Prostaglandin D₂ Synthase Inhibits the Exaggerated Growth Phenotype of Spontaneously Hypertensive Rat Vascular Smooth Muscle Cells* 

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Lipocalin-type prostaglandin D₂ synthase (L-PGDS) has recently been linked to a variety of pathophysiological cardiovascular conditions including hypertension and diabetes. In this study, we report on the 50% increase in L-PGDS protein expression observed in vascular smooth muscle cells (VSMC) isolated from spontaneously hypertensive rats (SHR). L-PGDS expression also increased 50% upon the differentiation of normotensive control cells (WKY, from Wistar-Kyoto rats). In addition, we demonstrate differential effects of L-PGDS treatment on cell proliferation and apoptosis in VSMCs isolated from SHR versus WKY controls. L-PGDS (50 μg/ml) was able to significantly inhibit VSMC proliferation and DNA synthesis and induce the apoptotic genes bax, bcl-x, and ei24 in SHR but had no effect on WKY cells. Hyperglycemic conditions also had opposite effects, in which increased glucose concentrations (20 mM) resulted in decreased L-PGDS expression in control cells but actually stimulated L-PGDS expression in SHR. Furthermore, we examined the effect of L-PGDS incubation on insulin-stimulated Akt, glycogen synthase kinase-3β (GSK-3β), and ERK phosphorylation. Unexpectedly, we found that when WKY cells were pretreated with L-PGDS, insulin could actually induce apoptosis and failed to stimulate Akt/GSK-3β phosphorylation. Insulin-stimulated ERK phosphorylation was unaffected by L-PGDS pretreatment in both cell lines. We propose that L-PGDS is involved in the balance of VSMC proliferation and apoptosis and in the increased expression observed in the hypertensive state is an attempt to maintain a proper equilibrium between the two processes via the induction of apoptosis and inhibition of cell proliferation.
examined that phosphoryl-induced apoptosis is mediated by L-PGDS phosphorylation and activation by protein kinase C, and is accompanied by an inhibition of the PI3K/PKB anti-
 apoptotic signaling pathways (19). In the present study, we report on the elevation of L-PGDS in VSMCs isolated from SHR and the stimulation of enhanced L-PGDS production upon cell differentiation. We demonstrate the differential effects of L-
PGDS on serum-induced cell proliferation and apoptosis in VSMCs isolated from spontaneously hypertensive rats (SHR) versus normotensive Wistar-Kyoto (WKY) controls. We also examined the effects of L-PGDS on Akt and glycogen synthase kinase-3β (GSK-3β) phosphorylation in response to insulin. We propose that L-PGDS is involved in maintaining a proper balance between cell proliferation and apoptosis in the hypertensive and hyperglycemic states.

EXPERIMENTAL PROCEDURES

Materials—Cell culture reagents, including fetal bovine serum, were purchased from Invitrogen. SDS/polyacrylamide gel electrophoresis and Western blot reagents were from Bio-Rad. Signal transduction antibodies were purchased from Cell Signaling Technology (Beverly, MA) and Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibody against L-PGDS was from Cayman Chemical (Ann Arbor, MI). Bicin
 chonic acid protein assay reagent was purchased from Pierce. Western blots were visualized with enhanced chemiluminescence reagent purchased from Amersham Biosciences. Porcine insulin was a kind gift from the Eli Lilly Co. Type 1 collagenase was from Worthington. The caspase-3 activity apoptotic detection kit was purchased from R & D Systems (Minneapolis, MN). All other reagents were purchased from Sigma.

Cell Culture—VSMCs were isolated by collagenase digestion of the aortic media from male normotensive WKY and SHR rats with body weights between 200 and 220 g, as described in our recent publications (3–5). VSMCs prepared from these rats were not contaminated with fibroblasts or endothelial cells, as evidenced by a greater than 99% positive immunostaining of smooth muscle α-actin with fluorescein isothiocyanate-conjugated α-actin antibody. Subcultures of VSMCs were maintained in α-minimum essential medium containing 10% FBS and 1% antibiotic/antimycotic. Unless otherwise stated, cells were grown to 70–80% confluency and studied at passages 4–5 for all experiments.

Western Blotting—Culture plates were washed four times with ice-
cold PBS followed by the addition of cell lysis buffer containing 50 mm to 70 mm and 1% antibiotic/antimycotic. Unless otherwise stated, cells were grown to 70–80% confluency and studied at passages 4–5 for all experiments.

Treatment of VSMCs — VSMCs were treated for 15 min with insulin (100 nM) followed by

Western Blotting—Culture plates were washed four times with ice-
cold PBS followed by the addition of cell lysis buffer containing 50 mm HEPEs, pH 7.6, 20 mm EDTA, 20 mm EGTA, 1% SDS, 1% benzamidine, 2.0 mg protein/μl, and 10 μg/ml each leupeptin, aprotinin, antipain, soybean trypsin inhibitor, and pepstatin A. After phosphorylation was detected, PBS and cell lysis buffer contained 2 mM sodium vanadate and 1 μM microcystin at 4 °C. The plates were scraped, and the cell lysate was sonicated and centrifuged at 2000 × g for 5 min. Typically, 50 μg of protein was mixed with Laemmli sample buffer containing 0.1% bromophen blue, 1 μM NaH₂PO₄, pH 7.0, 50% glycerol, and 10% SDS, boiled for 5 min, and loaded on a SDS-polyacrylamide gel. The separated proteins were transferred to polyvinylidene difluoride membrane and probed with the proper antibody followed by detection with enhanced chemiluminescence reagent and subsequent autoradiography. The intensity of the signal was quantitated by densitometric analysis of the autoradiograms.

Apoptotic Activity Assay—Apoptosis was quantitated as described previously (19) by the colorimetric measurement of caspase-3 activity as per the manufacturer’s procedure. In addition, conformation of apoptosis was determined by TUNEL assay as described previously (19) using the ApopDetek Cell Death Assay Kit (Enzo, Farmingdale, NY).

Apopotic Gene Expression by Gene Array—Cells were grown as described above in the presence or absence of L-PGDS (50 μg/ml) for 15 h, and total RNA was isolated using Trizol reagent (Invitrogen). Typically, 10 μg of RNA was used as a template for [32P]cDNA probe synthesis using α-32P-dCTP (Amersham Biosciences). The gene array membrane (SuperArray, Frederick, MD) was prehybridized for 2 h at 68 °C, and then denatured cDNA probe was added and hybridized for 15 h at 68 °C. The membrane was washed twice with solution 1 (2× SSC, 1% SDS) and twice with solution 2 (0.1× SSC, 0.5% SDS) for 20 min each at 68 °C with shaking. The membrane was wrapped with plastic wrap and exposed to x-ray film with an intensifying screen at −70 °C.

Cell Growth and DNA Synthesis—Cell proliferation was assessed by counting cell numbers. After the specified incubation period, cells were washed twice with ice-cold PBS, harvested with trypsin, and

FIG. 1. L-PGDS protein expression increases upon VSMC differentiation. VSMCs isolated from WKY and SHR rats were cultured as described under “Experimental Procedures.” Proteins were extracted after either 3 days (undifferentiated) or 10 days (differentiated) of culture, and 50 μg was separated by SDS-PAGE on a 12% gel. Proteins were transferred to a PVDF membrane and probed with both L-PGDS and actin antibodies (A). Panel B represents the corrected L-PGDS expression based upon actin expression. Results are the mean ± S.E. of four experiments. *p < 0.05 compared with day 3 WKY cells.

RESULTS

L-PGDS Expression Is Elevated in SHR and Increases upon VSMC WKY Differentiation—Because L-PGDS metabolism appears to be associated with hypertension and vascular complications, we decided to look at L-PGDS expression in VSMCs isolated from WKY and SHR. Interestingly, L-PGDS levels were ~50% higher in SHR cells when compared with undifferen-
tiated WKY cells (Fig. 1A, lane 3 versus lane 1). Furthermore, upon differentiation (day 10), L-PGDS expression increased 50% in WKY cells (Fig. 1A, lane 2 versus lane 1) and slightly in SHR cells (Fig. 1A, lane 4 versus lane 3). Fig. 1B represents the quantitation of L-PGDS intensity corrected for α-actin expression.

L-PGDS Inhibits the Exaggerated VSMC Proliferation Observed in SHR—To determine the effect of L-PGDS on cell

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proliferation, we measured total cell numbers by cell counting and DNA synthesis by \[^{3}H\]thymidine incorporation in response to exogenously added L-PGDS. As seen in Fig. 2A, L-PGDS treatment had a slight inhibitory effect on WKY cell doubling. A 50% inhibition of growth, however, was observed in SHR cells after 5 days in culture with L-PGDS (Fig. 2B). Concomitantly, the 2-fold stimulation of DNA synthesis observed in the presence of serum, in both WKY and SHR, was completely inhibited in the presence of L-PGDS in SHR, but not WKY, VSMCs (Fig. 3). It is worth noting that the basal level of DNA synthesis was 3-fold higher in SHR as compared with WKY cells.

Effect of L-PGDS Exposure on Apoptosis—Recently, we observed the induction of apoptosis by L-PGDS in several cell lines (17, 18). Because L-PGDS significantly inhibited the serum-induced proliferation of SHR VSMCs, we decided to examine the effect of L-PGDS on apoptosis in VSMCs isolated from both WKY and SHR rats. Fig. 4 demonstrates a 55% increase in the induction of caspase-3 activity by L-PGDS in serum-induced SHR VSMCs when compared with serum alone. Caspase-3 activity was not significantly altered by L-PGDS in WKY cells in the presence or absence of serum (Fig. 4). It is worth noting that the absolute level of apoptosis, as measured by caspase-3 activity, was nearly 2-fold higher in SHR when compared with WKY cells and that similar results were observed using the TUNEL assay to measure apoptosis (data not shown).

Furthermore, we examined the effect of L-PGDS exposure on the induction of known apoptotic genes using gene array hybridization. Exposure of WKY cells to L-PGDS for 15 h, in the presence of serum, had no effect on the induction of any of the apoptotic genes studied (Fig. 5A), whereas exposure of SHR cells to L-PGDS activated the expression of *bax* (coordinates C1 and D1), *bcl-x* (coordinates C2 and D2), and *ei24* (coordinates C7 and D7), three apoptosis-related genes (Fig. 5B).

L-PGDS Pretreatment Inhibits the Insulin-induced Phosphorylation of Akt and GSK-3β and Induces Apoptosis in Insulin-treated WKY VSMCs—The PI3K pathway has been shown to be involved in the apoptosis of VSMCs (21–24). Previous work from this laboratory has linked both Akt and GSK-3β phosphorylation to L-PGDS-induced apoptosis (19). Insulin, a known inducer of Akt and GSK-3β phosphorylation, has been shown to stimulate proliferation and inhibit VSMC apoptosis (22). Be-
cause insulin resistance is a common phenomenon associated with hypertension (25), we decided to examine the effects of L-PGDS on insulin treated VSMCs. Fig. 6 demonstrates the induction of caspase-3 activity in the presence of insulin and L-PGDS in WKY cells but not in SHR cells. Furthermore, we examine the effect of L-PGDS pretreatment on insulin-stimulated Akt/GSK-3β phosphorylation. Fig. 7A demonstrates an approximate 2-fold induction of Akt phosphorylation in response to insulin (WKY, lane 2 versus lane 1; SHR, lane 5 versus lane 4). Insulin-stimulated Akt phosphorylation was blocked when WKY cells were pretreated with L-PGDS (Fig. 7A, lane 3 versus lane 2) but remained elevated in SHR, even with L-PGDS pretreatment (Fig. 7A, lane 6 versus lane 5). Fig. 7B represents the quantitation of Akt phosphorylation corrected for Akt protein. Similar observations were observed with GSK-3β phosphorylation. Insulin stimulated a 45% increase in GSK-3β phosphorylation in both cell lines (Fig. 8A, WKY, lane 2 versus lane 1; SHR, lane 5 versus lane 4). Pretreatment of WKY cells with L-PGDS completely inhibited phosphorylation (Fig. 8A, lane 3 versus lane 2), whereas SHR cells pretreated with L-PGDS exhibited no inhibition of insulin-stimulated GSK-3β phosphorylation (Fig. 8A, lane 6 versus lane 5). Both processes appear to be independent of the ERK pathway because insulin-stimulated ERK phosphorylation was unaffected by L-PGDS pretreatment in both WKY and SHR (Fig. 9).

Effect of Hyperglycemia on L-PGDS Protein Expression—Hyperglycemia, associated with increased VSMC proliferation and the inhibition of apoptosis (26, 27), has been linked to hypertension and insulin resistance (28, 29). Blood sugar control has been associated with urinary L-PGDS excretion in Type II diabetics (30). We therefore examined the effect of hyperglycemia on L-PGDS expression in VSMCs. Fig. 10 demonstrates the inhibition of L-PGDS expression in response to high glucose (20 mM) in WKY cells. Interestingly, the opposite was observed in SHR cells, where growth under hyperglycemic conditions actually increased L-PGDS expression 2-fold.

DISCUSSION

In this study, we examined L-PGDS expression in VSMCs isolated from normotensive and hypertensive rats and found a 50% elevation in L-PGDS expression in SHR compared with WKY controls (Fig. 1). Interestingly, patients with essential hypertension have a significant elevation in their serum L-PGDS levels (14). This SHR phenotype was maintained up through the sixth passage of our primary VSMC cultures,
whereas subsequent passages had L-PGDS levels more comparable with WKY. We also examined the effect of exogenously added L-PGDS on cell proliferation and apoptosis and demonstrated the ability of L-PGDS to significantly inhibit SHR VMSC proliferation (Figs. 2 and 3) and concomitantly stimulate SHR apoptosis (Fig. 4). Caspase-3 activity as well as the TUNEL assay (data not shown) were used to confirm apoptosis. In addition, gene array analysis confirmed the activation of apoptosis in SHR by the induction of bax, bcl-x, and e124 (Fig. 5). We have confidence in the array technique because the negative bacterial plasmid controls (coordinates G1 and G2) as well as the positive actin and glyceraldehyde-3-phosphate dehydrogenase controls (coordinates G3 and G4 and G5-G8, E8, F8, respectively) all gave the predicted results (Fig. 5). These observations are also consistent with the increased apoptotic levels observed in target organs of SHR rats (31, 32). It is noteworthy that L-PGDS expression increased in both SHR and WKY upon differentiation, a stage of development known to have increased levels of apoptosis.

Recent evidence suggests that arachidonic acid metabolites, namely prostaglandins, are involved in regulating cell proliferation. Inhibition of 85-kDa phospholipase A2, the enzyme responsible for hydrolyzing membrane phospholipids into arachidonic acid, resulted in a dose-dependent inhibition of VSMC proliferation (33). 15d-PGJ2, which is quickly formed from PGD2, is known to inhibit basic fibroblast growth factor-induced DNA synthesis in rat VSMCs (34). 15d-PGJ2 also inhibits platelet-derived growth factor-directed migration (34) and induces G1 arrest and differentiation (35) in VSMCs. Similar effects have been observed with the synthetic PPARy activator, troglitazone, which inhibits VSMC proliferation (36), decreases the intimal and medial thickness of carotid arteries in humans (37), and inhibit the development of atherosclerosis (38). It is our hypothesis that L-PGDS functions to stimulate the production of these PGs and thereby inhibits cell proliferation and

**Fig. 5.** Effect of L-PGDS on apoptotic gene expression. Total RNA was isolated from VMSCs cultured as described under “Experimental Procedures” in the presence or absence of (50 μg/ml) L-PGDS for 18 h. 10 μg of RNA was used as a template for [32P]cDNA probe synthesis. The gene array membranes (A, WKY; B, SHR) were hybridized for 15 h at 68 °C and washed as described under “Experimental Procedures.” The membranes were exposed to film at −70 °C for several days and the autoradiogram scanned with SigmaGel software. Apoptotic gene coordinates: bax, C1/D1; bcl-x, C2/D2; e124 (pig8), C7/D7.

**Fig. 6.** Effect of insulin on L-PGDS-induced apoptosis. VMSCs were cultured as described under “Experimental Procedures” in medium containing 10% FBS in the presence or absence of L-PGDS (50 μg/ml) for 2 h. Where indicated, insulin (100 nM) was added for 10 min. Proteins were extracted, and 50 μg of protein lysate was assayed for caspase-3 activity. Values are the mean ± S.E. of at least three experiments performed in duplicate and expressed as percent of basal. *, p < 0.05 compared with control cells.

**Fig. 7.** Effect of L-PGDS on insulin-stimulated AKT phosphorylation. VMSCs were cultured as described under “Experimental Procedures” in medium containing 10% FBS in the presence or absence of L-PGDS (50 μg/ml) for 2 h. Where indicated, insulin (100 nM) was added for 10 min. Protein lysates (50 μg) were separated by SDS-PAGE on a 12% gel, transferred to a PVDF membrane, and probed for the 60-kDa phospho-Akt (pAKT) and Akt (A). Panel B represents the phosphorylated AKT intensity corrected for AKT expression. Values are the mean ± S.E. of four experiments. *, p < 0.05 when compared with insulin-stimulated cells.
stimulates apoptosis in hypertension and diabetes.

The role of PGs in VSMC proliferation has also been demonstrated directly in SHR VSMCs. For example, the addition of indomethacin, a cyclooxygenase inhibitor, leads to increased proliferation of VSMCs in SHR rats (39). In addition, the inducible gene responsible for NO synthesis in VSMCs, nitric oxide synthase, is inhibited by both PGD2 and 15d-PGJ2 but not by PGE2, PGI2, or PGF2 (40). In addition, the effect of retinoic acid metabolism on cell growth has been investigated for its potential as an anticancer agent because of its ability to inhibit cell proliferation, migration, and differentiation of VSMCs (41). In human coronary artery VSMCs, the antiproliferative effects of 15d-PGJ2 were enhanced 4-fold with the addition of 9-cis-retinoic acid, a retinoid X receptor ligand (42).

Although this study (42) does not address the apoptotic state of the cells, it does demonstrate the effectiveness of the combination of 15d-PGJ2 and retinoic acid in terms of the inhibition of VSMC proliferation. It is worth noting that in addition to its function as the enzyme responsible for the eventual synthesis of 15d-PGJ2, L-PGDS is also a retinoid transporter.

Several growth factors have been shown to alter the balance between VSMC proliferation and apoptosis. For example, insulin-like growth factor (IGF-1) has been shown to inhibit apoptosis and promote cell proliferation (43). Bai et al. (44) have shown that apoptosis is prevented by IGF-1 via the PI3K signaling pathway, which inactivates the proapoptotic gene, Bad, by phosphorylation presumably via Akt. In addition, protein kinase C and p38 MAPK activation have been reported to cause an increase in PG formation (45), and elevated protein kinase C levels have also been reported in VSMCs of diabetic rats (46). Similarly, we have observed the decreased activation of PI3K

![Fig. 8. Effect of L-PGDS on insulin-stimulated GSK-3β phosphorylation.](image)

![Fig. 9. Effect of L-PGDS on insulin-stimulated ERK phosphorylation.](image)

![Fig. 10. Effect of hyperglycemia on L-PGDS protein expression.](image)
(3) and increased p38 MAPK activation and phosphorylation in VSMCs isolated from SHR rats (47). Our present results suggest that when VSMCs are in a hyperproliferative state, such as in serum-induced SHR or upon differentiation, L-PGDS expression is elevated to inhibit cell proliferation and stimulate cellular apoptosis. It is only when proliferation pathways are active that L-PGDS can induce apoptosis and inhibit cell proliferation. This is consistent with our previous findings in other cell lines, where L-PGDS-induced apoptosis is more pronounced in the presence of serum (19). In addition, L-PGDS pretreatment appears to inhibit insulin-stimulated proliferation by blocking any further Akt/GSK-3β phosphorylation in wild-type VSMCs (Figs. 7 and 8), inducing a beneficial “insulin resistance” environment. L-PGDS may act as a moderator for the effects of insulin on cell proliferation and apoptosis, and it may help control WKY proliferation, despite the presence of insulin. This observation is made in differentiated WKY VSMCs, which express significant levels of L-PGDS. It appears that L-PGDS expression increases to inhibit cell proliferation and stimulate apoptosis. It is only when proliferation pathways are active that L-PGDS can induce apoptosis and inhibit cell proliferation. This is consistent with our previous findings in other cell lines, where L-PGDS-induced apoptosis is more pronounced in the presence of serum (19). In addition, L-PGDS pretreatment appears to inhibit insulin-stimulated proliferation by blocking any further Akt/GSK-3β phosphorylation in wild-type VSMCs (Figs. 7 and 8), inducing a beneficial “insulin resistance” environment. L-PGDS may act as a moderator for the effects of insulin on cell proliferation and apoptosis, and it may help control WKY proliferation, despite the presence of insulin. This observation is made in differentiated WKY VSMCs, which express significant levels of L-PGDS.
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