the 4 kb cross-hybridizing message observed with a PGS-1 cDNA probe in C127 cells, as well as the labelling of the cyclooxygenase doublet in these cells, was also reduced in the presence of dexamethasone [22].

Once the identity of a second PGS-2 gene was clearly established, the inhibition of mRNA accumulation for this gene was unequivocally established in murine fibroblast cell lines [27, 40]. Dexamethasone inhibits accumulation of PGS-2 message in response to serum [29], TPA [40], and forskolin [40]. These results suggest that the mechanism of glucocorticoid inhibition is likely to be distal to the signal transduction pathways that activate gene expression, since these three inducers activate distinct pathways involving tyrosine kinases, protein kinase C, and protein kinase A, respectively. PGS-2 expression is exquisitely sensitive to glucocorticoid inhibition; nanomolar dexamethasone can block TPA-induced PGS-2 message accumulation [40]. Nuclear run-on experiments suggest that the glucocorticoid inhibitory effect is at least in large part at the transcriptional level [41]. Dexamethasone treatment of Swiss 3T3 cells also blocks the TPA-induced production of PGE_2, despite the presence of PGS-1 message and protein in these cells [40]. These data suggest that the synthesis of PGS-2 is required for mitogen-induced prostaglandin synthesis.

PGS-2 can be induced in a variety of cell types, in response to cell-type specific stimuli

Prostanoid synthesis occurs in a wide variety of biological contexts, in response to a number of different physiological challenges. A PCR-based analysis of PGS-1 and PGS-2 messages in human tissues suggests that many different organs can express both messages, but does not address either differential cell-expression of these genes or their inducibility by physiologic and or pharmacologic agents [42].

Since the existence of a second gene for PGS was discovered, induced PGS-2 expression in a number of different contexts has been demonstrated.

Biochemical and immunochemical studies indicated that a second PGS is induced in ovarian granulosa cells in response to pituitary glycoprotein hormones [19, 20]. Purification and amino-terminal sequence analysis of the PGS isoform induced by human chorionic gonadotrophin in granulosa cells of rat preovulatory follicles demonstrated that this PGS is the rat homologue of PGS-2 [20]. Northern analysis then demonstrated that the exposure of preovulatory follicles to FSH or LH rapidly and transiently induced the PGS-2 message [43]. Prostaglandin synthesis in the female reproductive cycle appears to depend on the expression of PGS-2 in response to pituitary glycoprotein hormones.

Monocytes and macrophages produce large amounts of prostanoids following activation by inflammatory stimuli. Although we were unaware of the identity of TIS10 as a second PGS at the time, we observed an increased expression of TIS10 message in the murine 32D monocyte cell line in response to both granulocyte-macrophage colony stimulating factor and TPA, as well as an increase in TIS10 message in human neutrophils in response to TPA [44]. Subsequently, induction of PGS-2 message and protein have been observed in endotoxin-treated tumor-derived macrophages and in the murine macrophage RAW 264.7 cell line [45], in IL-1b treated human monocytes [27], endotoxin-treated alveolar macrophages [46], and TPA-treated U937 human monocytes [47]. In each of these cases the induction of PGS-2 message and/or protein is inhibited by glucocorticoids. This summary is not meant to be inclusive for the many recent studies of induced PGS-2 expression in monocytes and macrophages.

The epithelial cells lining the airway and gut are two of the three interfaces between our environment and our bodies. Alterations in these cells are associated with asthma, inflammatory bowel disease, and lung and colon cancer. Dysregulated prostaglandin production is associated with many of the pathophysiological conditions of these epithelial cells. Tracheal epithelial cells exposed to either TPA or EGF demonstrate both elevated levels of PGE_2 and rapidly and transiently elevated levels of PGS-2 message [48]. The rat intestinal epithelial IEC-6 cell produces elevated levels of prostacyclin as well as increased levels of PGS-2 message and protein in response to either TPA or IL-1 beta exposure (R. Gilbert and H. Herschman, in preparation).

PGS is thought to play a major role in the pathophysiology of rheumatoid arthritis and osteoarthritis. Sano *et al.* [49] demonstrated elevated levels of PGS antigen in the synovium lining layer, stromal fibroblasts, and cartilage chondrocytes, as well as in mononuclear inflammatory cells from both patients with rheumatoid arthritis and osteoarthritis, and from the joints of rats with experimentally induced arthritis. The antiserum used in this study was a polyclonal serum prepared against PGS-1, likely to cross react with PGS-2. In contrast to the elevation observed for PGS immunoreactivity, no increase occurred in the level of PGS-1 message. The authors suggest that the increase in PGS immunoreactivity is likely to result from an elevation in PGS-2 message and protein as a result of inflammatory stimuli. Consistent with this proposal, PGS-2 message is induced by the inflammatory cytokine IL-1 in cultured rabbit articular chondrocytes [50].

Prostacyclin production by vascular endothelial cells plays a vital role in maintaining both normal hemostasis and vascular tone. Prostacyclin production is elevated in vascular endothelial cells by IL-1, thrombin, tumor necrosis factor, and histamine, as well as by pharmacologic agents such as calcium ionophore or TPA. Jones *et al.* [35] isolated the human homologue of PGS-2 from a cDNA library prepared from human umbilical vein cells. Message levels for PGS-2 were elevated in these cells following treatment with IL-1, TPA, endotoxin, or TNF. Immunohistochemical studies using an antibody directed to the unique peptide of human PGS-2 has also demonstrated that IL-1 or TPA treatment of human umbilical vein cells in culture stimulates expression of the PGS-2 gene [51].

Many of the 'primary response genes' [52] cloned from murine fibroblasts are also expressed in response to either nerve growth factor-induced differentiation of peripheral neuronal precursors or in response to pharmacological or electroshock induced

seizures in brain. A number of physiological stimuli and behavioral paradigms are also able to modulate the expression of primary response genes such as c-fos, c-jun, egr-/TIS8, and nur77/TIS1 in specific neuronal subsets [53]. Yamagata *et al.* [54] identified the rat homologue of PGS-2 during a differential screen of a subtracted cDNA library prepared from the hippocampi of rats subjected to electroconvulsive stimulation. Using both northern analysis and immunohistochemical studies, these authors demonstrated that PGS-2 is induced in neurons in anatomically discrete regions of the cortex and limbic system. Expression appears to be regulated by NMDA-dependent receptor activation, and is glucocorticoid inhibitable. We have now observed that depolarization of cortical neurons in culture dramatically induces the expression of the PGS-2 gene (S. Reddy and H. Herschman, in preparation). Although outside the scope of this review, there is substantial suggestive evidence that prostaglandins may play a role in neuronal plasticity and in direct modulation of pain perception (as opposed to a secondary effect of prostaglandin production during inflammation). These observations may be relevant to some of the currently unexplained paradoxes in NSAID analgesia.

Prostaglandins are well-known as modulators of bone resorption. The murine osteoblast MC3T3-E1 cell produces substantial amounts of PGE_2 in response to stimulation by EGF [55] or norepinephrine [56]. The catecholamine induction is modulated by an increase in intracellular cAMP [57]. Using a polyclonal antiserum, a norepinephrine-mediated increase in immunoprecipitable PGS antigen was demonstrated [57]. Although small increases in the PGS-1 message level were observed, it seems likely that this increase in enzyme activity, immunoprecipitable PGS protein, and prostaglandin production is likely to result primarily from the induced expression of PGS-2. More recently, induction of PGS-2 in these cells by serum, TPA, transforming growth factor beta, PGE_2, and forskolin has been reported [58].

It is clear that the PGS-2 enzyme can be induced in a variety of distinct cell types, in response to a wide range of physiologic, pathologic, and pharmacologic stimuli. The role of induced prostaglandin synthesis in many of these biological contexts is currently obscure. The identification of a second gene encoding a highly regulated PGS should result in new hypotheses for the roles of these induced changes in prostanoid levels, and permit the design of a variety of experiments to test such speculations.

PGS-1 expression can also be induced in response to extracellular stimulation, but not in as wide a variety of cell types, or to as great a degree as PGS-2 induction

Prior to the discovery of the inducible PGS-2 gene, several reports suggested that the level of PGS-1 message could be induced in response to appropriate inducers in 3T3 cells [59], osteoblasts [57], and human endothelial cells [60]. Subsequent experiments have demonstrated that the elevation in PGS-1 message in response to mitogens in 3T3 cells is modest at best, and occurs only after a substantial increase in prostaglandin production [25]. Moreover, the elevation in prostaglandin production [40] and the production of PGS-2 message [40] and protein [31] are all inhibited by dexamethasone. In contrast, dexamethasone has no effect on the level of PGS-1 message. Similarly, although a small increase in PGS-1 message may occur in IL-1 treated human endothelial cells [60], analysis of the synthesis of the PGS-1 and PGS-2 proteins revealed a 20-fold increase in the cytokine-induced synthesis of PGS-2 and a less than 2-fold increase in PGS-1 synthetic rate [51].

Treatment of tracheal epithelial cells with TPA produces a rapid elevation of PGS-2 message, reachng near peak values at 30 minutes at about four-six fold over that observed in untreated cells. In contrast, PGS-1 message is elevated only after about two to three hours, and is increased only two to three fold. Dexamethasone blocked accumulation of both PGS-1 and PGS-2 message in these cells [48]. Prostaglandin production may, therefore, be mediated by both PGS enzymes in these cells.

The expression of PGS protein and message in human model cell systems in which monocytic cell lines can be induced to differentiate was the subject of a number of papers published prior to the discov-

ery of PGS-2. I will discuss here only those reports in which an analysis of both PGS species has been performed. PGS-1 message and protein are present in untreated U937 monoblastoid cells [61]. PGS-2 message and protein are undetectable in these cells. When treated with TPA, U937 cells differentiate into a macrophage-like cell. In the TPA-differentiated cells, the level of PGS-1 message remains unchanged, but the level of PGS-1 protein rises 13 fold over three days. A substantial amount of PGS-2 message and protein are also present in U937 three days after TPA treatment. Because basal levels are undetectable in untreated cells, fold-inductions cannot be calculated for PGS-2 message and protein. Dexamethasone abolished PGS-2 message and protein completely, and attenuated but did not eliminate PGS-1 induction. These complicated results suggest that there are both transcriptional and translational modulations of the two PGS genes that accompany monocyte/macrophage differentiation, and that the roles of these two genes will require additional study in U937 cells. In contrast, when the human monocytic THP-1 cell line is induced to a more macrophage-like phenotype by TPA, a substantial increase in PGS-1 message is observed, with no detectable PGS-2 message [62]. Western blot analysis for PGS-1 and PGS-2 protein confirmed the observation that TPA-differentiated cells had elevated levels of PGS-1 protein relative to control cells, and little or no PGS-2 was present in either cell type. These data differ from those with human monocytes, in which LPS and TPA cause the rapid induction of PGS-2 message [34]. Although the half-life of PGS-2 is about four hours [31, 32a], no clear and unequivocal measurement of the half life of PGS-1 has been described since the identification of PGS-2 as a related, likely cross-reacting protein. The human THP-1 cell line may be an excellent cell type in which to address this question.

The data presented in this section suggest that the induction of PGS-1 is neither as widely observed nor as extensive as the induction of PGS-2 in response to acute stimuli. However, induction of PGS-1 message accumulation and protein do occur in response to acute stimuli in some cells, and in response to agents that stimulate differentiation in other cells. An alternative form of regulation of PGS-1, differential splicing in response to alternative ligands to produce products with different open reading frames, has also been suggested [63]. The roles of the PGS-1 and PGS-2 enzymes, and the requirements for and consequences of their differential induction should occupy researchers in the eicosanoid field for some time.

Endogenous glucocorticoids chronically modulate PGS-2 gene expression in murine peritoneal macrophages *in vivo*

Glucocorticoid attenuation of ligand-induced PGS induction and prostaglandin production is a nearly ubiquitous pharmacologic phenomenon. What role do endogenous glucocorticoids play in PGS regulation? Adrenalectomy results in increased production of thromboxanes, prostaglandins, and prostacyclins in the peritoneal macrophages from otherwise untreated animals [64]. Hormone replacement therapy with dexamethasone completely blocks the increase in prostanoids in response to adrenalectomy. Using a polyclonal antiserum that cannot distinguish between PGS-1 and PGS-2, Masferrer *et al.* [64] showed (i) that adrenalectomy caused an increase in the synthesis of immunoprecipitable PGS protein and (ii) that hormone replacement could also completely block the adrenalectomy-induced synthesis of PGS protein, suggesting that glucocorticoids exert a tonic negative regulatory effect on PGS expression.

We have reanalyzed this experimental paradigm, using antibodies and cDNA probes that distinguish PGS-1 and PGS-2 protein and message [65]. We find that the constitutive levels of PGS-1 message and protein are essentially unchanged in control, adrenalectomized, and sham-operated animals, or in adrenalectomized animals receiving hormone replacement. In contrast, the levels of both PGS-2 message and protein are undetectable in peritoneal macrophage from untreated animals, are elevated following adrenalectomy, and are not detectable in adrenalectomized animals that receive dexamethasone replacement therapy. These data demonstrate that, under normal physiological conditions glucocorticoids play a tonic inhibitory role, suppressing

the expression of the PGS-2 gene. It will be of interest to determine what other cells show elevated PGS-2 levels *in vivo* in the absence of circulating glucocorticoids following adrenalectomy.

The existence of two forms of prostaglandin synthase raises questions about the roles of these two gene products in normal and abnormal cellular physiology

We now know that many cell types contain both a constitutive enzyme, PGS-1, and the potential to express an inducible enzyme, PGS-2, in response to specific cell stimulation. The inducible PGS-2 enzyme and the constitutive PGS-1 enzyme appear to both be localized to the endoplasmic reticulum in 3T3 cells, at least at the level of the resolution of immunofluorescence microscopy [31, 66]. If both enzymes are accessible to arachidonic acid, what are the differential roles of the constitutive and inducible enzymes?

The activity of the PGS-1 enzyme in 3T3 cells can be demonstrated by providing untreated cells with exogenous arachidonic acid, and measuring prostaglandin production. However, inhibition of protein synthesis following mitogen stimulation blocks not only PGS-2 protein production [31], but also prostaglandin accumulation from endogenous arachidonic acid stores in 3T3 cells [40]. Why is the constitutive PGS-1 present in these cells not able to convert arachidonic acid, released in response to mitogen stimulation, to prostaglandin? Similarly, although glucocorticoids are able to block the induction of PGS-2 message [40] and protein [31] production in 3T3 cells, why do they block mitogen-induced prostaglandin synthesis, if constitutive PGS-1 is present in these cells [25, 59]? The conventional explanation has been that glucorticoids prevent the release of arachidonic acid from endogenous stores by blocking mitogen-induced phospholipase activation. However, it is also reasonable to assume that the glucocorticoid inhibition of PGS-2 induction may be responsible for the inhibition of mitogen-induced prostaglandin production.

Three approaches to determine the different roles of the PGS-1 and PGS-2 genes and their products exist

First, pharmacologic inhibitors that can discriminate between PGS-1 and PGS-2 could be used to dissect the roles of these two enzymes in cellular functions. Pharmacologic differences between the PGS-1 and PGS-2 enzymes have been observed [67]; moreover this is currently a very active area of research in the prostaglandin field. At the time of preparation of this review, however, no pharmacologic experiments that help to unravel the distinct roles of these two PGS enzymes have been described. Second, mice with homologous gene disruptions of the PGS-1 and/or PGS-2 genes can be created and the physiological consequences of the inability to express either of these two enzymes can be explored. While work on this approach is proceeding rapidly in several laboratories, no results have yet been reported. Finally, antisense experiments can be performed to acutely inhibit the expression of the PGS-2 gene and the consequences can be observed.

We have used transfection of antisense oligonucleotides to block both the TPA- and PDGF-induced expression of PGS-2 in 3T3 cells and in primary cultures of murine embryo fibroblasts [68]. Antisense oligonucleotides block mitogen-induced production of prostaglandins from endogenous arachidonic acid. In contrast, sense and random oligonucleotides had no effect on mitogen-induced prostaglandin synthesis. The constitutive PGS-1 present in these cells is still able to convert exogenously supplied arachidonic acid to prostaglandin. The data suggest (i) that endogenous arachidonic acid released from membrane stores following mitogen stimulation is not available to PGS-1 and (ii) that PGS-2 synthesized in response to mitogen stimulation is required for and able to convert the released arachidonic acid to prostaglandin. If membrane lipids are first prelabelled with radioactive arachidonic acid, mitogen-treated 3T3 cells that have been transfected with antisense PGS-2 oligonucleotides are able to release free arachidonic acid, demonstrating that the antisense oligonucleotides do not inhibit TPA or PDGF activation of phospholipase

and consequent arachidonic acid release. We conclude that endogenous arachidonic acid released from membrane stores following mitogen stimulation in fibroblasts is 'channeled' in some way to PGS-2, and is not accessible to PGS-1. Similar results are true for LPS stimulated macrophages [68]. Precedent for a restricted pathway of access to endogenous arachidonic acid exists; arachidonic acid in cells must be presented to 5′-lipoxygenase by the 5′-lipoxygenase activating protein, or FLAP, in order for leukotriene biosynthesis to occur [69–71].

The 5′ regulatory regions of the PGS-1 and PGS-2 genes have been cloned and shown to act as promoters in transient transfections with reporter gene constructs

A 2.5 kb region upstream of the start site of transcription of the murine PGS-1 gene has been cloned, and a potential AP-1 site has been observed. In addition, a possible dioxin responsive element, the sequence that confers dioxin and polyaromatic hydrocarbon inducibility to the cytochrome P450IA1 gene, is present at position – 400 of the murine PGS-1 gene [36]. However, no analysis of these regions for regulatory activity has been reported at the time of preparation of this review. The first kilobase 5′ of the transcription start site of the human PGS-1 gene has also been sequenced. This region contains putative AP-2, GATA-1, Sp1, and PEA3 cis-acting elements. Constructs of 0.8 and 0.4 kb of the regulatory region of the human PGS-1 gene fused to a luciferase promoter are modestly active following transfection into NS-20 cells [72]. No deletional or mutagenic analysis of the potential cis-acting elements has been reported, however.

The first kilobase of DNA 5′ to the start site of transcription of the murine PGS-2 gene is able to confer both basal and serum (14-fold) or phorbol ester (4-fold) induction to a luciferase reporter gene transfected into NIH 3T3 cells [26]. These characteristics are retained in a construct that begins at – 371 of the gene. Within the first 1.6 kb of chicken PGS-2 gene there are a potential serum response element, as well as an AP-1 site, an NF-kB sequence, and several Sp1 and AP-2 sites [38]. A promoter fusion from nucleotide – 1586 could drive the chloramphenicol acetyl transferase (CAT) gene in transient transfection assays. Results on serum inducibility in this study were inconclusive [38]. When the first 2.6 kb of the rat PGS-2 gene were fused to a CAT reporter gene and transfected in the culture ovarian granulosa cells, FSH, LH, and forskolin were able to induce three to four fold elevations in the expression of CAT activity [33].

Deletion and mutation analyses have identified the cis-acting elements regulating gonadotrophin induction of the PGS-2 gene in granulosa cells and *v-src* induction in 3T3 cells: The early experiments delineating the promoter region of the PGS-2 gene that can confer ligand-dependent induction to chimeric reporter gene constructs [26, 33] have created the experimental tools for fine-scale analysis of the cis-acting regions of the gene regulating expression in a variety of cell types, and for identifying their trans-acting transcriptional regulatory proteins. In contrast to the 'primary response' gene nature of PGS-2 induction by mitogens and oncogenes in 3T3 cells, gonadotrophin/cAMP induction of PGS-2 transcription in granulosa cells requires intervening protein synthesis [33, 73]. Deletion analysis identified the sequence 5′-TTATGCAAT-3′ between nucleotides – 140 and – 131 of the rat PGS-2 promoter as a key element in the induction of this gene by pituitary glycoprotein hormones and forskolin. Gel-shift analysis demonstrated that the CAAT box/enhancer binding protein C/EBP-beta recognizes this sequence. Mutation of this sequence reduced forskolin induction by 50%, demonstrating the likely causal role of this cis-acting element in induced PGS-2 gene expression.

Cotransfection of NIH 3T3 cells with a *v-src* pp60src expression vector along with a deletion series of the murine PGS-2 promoter fused to a luciferase reporter gene demonstrated a 40-fold drop in *v-src* induced luciferase activity between the – 80 and – 40 constructs [73a]. Within this region is a 9 nucleotide sequence, 5′-CGTCACGTG-3′, that contains overlapping ATF/CRE (5′-CGTCA-3′) and E-box (5′-CACGTG-3′) transcription factor recognition sequences. Mutation of the proximal and distal two nucleotides of this nine base-pair sequence and construction of reporter plasmids dem-

onstrated that the ATF/CRE element is essential for *v-src* induction, while the E-box is not necessary. At least two proteins, one of which was shown to be CREB by antibody supershift experiments, are present in nuclear extracts and can bind to the ATF/CRE element of the PGS-2 gene. We conclude that the ATF/CRE element of the PGS-2 gene is necessary for *v-src* induction of the murine PGS-2 gene.

In the next year the cis-acting elements and trans-acting factors that regulate the expression of the PGS-2 gene by a variety of inducers (endotoxin, depolarization, IL-1b, serum, etc.) should begin to be identified. However, dexamethasone inhibition has not been demonstrated for any of the transfected gene constructs. The mechanism of glucocorticoid suppression of induction of the PGS-2 gene remains a major challenge for researchers in this area.

Transforming growth factor beta modulates PGS-2 gene expression in fibroblasts, macrophages, and epithelial cells

Transforming growth factor beta (TGF-beta) is one of the most powerful cytokines known, modulating embryonic development, wound healing, bone formation, hematopoiesis, cell proliferation, and inflammatory processes. TGF-beta is a potent attenuator of macrophage activation. Although TGF-beta alone is unable to modulate prostaglandin production or PGS-2 gene expression in murine macrophages, this cytokine can – like the glucocorticoids – attenuate both prostaglandin production and PGS-2 message and protein accumulation in murine macrophages [74]. The inhibitory effect of TGF-beta on endotoxin induction of PGS-2 in macrophages occurs at the level of transcription.

TGF-beta also modulates fibroblast growth. TGF-beta is unable to alter prostaglandin production or PGS-2 gene expression when given alone to 3T3 cells or primary cultures of murine embryo fibroblasts. However, in contrast to the results observed for macrophages, TGF-beta augments the prostaglandin production of serum- or TPA-treated fibroblasts, and also enhances mitogen induction of PGS-2 protein synthesis and accumulation, as well as message transcription and accumulation [75]. TGF-beta thus modulates the ligand-induced response of the PGS-2 gene in opposite fashions in macrophages and fibroblasts, attenuating the PGS-2 response in the former cells and augmenting the PGS-2 response in the latter.

Treatment of the rat intestinal epithelial cell line IEC-6 with TPA induces both the expression of PGS-2 and the accumulation of prostacyclin. TGF-beta has substantial effects on epithelial cells, often preventing their division and stimulating their differentiation. Although TGF-beta alone had no effect, it augmented both prostacyclin secretion and the expression of the PGS-2 gene in response to TPA (R. Gilbert and H. Herschman, in preparation). A variety of other cytokines and interleukins are also likely to modulate expression of the PGS-2 gene. At present, the signal transduction pathways and molecular targets for TGF-beta modulation of PGS-2 gene expression are completely unknown.

There are substantial similarities in the regulation of the inducible forms of prostaglandin synthase and nitric oxide synthase in fibroblasts and macrophages

The gene for one form of nitric oxide synthase, the enzyme that produces NO from arginine, can be induced in murine macrophages by endotoxin. Induction is inhibited by glucocorticoids. The initial similarities in the induction kinetics, and the role of both these enzymes (PGS-2 and iNOS) in mediating the synthesis of small molecule paracrine regulators of cell-cell communication, stimulated us to examine the expression if iNOS in murine fibroblasts and its regulation by TGF-beta and to compare these results with PGS-2 induction. iNOS can be induced in 3T3 cells by an overlapping, but not identical, set of ligands that induce PGS-2 [76]. TPA, serum, PDGF, and forskolin all induce both genes. Endotoxin, however, is a much better inducer of iNOS than of PGS-2. *v-src*, a potent PGS-2 inducer, is unable to induce iNOS mRNA accumulation [76].

Like PGS-2, iNOS induction is almost completely blocked in both 3T3 cells and macrophages by dexamethasone [76]. TGF-beta is unable to modulate iNOS message levels in either fibroblasts or

macrophages. However, this cytokine can attenuate endotoxin induction of iNOS message accumulation in macrophages [77], just as it does for PGS-2 [74]. Moreover, TGF-beta instead augments serum or PDGF induction of iNOS message accumulation in 3T3 cells [77], just as it did for mitogen induction of PGS-2 [75]. The iNOS and PGS-2 genes thus share substantial regulatory similarity to one another; (i) they are induced by many of the same agents in macrophages and fibroblasts, (ii) their induction is inhibited in both cells by dexamethasone (in contrast to most of the other primary response genes), and (iii) the induction of both genes is attenuated by TGF-beta in macrophages and augmented by TGF-beta in fibroblasts. While these data suggest that there are strong links in the regulation of these genes, the molecular nature of these regulatory links remains completely unknown at the present.

Conclusions

Three years ago, when David DeWitt definitively and exhaustively reviewed the regulation of prostaglandin synthase enzyme expression [13], a second pool of prostaglandin synthase had been tentatively suggested [17]. However, the nature of the second PGS gene, its relatively widespread expression, its dramatic inducibility, and its susceptibility to modulation by glucocorticoids and cytokines were all unknown. It is clear now that PGS-2, the product of a gene distinct from PGS-1, satisfies all the criteria for the hypothesized 'second pool' of PGS: (i) it has cyclooxygenase and hydroperoxidase activity, (ii) it is inducible by growth factors, tumor promoters, endotoxin, and other ligands known to elevate prostaglandin levels in target cells, (iii) it is rapidly and transiently expressed, leading to a bolus of prostaglandin production in response to stimulation, (iv) its induction is substantially attenuated by glucocorticoids, and (v) it appears to serve at least in some cases as the rate-limiting step in the production of prostanoids in response to ligand stimulation in intact cells.

The discovery of the second form of PGS-2, and its dramatic inducibility by endotoxin, IL-1, and TNF in cells mediating the inflammatory response, has rekindled the search for and development of NSAIDs that inhibit PGS. A pharmaceutical preparation that can inhibit PGS-2 activity without inhibiting PGS-1 catalysis might result in an anti-inflammatory drug with substantially reduced side effects. The rapid inducibility of the PGS-2 gene and its global susceptibility to modulation by agents such as steroids and cytokines suggests that therapeutic approaches that emphasize preventing expression of the PGS-2 gene, rather than inhibition of its product, might also be an avenue of pharmacologic intervention.

Characterization of the cis-acting sequences that regulate basal and induced expression of the PGS-1 and PGS-2 genes, and identification of their DNA-binding transcriptional modulators, should be a set of relatively straightforward problems in molecular biology. Understanding the more global inhibitory effects of glucocorticoids and the modulatory effects of cytokines such as TGF-beta may be more difficult. However, these mechanisms may be the key to alternative therapeutic approaches to problems of inflammation and analgesia. It is of interest that iNOS and PGS-2 are modulated so similarly in macrophages and fibroblasts. In contrast to the results with iNOS and PGS-2, induction of the majority of primary response genes is not substantially modified by glucocorticoids or TGF-beta. We suspect that there are likely to be other mitogen and endotoxin-induced primary response genes whose products are involved in intercellular communication, and that these genes may show similar global modulation by glucocorticoids and/or TGF-beta and related molecules.

The potential role of prostaglandins in colon cancer has been highlighted both by epidemiologic studies of men and women who take aspirin routinely, and by laboratory studies on the amelioration of colon cancer induction by NSAIDs, and is discussed in several places in this collection of reviews. It is of interest that human PGS-2 maps to chromosome 1 [35], one of the loci associated with colon cancer, and that PGS-2 can be expressed in a number of different epithelial cell types, including those of the digestive tract. It seems likely that the

PGS-2 gene product and its catalytic activity plays some role in the etiology of colon cancer.

One of the major questions raised by the discovery of the second form of PGS is the role of the two enzymes in normal cellular physiology. Why do cells have both a constitutive and an inducible PGS? Our data with antisense oligonucleotides suggests that, in at least some cases, endogenous and exogenous arachidonic acid are differentially available to the two enzymes. Perhaps PGS-2 produces prostanoid precursor from endogenous arachidonic acid released from membranes following acute stimulation by ligands or other signals, while PGS-1 utilizes arachidonic acid produced by neighboring cells to make prostanoids. However, it is clear that PGS-1 levels are modulated by alterations in the cell environment in some cases, and that pharmacologic stimulation of cells can often result in the rapid production of prostaglandins in the absence of protein synthesis. The development of pharmacologic agents that differentially extinguish the activities of PGS-1 and PGS-2, antisense approaches to acute suppression of the induced PGS-2 response, and mice unable to express the PGS-1 or PGS-2 proteins should provide tools that will enable us to determine the roles of these enzymes and their products in normal and abnormal physiology.

Another problem with which we are now confronted is to determine the roles of phospholipase activation and consequent arachidonic release, versus PGS-2 induction and consequent utilization of substrate, in the control of prostanoid biosynthesis. It is clear that arachidonic acid must be released from membrane stores for cells to produce prostanoids in response to ligand stimulation. It is likely that the relative roles of phospholipase activation and PGS-1 or PGS-2 synthesis in response to ligand may differ from cell type to cell type. This is likely to be a difficult set of problems to resolve, since we now know there are several forms of phospholipase A2, one of which is inducible. Moreover, activity of at least one of the phospholipase A2 forms can be modulated by phosphorylation. The fact that there are several forms of phospholipase and of PGS will certainly complicate our unraveling of the regulation of prostanoid synthesis; however, knowing of their existence and characteristics gives hope that we can better manipulate the relevant variables in order to understand their relationships.

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