Accelerated Glucose Intolerance, Nephropathy, and Atherosclerosis in Prostaglandin D₂ Synthase Knock-out Mice*

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Louis Ragolia‡§, Thomas Palaiak, Christopher E. Hall‡, John K. Maesaka‡§, Naomi Eguchi‡§, and Yoshihiro Urade‡

From the ¶Vascular Biology Laboratory, Winthrop-University Hospital, Mineola, New York 11501, §Stony Brook University School of Medicine, Stony Brook, New York 11794, and the ¶Osaka Bioscience Institute, Osaka, Japan

Type 2 diabetics have an increased risk of developing atherosclerosis, suggesting the mechanisms that cause this disease are enhanced by insulin resistance. In this study we examined the effects of gene knock-out (KO) of lipocalin-type prostaglandin D₂ synthe (L-PGDS), a protein found at elevated levels in type 2 diabetics, on diet-induced glucose tolerance and atherosclerosis. Our results show that L-PGDS KO mice become glucose-intolerant and insulin-resistant at an accelerated rate when compared with the C57BL/6 control strain. Adipocytes were significantly larger in the L-PGDS KO mice compared with controls on the same diets. Cell culture data revealed significant differences between insulin-stimulated mitogen-activated protein kinase phosphatase-2, protein-tyrosine phosphatase-1D, and phosphorylated focal adhesion kinase expression levels in L-PGDS KO vascular smooth muscle cells and controls. In addition, only the L-PGDS KO mice developed nephropathy and an aortic thickening reminiscent to the early stages of atherosclerosis when fed a “diabetogenic” high fat diet. We conclude that L-PGDS plays an important role regulating insulin sensitivity and atherosclerosis in type 2 diabetes and may represent a novel model of insulin resistance, atherosclerosis, and diabetic nephropathy.

Cardiovascular disease is the primary cause of morbidity and mortality in people with non-insulin-dependent diabetes mellitus (1). Type 2 diabetics have a significantly increased risk of developing hypertension, atherosclerosis, and restenosis after angioplasty or stent implantation (2, 3). These phenomena are partially attributable to the abnormal accumulation of vascular smooth muscle cells (VSMCs) within the intima of blood vessels resulting from alterations in migration, proliferation, and apoptosis (4, 5).

Previously, we have demonstrated that lipocalin-type prostaglandin D₂ synthase (L-PGDS) inhibits the exaggerated growth phenotype of VSMCs isolated from hypertensive rats (6). The mechanism appears to involve the inhibition of insulin-stimulated protein kinase B (Akt) and glycogen synthase kinase-3β phosphorylation (6). We have also shown that VSMC apoptosis and migration are both altered by L-PGDS and that any beneficial effects of L-PGDS are absent in VSMCs isolated from diabetic animals (7). In addition, L-PGDS is ultimately responsible for the synthesis of the naturally occurring peroxisome proliferator activator receptor γ ligand, 15-deoxy-Δ12,14-prostaglandin J₂, already known to induce apoptosis (8). Moreover, thiazolidinediones, which are synthetic peroxisome proliferator activator receptor γ ligands, have been shown to increase insulin sensitivity and enhance insulin-stimulated glucose transport into muscle (9) and exert other beneficial cardiovascular effects such as blocking VSMC growth and migration to delay the onset of atherosclerosis (10, 11).

Several other findings have emerged suggesting that L-PGDS has important vascular functions. Inoue et al. (12) have demonstrated that elevated serum L-PGDS levels correlate with a decreased occurrence of restenosis after coronary angioplasty. A polymorphism in the L-PGDS gene appears to be associated with carotid atherosclerosis in a population of hypertensive Japanese individuals (13); the balance between L-PGDS and prostaglandin (PG) E synthase has been shown to be a major determinant of atherosclerotic plaque instability (14); laminar shear stress stimulates vascular endothelial cells to up-regulate L-PGDS expression (15); human L-PGDS mRNA expression has been measured to be highest in heart tissue, with enzyme immunoreactivity localized in myocardial cells, arterial endothelial cells, and the synthetic state of smooth muscle cells in arteriosclerotic plaques (16); chicken apolipoprotein D and L-PGDS share similar protein sequences (17); urinary L-PGDS excretion has been shown to increase in the early stages of diabetes mellitus (18). Collectively, these studies illustrate the need to clarify the role of L-PGDS in the vasculature, especially in atherosclerosis, hypertension, and diabetes.

The C57BL/6 mouse strain represents one model to study diet-induced atherosclerosis and diabetes (19). After 20 weeks on a high fat (42% kcal) diabetogenic diet the mice become hyperglycemic, insulin-resistant, and obese. Given our previous observations on the effects of L-PGDS and the vascular implications of L-PGDS, we decided to examine the effects of a high fat diet on insulin resistance and atherosclerosis in L-PGDS knock-out (KO) mice.

MATERIALS AND METHODS

Cell culture reagents, including fetal bovine serum, were purchased from Invitrogen. SDS/polyacrylamide gel electrophoresis and Western blot reagents were from Bio-Rad. Antibodies to mitogen-activated pro-
tein kinase phosphatase (MKP)-2 and phosphotyrosine phosphatase-1D (PTP-1D) were purchased from BD Biosciences and used at dilutions of 1:500 and 1:2500, respectively. Focal adhesion kinase (FAK) and pFAK antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and used at a dilution of 1:200. Bicinchoninic acid protein assay reagent was purchased from Pierce. Western blots were visualized with enhanced chemiluminescence reagent purchased from Amersham Biosciences. Type-1 collagenase was from Worthington Biochemical Co. (Freehold, NJ). All other reagents were of reagent grade or better and purchased from the Sigma.

Animals and Diets—Male L-PGDS transgenic KO C57BL/6 mice, obtained from the Osaka Bioscience Institute (Osaka, Japan) (20), and C57BL/6NHla mice, purchased from Hilltop Lab Animals, Inc. (Scottsdale, PA), were maintained in temperature-controlled rooms (22 °C) with a 12-h light/dark cycle and given free access to food and water. Mice were fed a pellet-form rodent chow diet (Harlan Teklad Test Diets #8604, Madison, WI) before the initiation of studies. At 6–8 weeks of age animals were switched to either a high fat diet providing 45% of the calories as fat (D12451) or the corresponding control diet (D12450B), with 10% of the calories as fat (Research Diets Inc., New Brunswick,
The fat source for both diets was identical, and equal numbers of mice were fed the low and high fat diets for up to 20 weeks.

Analytical Procedures—Mice were fasted for 5 h before drawing tail blood for all experiments except glucose tolerance tests, when 16 h fasts were implemented. Fresh blood was assayed for glucose using a Bio-Scanner 2000 (Polymer Technology Systems, Inc., Indianapolis, IN), and the concentration was expressed as mg/dl. Serum concentrations of insulin were determined using the ultrasensitive rat/mouse insulin

![Graph A](image1)

**Fig. 2.** Mouse intraperitoneal insulin tolerance tests. Control C57BL/6 ($n = 12$) and L-PGDS KO ($n = 12$) male mice at 6–8 weeks of age (A), after 20 weeks on a low fat diet (B) and after 20 weeks on a high fat diet (C), were fasted for 5 h. Blood was collected before (T0) and after intraperitoneal injection of insulin (1 units/kg body wt) at the indicated times, and serum glucose was measured. Data are expressed as the means ± S.E. Asterisks (*) represent a $p < 0.01$ when compared with corresponding control C57BL/6 mice.
Intraperitoneal Glucose Tolerance Test and Acute Insulin Response—
Intraperitoneal glucose tolerance tests were performed in animals fasted for 16 h. Blood was collected before (0 min) and after intraperitoneal injection of glucose (2 g/kg body wt) at 15, 30, 60, 90, and 120 min. In a separate series of experiments, overnight fasted mice (both control and L-PGDS KO) had initial bloods collected (T0) followed by glucose injection (2 g/kg body wt intraperitoneal) and another blood collection after 10 min (T10).

Intraperitoneal Insulin Tolerance Test—An intraperitoneal insulin tolerance test was performed after animals were fasted for 5 h. Blood glucose levels were measured before the injection of insulin (1.0 units/kg body wt; NovolinR, Novo Nordisk Pharmaceuticals, Inc., Princeton, NJ) and at 15, 30, 60, 90, and 120 min after injection.

Cell Culture—VSMCs were isolated by collagenase digestion of the aortic media from mice as described previously (7). VSMCs prepared from these mice 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-minum Eagle’s medium containing 10% fetal bovine serum and 1% antibiotic-antimycotic. Cells were synchronized in G0 by incubating in serum-free medium for 24 h. Cells were grown to confluence and studied at passages 5–6 for all experiments.

Western Blotting—Cells were lysed as previously described. When phosphorylation was detected, phosphate-buffered saline and cell lysis buffer contained 2 mM sodium vanadate and 1 mM microcystin at 4 °C. Typically, 50 μg of protein was mixed with Laemmli sample buffer containing 0.1% bromophenol blue, 1.0 mM 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 primary antibodies followed by 1:2000 diluted secondary antibody and detection with enhanced chemiluminescence reagent and subsequent autoradiography. The intensity of the signal was quantitated by densitometric analysis of the autoradiograms and normalized against β-actin or, in the case of phosphorylations, the non-phosphorylated form of the protein.

Serum Creatinine—A creatinine diagnostic kit (Thermo Electron, Louisville, CO) was used to determine serum creatinine based on the formation of an alkaline picrate product with absorption at 500 nm.

RESULTS

Glucose and Insulin Tolerance in L-PGDS KO Mice—The effects of a high fat (diabetogenic) diet on glucose and insulin tolerance were measured in L-PGDS KO mice as well as the C57BL/6 control strain. As seen in Fig. 1A, both mouse strains show rather similar initial glucose profiles in response to an intraperitoneal glucose tolerance test, with the L-PGDS KO curve shifted slightly higher than the control. Interestingly, although the control C57BL/6 mice seem to display typical glucose tolerance after 20 weeks on a low fat diet, the L-PGDS KO mice have already developed impaired glucose tolerance (Fig. 1B). Both strains exhibit impaired glucose tolerance after 20 weeks on the high fat diet (Fig. 1C).

An analogous situation is observed with insulin tolerance. Although both strains seem responsive to intraperitoneal insulin injection initially (Fig. 2A), only the L-PGDS KO mice are significantly less responsive to insulin after 20 weeks on the low fat diet (Fig. 2B). Insulin resistance is observed in both strains after 20 weeks on the high fat diet (Fig. 2C).

Blood Glucose and Insulin Levels in Response to Glucose—To determine whether defective insulin secretion contributed to the glucose intolerance observed in the L-PGDS KO mice, insulin levels were measured in response to intraperitoneal glucose injections. As seen in Fig. 3, glucose injection increased insulin secretion in L-PGDS KO mice initially; however, after 10 weeks on either diet, both sets of mice were hyperinsuline-
mic and hardly responsive to glucose injection (Fig. 3).

**Aorta Fat Staining and Cross-section Thickness**—The effects of a high fat diet on fat deposition and aorta thickness were measured in L-PGDS KO mice as well as controls. As seen in Fig. 4, the aorta of control C57BL/6 mice had a 2% fat deposition on a low fat diet and a 10% fat deposition after 20 weeks on a high fat diet. This is in sharp contrast to the L-PGDS KO mice, which already had 33 and 50% fat deposition after 20 weeks on the low fat and high fat diets, respectively (Fig. 4).

The effects of the diabetogenic diet on aortic cross-section thickness closely mimicked the fat deposition results. Although the control C57BL/6 aortic thicknesses were unaffected by diet, the L-PGDS KO mice aortic thicknesses were significantly increased after 20 weeks on both the low fat (14% over control) and high fat diets (34% over control) (Fig. 5).

**Diabetic Nephropathy and Transforming Growth Factor-β (TGF-β) Staining of Kidney Sections**—Microvascular complications of type 2 diabetes such as diabetic nephropathy have been tied to an increase in TGF-β activity (22). Therefore, the effects of a high fat diet on kidney morphology and TGF-β staining were examined. As seen in Fig. 6A, there was a significant increase in C57BL/6 glomerular size as well as increased fibrosis after 20 weeks on the diabetogenic diet. The L-PGDS KO kidneys had significant glomerular hypertrophy and fibrosis even after 20 weeks on the low fat diet, which progressed even further on the high fat diet (Fig. 6A). The C57BL/6 control kidneys had normal tubule formation and almost no TGF-β staining when fed a low fat diet (Fig. 6B). When this same strain was fed a high fat diet we observed the initial stages of tubule damage and fat deposition (red arrows) and TGF-β staining (green arrows). The L-PGDS KO mice already had significantly increased tubule damage and fat deposition as well as higher TGF-β staining on the low fat diet which was exacerbated on the high fat diet (Fig. 6B).

Histopathology results were confirmed by the measurement of serum creatinine after 20 weeks on respective diets. The
serum creatinine levels of the L-PGDS KO mice were nearly 2-fold higher than C57BL/6 controls (Fig. 6C).

Adiponectin Levels—Adiponectin appears to have substantial anti-inflammatory properties and may be a major modulator of the insulin resistance and increased atherosclerotic risk found in type 2 diabetic patients (23). We measured the level of adiponectin in mice on the low fat and diabetogenic diets and found that there was a significantly lower level of adiponectin in the L-PGDS KO mice compared with control C57BL/6 mice under either dietary condition (Fig. 7).

Adipocyte Size—It is well accepted that muscle and adipose represent the two major tissues of insulin-stimulated glucose disposal. As seen in Fig. 8, visceral adipocytes isolated from C57BL/6 on a high fat diet were 2.5-fold larger in size than those isolated from control mice on a low fat diet. Adipocytes isolated from L-PGDS KO mice on a low fat diet were comparable in size to those isolated from C57BL/6 on a high fat diet and became an additional 40% larger when fed the diabetogenic diet (Fig. 8).

Insulin-stimulated Expression of MKP-2, PTP-1D, and pFAK—Gene array screening of L-PGDS KO and C57BL/6 aorta provided some insight into which signal transduction proteins might be affected by diet (data not shown). We decided to study the effects of insulin stimulation on a select group of these signaling molecules in primary VSMC cultures isolated from the aorta of C57BL/6 and L-PGDS KO mice.

MKP are involved in the feedback regulation of mitogen-activated protein kinase gene expression and have been linked to insulin resistance (24), diabetes (25), and atherosclerosis (26). As seen in Fig. 9A, insulin caused a 2.5-fold stimulation of MKP-2 expression in C57BL/6 (lane 2 versus lane 1), which was further unaffected by exogenously added L-PGDS (lane 3 versus lane 2). The opposite effect of insulin was observed in L-PGDS KO cells. Insulin caused a 40% reduction in MKP-2 expression (lane 5 versus lane 4), which was nearly abolished with exogenously added L-PGDS (Fig. 9A, lane 6 versus lane 5).

FAK phosphorylation, which is involved in growth and migration, has been shown to be affected by insulin (27). We
FIG. 6. Trichrome and TGF-β staining of kidney cross-sections. Control C57BL/6 (n = 8) and L-PGDS KO (n = 8) male mice were sacrificed after 20 weeks on either a low fat or high fat diet. Kidneys were removed and sectioned as described under “Materials and Methods” and either stained with trichrome (A) and viewed at 100× magnification or probed for TGF-β (1 μg/ml) and viewed at 40× magnification (B). Arrows highlight fat deposition (red) and TGF-β (green). C represents the mean serum creatinine ± S.E. after 20 weeks. Asterisk (*) represents a p < 0.01 when compared with controls.
observed a 2-fold increase in insulin-stimulated FAK phosphorylation in C57BL/6 cells (Fig. 9B, lane 2 versus lane 1), whereas there was no change in L-PGDS KO (Fig. 9B, lane 5 versus lane 4). Exogenous L-PGDS stimulated FAK phosphorylation in C57BL/6 cells (Fig. 9B, lane 3 versus lane 2) but actually inhibited FAK phosphorylation in the L-PGDS KO VSMCs (Fig. 9B, lane 6 versus lane 5).

Insulin signal transduction involves the binding of the insulin receptor to PTP-1D. Expression of PTP-1D was stimulated 3-fold by insulin in C57BL/6 cells (Fig. 9C, lane 2 versus lane 1) yet unaffected in L-PGDS KO cells (Fig. 9C, lane 5 versus lane 4). Exogenously added L-PGDS slightly enhanced PTP-1D expression in C57BL/6 cells (Fig. 9C, lane 3 versus lane 2) and greatly stimulated PTP-1D expression in L-PGDS KO cells (Fig. 9C, lane 6 versus lane 5).

**DISCUSSION**

Central obesity is a predisposing factor for the development of type 2 diabetes and is also associated with insulin resistance (28). The precise mechanisms linking a high fat diabetogenic diet and diabetes, however, are unclear. The fact that urinary and serum L-PGDS concentrations increase in people with type 2 diabetes and hypertension led us to examine the role of this protein in diabetes. It is apparent from our data that L-PGDS depletion accelerated glucose intolerance and insulin resistance, since only the L-PGDS KO mice developed these characteristics on the low fat diet (Figs. 1 and 2). That the serum insulin concentrations were elevated in L-PGDS KO mice implies it was the insulin resistance that was responsible for the glucose intolerance rather than any defects in insulin secretion (Fig. 3).

Atherosclerosis and diabetic nephropathy are two common pathologies associated with type 2 diabetes (22, 29). Generally, it is a high cholesterol diet, not a diabetogenic diet, that induces atherosclerosis in the C57BL/6 mouse model (19). It is apparent from our histological data that L-PGDS KO mice had increased fat deposition as well as a thickening of the aortic media, which was not evident in C57BL/6 mice after 20 weeks on the diabetogenic diet (Figs. 4 and 5). This is consistent with our tissue culture observations, where we demonstrated that exogenously added L-PGDS could suppress the hyperproliferation of VSMCs isolated from hypertensive rats and inhibit VSMC migration and cell cycle progression (6, 7). Collectively, these data support the use of thiazolidinediones in the management of diabetes and the associated cardiovascular diseases and may explain why elevated serum levels of L-PGDS predict a decreased occurrence of restenosis (12) and why polymorphisms in L-PGDS result in carotid atherosclerosis (13). In addition, since L-PGDS can bind retinoids, it is quite possible that L-PGDS transports a ligand that activates the retinoid X receptor, which has been reported to reduce atherosclerosis in apoE KO mice (21).

Nephropathy has been associated with the severity of hyperglycemia, which itself may contribute to early alterations in kidney structure and function, before the clinical detection of diabetes (30, 31). Structural changes indicative of diabetic nephropathy, such as glomerular hypertrophy, fibrosis, and basement membrane thickening, were observed in the L-PGDS KO mice on the low fat diet and were further exacerbated on the diabetogenic diet (Fig. 6A). In addition, the deposition of fat and TGF-β was also more pronounced in the L-PGDS KO and appeared in mice fed the low fat diet (Fig. 6B). These data along with the increase in L-PGDS KO serum creatinine (Fig. 6C) would suggest that the L-PGDS KO mouse is a novel model of insulin resistance as well as fat-induced atherosclerosis and diabetic nephropathy.

Adipocyte hypertrophy was obvious in the L-PGDS KOs on either diet (Fig. 8). Although adiponectin levels were significantly lower in C57BL/6 controls (Fig. 7), leptin levels were surprisingly unaffected (data not shown). It should be noted that Fisher et al. (32) have demonstrated that it is the high molecular weight adiponectin complex that better correlates with glucose tolerance. Maier et al. (33) have proposed that soluble N-ethylmaleimide attachment receptor (SNARE) proteins such as syntaxin 4 may be altered in insulin-resistant states and that their levels are modulated by agents that increase insulin sensitivity. Preliminary observations from our laboratory suggest that alterations in the expressions of Glut4 and syntaxin 4 may be involved in the adipocyte hypertrophy observed in L-PGDS KO mice; however, further studies are necessary to confirm these observations. We believe that the role L-PGDS has transporting retinoids is involved in this process since a link exists between the retinoid X receptor and adipogenesis (34).

Reddy et al. (26) have shown that MKP-1 expression is associated with hypercholesterolemia and atherosclerosis and that inhibition of MKP-1 activity may prevent atherosclerotic lesion development in mice. MKP-1 protein is expressed in the atherosclerotic lesions of mice, and inhibition of tyrosine phosphatase activity and MKP-1 protein reduce atherosclerotic lesions in mouse models. Our data demonstrate that basal MKP-2 expres-
tion was elevated in L-PGDS KO VSMCs, and the addition of exogenous L-PGDS abolished any MKP-2 expression (Fig. 9A). The fact that exogenously added L-PGDS had no effect on insulin-stimulated MKP-2 expression in C57BL/6 VSMCs is probably related to the fact that the endogenous L-PGDS is at a sufficient concentration to regulate MKP-2.

Toriumi et al. (35) have demonstrated that pioglitazone treatment significantly reduced FAK phosphorylation, indicating a novel anti-inflammatory role for this compound. Protein kinase C is an important signaling protein during insulin-mediated cell spreading, and its activation is known to increase FAK-associated phosphatidylinositol 3-kinase activity (36, 37). Previously, we have demonstrated that phorbol ester-induced apoptosis is mediated by L-PGDS phosphorylation and activation by protein kinase C and is accompanied by inhibition of the phosphatidylinositol 3-kinase/protein kinase B anti-apoptotic signaling pathways (38). The observation that insulin-stimulated FAK phosphorylation was enhanced in the presence of L-PGDS leads us to believe that L-PGDS has a definite role in this process, which is absent in the L-PGDS KO strain (Fig. 9B). Furthermore, FAK also regulates insulin-stimulated activity of protein kinase B and glycogen synthase kinase-3β (39), two enzymes we have shown to be regulated by L-PGDS (6).

PTP-1B overexpression decreases glucose uptake as well as GLUT4 translocation to the plasma membrane (40). PTP-1D

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**Fig. 8. Adipocyte size.** Control C57BL/6 (*n* = 9) and L-PGDS KO (*n* = 9) male mice were sacrificed after 20 weeks on a low fat or high fat diet. Adipose tissue excised from epididymal fat pads was removed as described under "Materials and Methods" and stained with hematoxylin and eosin (A). Adipocytes were digitized at 40× magnification, and their size was quantitated with the aid of the SigmaGel program. Data are expressed as mean pixels ± S.E. (B). Asterisks (*) represent a *p* < 0.05.
Representative blots from at least three experiments are provided.

L-PGDS (50 nM) for 10 min where indicated in the absence or presence of a2h using mice deficient in L-PGDS expression. We propose that the elevation of L-PGDS in diet-induced diabetes using mice deficient in L-PGDS had low levels of PTP-1D expression which were reversed by increasing PTP-1D expression. Our data revealed that insulin stimulation led to increased VSMC PTP-1D expression in C57BL/6, which was further enhanced with exogenous L-PGDS (Fig. 9C). The L-PGDS KO VSMCs had low levels of PTP-1D expression which were reversed by exogenous L-PGDS (Fig. 9C). It appears from our signal transduction data that L-PGDS helps sensitize cells to insulin by increasing PTP-1D expression.

In this study, we have demonstrated an important role for L-PGDS in diet-induced diabetes using mice deficient in L-PGDS expression. We propose that the elevation of L-PGDS in diabetes and hypertension is a compensatory mechanism to correct insulin resistance and alleviate the cardiovascular problems associated with the diseases by suppressing vessel media overgrowth. Furthermore, we propose that the L-PGDS KO mouse represents a model of insulin resistance and diabetic nephropathy as well as fat-induced atherosclerosis.

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