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## 2 Effects of Diabetes and Insulin Resistance on Endothelial Functions

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### INTRODUCTION

Cardiovascular complications have been the leading cause of mortality and morbidity in patients with diabetes and affect a variety of tissue and organs including retina, myocardium, nerves, skin, and kidney (1–3). The incidence of coronary artery disease (CAD) in patients with diabetes or insulin-resistance syndrome is increased in subjects older than 30 years (4,5). The Framingham study, which surveyed longitudinally more than 5000 patients with 18 years of follow-up indicated that major clinical manifestation of CAD were increased in diabetic patients, especially in women (2). The risk of CAD increases with duration, reflecting the effect of the aging process, whereas in diabetic patients, both aging and duration of diabetes increased the risk of cardiac mortality: more than 50% of mortality in diabetic patients is related to cardiovascular disease (CVD). The incidence of cardiac or cerebrovascular disease is two to four times higher in diabetic patients than those of the general population (6,7).

In patients with insulin-dependent diabetes mellitus (IDDM) who were followed for 20–40 years, the mortality as a result of CAD between the age of 30 and 55 years was 33%, whereas only 8% of the men and 4% of the women had died in nondiabetic population (5). Unlike that of the general population, the risks of CAD in patients with IDDM are similar in men and women and increase at the same rate after age of 30. The incidence of CAD is also increased in noninsulin-dependent diabetes mellitus (NIDDM) and frequently occurs in families with CAD and NIDDM. Hyperinsulinemia and insulin resis-

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tance, which often precedes NIDDM, are risk factors for CAD. Several studies have shown that patients with NIDDM treated with insulin have a higher risk of CAD than those without insulin therapy, suggesting that severity of disease; loss of islet cell functions, or exogenous insulin treatment may also have an impact on CAD.

The influence of diabetes on CAD is synergistic with other factors, such as age, hypercholesterolemia, hypertension, and smoking. Additionally, diabetes itself is also an independent risk factor (2,8–10). Although the increase in cardiovascular mortality probably has several causes, one of the specific factors pertaining to diabetes in the pathogenesis of diabetic vascular complications is hyperglycemia. This is well supported by both the Diabetes Control and Complication Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS). The DCCT has clearly established that better glycemic control can prevent diabetic microangiopathy, such as retinopathy, nephropathy, and neuropathy, with improving trends observed in cardiovascular complications (11). In the UKPDS (3,12), more intensive glucose control was associated with a 12% reduction in the risk of pooled macrovascular and microvascular events. In a Japanese study, intensive insulin therapy in type 2 diabetic patients who were newly diagnosed, nonobese, and insulin sensitive (the Kumamoto trial) reduced the progression of retinopathy, nephropathy, and neuropathy, but too few events were seen to assess the impact of intensive glucose management on cardiovascular complications (13). These results suggest that glycemic control with insulin can increase the survival of diabetic patients.

The second major risk factor specific for patients with diabetes or glucose intolerance is abnormalities of insulin actions in the vascular tissues. A substantial body of evidence exists for a relationship between insulin resistance and cardiovascular morbidity and mortality, suggesting an association among insulin sensitivity, hypertension, and endothelial function (14–18). In this chapter, we first review the role of insulin resistance, hyperglycemia, and hypercholesterolemia in the vasculature and then describe cellular and functional abnormalities in endothelial cells.

Specific tissue responses or local factors are as important as systemic factors such as hyperglycemia in diabetes. The importance of tissue-specific responses or factors is demonstrated by differences in changes of vascular cells in the retina, renal glomeruli, and arteries (Table 1). In the retina, the number of endothelial cells appears to be increased, as exemplified by the formation of microaneurysms and neovascularization (19). In contrast, endothelial cells in macrovessels are injured, as shown by pathological studies leading to the initiation of acceleration of the atherosclerotic process (20,21).

## INSULIN RESISTANCE

Hyperinsulinemia and insulin resistance have been shown to increase the risk of CVDs or atherosclerosis in diabetic states, and being a potential risk factor in the development of hypertension, not only in diabetic patients but also in the general population. The mechanism by which hyperinsulinemia or insulin resistance increases the risk of atherosclerosis is still unclear. Many theories have been suggested, including insulin-induced salt retention, directly enhancing proliferation of vascular smooth muscle cells (VSMCs) (22,23), and indirectly regulating of endothelial cell homeostasis via the alteration of growth factors and cytokines in cells share extensive interaction with endothelial cells, such examples include fibroblasts, epithelial cells, VSMCs, and cardiomyocytes (24–26).

We have characterized insulin receptors on the vascular cells and reported that they are identical to those in the nonvascular cells with respect to binding, structure, and tyrosine

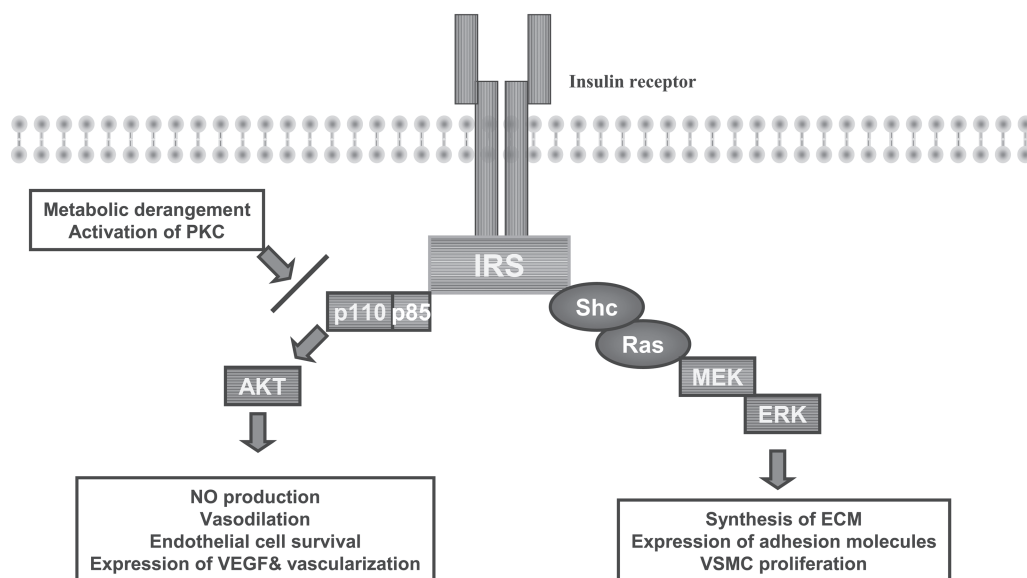
Table 1  
Alterations of Cell Numbers Observed in Various Vascular Tissues in Diabetes

	<i>Retina</i>	<i>Glomeruli</i>	<i>Macrovessels</i>
Endothelial cells	↑	↓	↓
Contractile cells	↓	↓	↑
Epithelial cells		↓	

phosphorylation activity (23,27). The insulin receptor is a member of the tyrosine kinase family, and the activation of the receptor by insulin-binding results in autophosphorylation of receptor and activation of tyrosine kinase (Fig. 1). As in other cells, insulin receptors in vascular cells can activate at least two different signal transduction pathways; one is PI 3-kinase (PI3K) cascades and the other is Ras-mitogen-activated protein (MAP) kinase cascades. These signaling processes mediate the many actions of insulin in vascular cells, such as the regulation of cell growth, gene expression, protein synthesis, and glycogen incorporation. However, insulin receptors can mediate unusual functions in endothelial cells. We have demonstrated that endothelial cells can internalize insulin via a receptor-mediated process and transport the insulin across the endothelial cell without degradation (28,29). In contrast, other types of endothelial cells, such as hepatocytes or adipocytes, will heavily degrade insulin when it is internalized. Another vascular-specific action of insulin is the activation or increased expression of nitric oxide (NO), resulting in localized vasodilation (30–33). Mice null for insulin receptor specifically in endothelial cells (VENIRKO mice) were recently established (34). Although only less than 5% of the insulin receptor mRNA expression was left in endothelial cells, these mice develop normally and did not show major differences in their vasculature as compared to their control litter mates except a mild reduction of gene expression for endothelial nitric oxide synthase (eNOS) and endothelin-1 in endothelial cells (34). However, when challenged with hypoxia, VENIRKO mice developed more than 50% reduction in retinal neovascularization (35). These results suggest that the alteration of insulin signaling might affect the expression of vascular regulators in endothelial cells and further affect vascular biology such as neovascularization.

Besides these actions, insulin has been reported to have many biological and physiological actions on vascular cells (Table 2). It is believed that hyperinsulinemia or insulin resistance can contribute to the acceleration of atherosclerosis by increasing the proliferation of aortic smooth muscle cells and the synthesis of extracellular matrix (ECM) proteins in the arterial wall (Fig. 2). However, the mitogenic actions of insulin on cells may not be significant in physiological conditions (36), because insulin can only stimulate the growth of vascular cells at concentrations greater than 10 nmol/L. Only in severe insulin-resistant or hyperinsulinemic state can the plasma level of insulin may exert its growth-promoting actions in smooth muscle cells (SMCs) by enhancing the mitogenic action of more potent growth factors, such as platelet-derived growth factor and insulin-like growth factors (37).

One of the best-characterized vascular effects of insulin is its vasodilatory action, which is mainly mediated by the production of NO (31). Baron (30) reported that blood flow to the leg increased by two fold after 4 hours of hyperinsulinemia during a euglycemic-hyperinsulinemic clamp study. With superimposed infusion of NG-monomethyl-L-arginine (L-NMMA), an inhibitor of NO synthase, into the femoral artery,

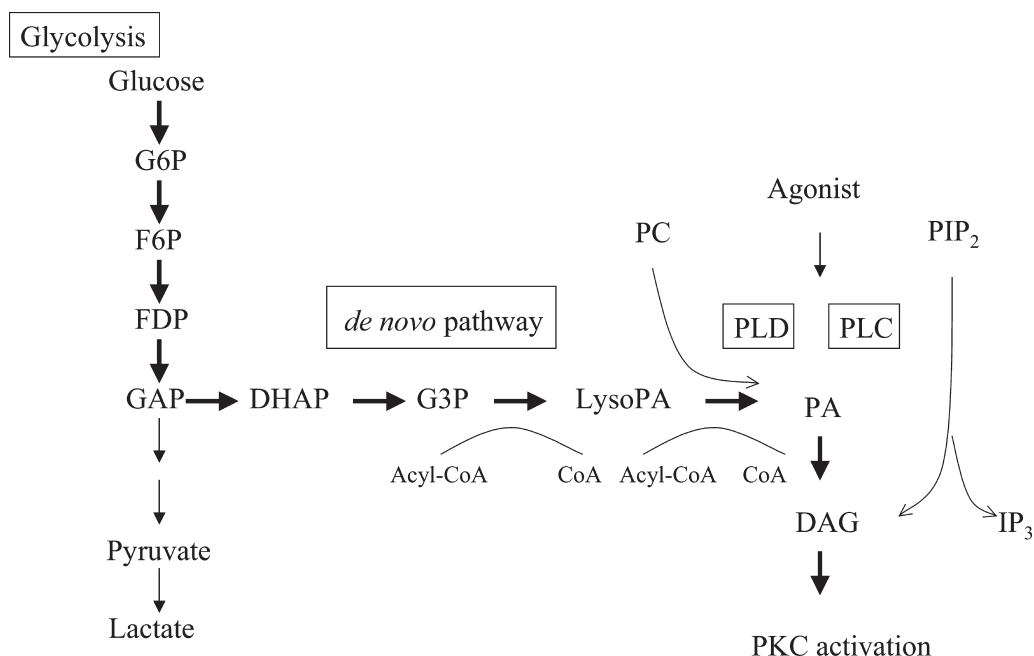


**Fig. 1.** Schematic diagram of the signaling pathways of insulin in vascular endothelial cells. Activation of either PI3K/Akt or Ras/MEK/MAP-kinase pathways can mediate most actions of insulin, with the former stimulating mainly anti-atherogenic effects, whereas the latter stimulating atherogenic actions. In diabetic or insulin-resistant states, metabolic derangements or activation of PKC has been suggested to selectively inhibit Insulin receptor-mediated activation of PI3K/Akt pathway, but spare the Ras/MEK/MAP pro-atherogenic arm of insulin's signaling cascade. This may in turn contribute to atherogenic lesion formation. IRS, insulin-receptor substrate; PI3K, phosphatidylinositol 3-kinase; MAPK, mitogen activated protein kinase.

Table 2  
List of Effects of Insulin in Vascular Cells

Glucose incorporation into glycogen
Amino acid transport
Endothelin expression
eNOS expression and activation
VEGF expression in vascular smooth muscle cells
Tyrosine phosphorylation of various proteins
Exocytosis and receptor-mediated transcytosis
Basement matrix synthesis
Increased plasminogen activator inhibitor I
c-myc, c-fos expression
Protein synthesis
DNA synthesis
Cellular proliferation

the vasodilation was completely abrogated. It has also been reported that insulin-mediated vasodilation is impaired in states of insulin-resistant states (38). Consistent with this observation, obese nondiabetic subjects often have impaired endothelium-dependent vasodilation, especially relative to the patients with type 2 diabetes (32). These findings suggest that endothelial cell dysfunction may have genetic base and is involved in the risk



**Fig. 2.** Mechanism of DAG synthesis and PKC activation in diabetes mellitus. Hyperglycemia activates the *de novo* synthesis of DAG and leads to PKC activation. Acy-CoA, acetyl-coenzyme A; CoA, coenzyme A; DAG, diacylglycerol; DHAP, dihydroxyacetone phosphate; FDP, fructose 1,6-diphosphate; F6P, fructose 6 phosphate; GAP, glyceraldehyde 3 phosphate; G3P, glycerol 3 phosphate; G6P, glucose 6 phosphate; IP<sub>3</sub>, inositol 1,4,5-triphosphate; LysoPA, lysophosphatidic acid; PA, phosphatidic acid; PC, phosphatidylcholine; PIP<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D.

of atherosclerosis in subjects with insulin resistance regardless whether they have diabetes (32).

The effect of insulin on NO production in the endothelial cells may be biphasic, with rapid and delayed components. Relative to other stimulants of NO production, insulin is rather weak, with 10 to 100 times less maximum effects than acetylcholine. However, it is possible that the delayed-positive effect of insulin on eNOS expression has an important consequence in sustaining the level of eNOS expression, which will have a general effect on all the stimulators of NO production. The mechanism of insulin's effect on NO production appears to be mediated by the activation of PI3K pathway (33). However, the acute effect appears to be an activation of eNOS, whereas the delayed effects are as a result of the upregulation of gene expression for eNOS.

Thus, in the vascular tissues, insulin has a variety of effects, which can be mediated by at least two signaling pathways involving PI3K and Ras-MAP kinase. At physiological concentrations, insulin mediates its effects through the activation of PI3K/Akt pathway, causing actions such as NO production. This effect can be interpreted as anti-atherogenic. In contrast, the effects mediated through Ras-MAP kinase pathway by insulin, for example, stimulation of ECM production; cell proliferation and migration, appears to be pro-atherogenic. The later effect requires the presence of high concentration of insulin that can be observed in insulin-resistant states. We have proposed that the increased risk of atherosclerosis in insulin-resistant states is caused by the loss of insulin's

Table 3  
Proposed Mechanisms of the Adverse Effect of Hyperglycemia

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Activation of the polyol pathway
Increases in the nonenzymatic glycation products
Activation of DAG-PKC cascade
Increases in oxidative stress
Enhanced flux via the hexosamine metabolism
Vascular inflammation
Altered expression and actions of growth factors and cytokines

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action on PI3K/Akt pathway activation and the subsequent production of NO, whereas the activation of Ras-MAP kinase pathway remain intact. In support of this theory, we have documented that the activation of PI3K/Akt pathway and eNOS expression by insulin are significantly reduced in microvessels from insulin-resistant Zucker obese rats as compared to that of the healthy lean control rats, whereas the activation of Ras-MAP kinase pathway was not affected (33,39). These results have provided a molecular explanation for the clinical findings that both insulin deficiency (as in type 1 diabetic patients) and insulin-resistant states (as in patients with metabolic syndrome and type 2 diabetes) can lead to an acceleration of CVD.

## HYPERGLYCEMIA

Hyperglycemia has been shown to be the main cause of microvascular complications in the DCCT (11) and UKPDS study (12). For cardiovascular complications, the contribution of hyperglycemia is probably also significant. Several biochemical mechanisms appear to explain the adverse effects of hyperglycemia on vascular cells (Table 3). This is not surprising because the metabolism of glucose and its metabolites can affect multiple cellular pathways. Glucose is transported into the vascular cells mostly by GLUT-1 transporters, which can be regulated by extracellular glucose concentration and other physiological stimulators, such as hypoxia (40). Once glucose is transported, it is metabolized to alter signal transduction pathways, such as the activation of diacylglycerol (DAG) and protein kinase C (PKC), or to increase flux through the mitochondria to change the redox potential (41–44). Lastly, another metabolic pathway (such as that of aldose reductase), which is normally inactive, can be used. In this review, we describe these theories and suggest that the common pathways for most of the adverse effects of hyperglycemia are mediated by alterations in signal transduction of such substances as DAG-PKC or other kinase and phosphatase.

### *Advance Glycation End-Products*

Extended exposure of proteins to hyperglycemia can result in nonenzymatic reactions, in which the condensation of glucose with primary amines forms Schiff bases. These products can rearrange to form Amadori products and advanced glycation end-products (AGE). The glycation process occurs both intracellularly and extracellularly. It has been reported that the glycation modification target to intracellular signaling molecules and extracellular structure proteins alike, and furthermore, alter cellular functions. Multiple forms of proteins subjected to glycation have been identified with N $\epsilon$ -(carboxymethyl)lysine (CML), pentosidine, and pyralline being the major form of AGEs presented in diabetic states.



A significant role for AGE in diabetic vascular complications is supported by their increased serum concentration in diabetic states (45,46). Infusion of AGE into animals without diabetes reproduces some pathological abnormalities in vasculature similar to that in diabetes (47). Furthermore, inhibition of AGE formation can partly prevent pathological changes in diabetic animals. Treatment of diabetic rats with aminoguanidine, an inhibitor of AGE formation and inducible nitric oxide synthase, can prevent the progression of both diabetic nephropathy (48) and retinopathy (49), evidenced by the reduction of albuminuria; mesangial expansion; endothelial cell proliferation; pericyte loss; and even the formation of microaneurysms. Other inhibitors of protein glycation, such as OPB-9195 (50) or ALT-711 (51) have yielded similar results in animals with diabetes.

Recently, receptor for advanced glycation end-products (RAGE) has received substantial attention in its role in endothelial cell dysfunction in diabetes, especially in the development of atherosclerosis (52). RAGE belongs to the immunoglobulin superfamily and has been reported to express in vascular cells including endothelial cells and SMCs (53). Accumulation of RAGE has been reported in the vasculature in diabetic states (46,47). Infusion of RAGE is associated with vascular hyperpermeability similar to that in diabetes and these changes can be neutralized in the presence of soluble RAGE (sRAGE) (47), the extracellular domain of RAGE that disrupt AGE–RAGE interaction. Additionally, when mice deficient for apolipoprotein (apo)E (apoE<sup>−/−</sup>) were induced to have type 1-like diabetes by streptozotocin injection, they developed much more advanced atherosclerotic lesions in their aorta as compared to the apoE<sup>−/−</sup> mice without diabetes (46) and the progression of the atherosclerotic lesion can be reversed by intraperitoneal injection of sRAGE (46). Although the molecular and cellular mechanisms underlying RAGE-induced vascular permeability change is still not fully understood, it is postulated that the induction of vascular oxidative stress (54); activation of PKC and other intracellular signaling events (55); and inflammation (56).

These results provide supportive evidence suggesting an important role for AGE formation and RAGE activation in the development of diabetic vascular complications. The AGE–RAGE axis could therefore potentially be a target for clinical interventions. Indeed, aminoguanidine is currently being evaluated in a clinical trial for its effect on the progression of nephropathy in type 2 diabetes in 599 patients across United States and Canada (57). However, majority of the results were obtained from animal studies and an affirmative role for AGE in the pathogenesis of diabetic vascular complications require further clinical evaluations.

### ***Activation of the Polyol Pathway***

Increased activity of the polyol pathway has been documented in culture studies using vascular cells exposed to diabetic level of D-glucose and in animals with diabetes (58,59). In these studies, hyperglycemia has been shown to increase the activity of aldose reductase and enhances the reduction of glucose to sorbitol, then further oxidized to fructose by sorbitol dehydrogenase. Abnormality in the polyol pathway has been suggested to cause vascular damage in the following ways: (a) osmotic damage by the accumulation of sorbitol (58); (b) induction of oxidative stress by increasing nicotinamide adenine dinucleotide phosphate (NADP)/NAD<sup>+</sup> ratio and the activation of Na<sup>+</sup>/K<sup>+</sup> adenosine triphosphate (ATP)ase (59); and (c) reduction of NO in the vasculature by decreasing cellular NADPH, a cofactor used by aldose reductase to reduce glucose to sorbitol (60). Multiple studies have shown that inhibition of aldose reductase, the key enzyme in the

polyol pathway, could prevent the some pathological abnormalities in diabetic retinopathy, nephropathy, and neuropathy (59). However, these results are not supported by data obtained from clinical trials using inhibitors of aldose reductase. A 3-year follow up of diabetic patients treated with Sorbinil (250 mg per day) failed to discern difference in retinopathy (61), although another aldose reductase inhibitor Zenarestat has been shown to improve nerve conduction in diabetic peripheral polyneuropathy (62). Based on the largely negative clinical data, a significant role for the activation of the polyol pathway in the pathogenesis of diabetic vascular complications has not been fully established.

### *Alteration in Oxidative Stress*

Increases of oxidative stress by metabolic derangement has long been reported in diabetic states and proposed to cause vascular complications (44,59,63,64). In diabetic states, induction of oxidative stress could be as a result of the increased production of superoxide anion via the induction of NADPH oxidase and mitochondrial pathway; decreases of superoxide clearance; lipid and protein modification; and the reduction of endogenous antioxidants such as ascorbic acid, vitamin E, and glutathione.

Several lines of evidence support a role of increased oxidative stress in the pathogenesis of diabetic vascular complications. Reactive oxygen species, an index of oxidative stress, has been reported to be increased and in diabetic patients with retinopathy (65) and other cardiovascular complications in the Framingham Heart Study (66) and correlate with the severity of these diseases. Furthermore, these results have been recapitulated in diabetic animals or even in vascular cells cultured in media containing high levels of D-glucose (59,64).

Induction of oxidative stress has been suggested to induce vascular dysfunctions via multiple approaches including cellular DNA damage by activating the poly(ADP-ribose) polymerase (67,68); reduction of NO bioavailability (59), and the activation of other mechanisms known to induce vascular cell damage such as AGE formation, PKC activation, and induction of polyol pathway (69). Additionally, evidence has shown that reactive oxygen species can cause severe disturbances in the regulation of coronary flow and cellular homeostasis, leading to the severe macrovascular lesions typically observed in diabetic patients after more than 10 years of disease (70,71). Inhibition of reactive oxygen species also prevent the generation of AGE products and the activation of PKC in cultured endothelial cells (69), suggesting that the auto-oxidative process plays an important role in the complex reaction cascade leading to AGE formation.

Several pathways in diabetic states, such as activation of PKC pathway, especially the  $\beta 2$  isoform (72,73); AGE formation (54), oxidized lipids (64,66), and altered polyol activity (59) can lead to the activation of NADPH oxidase or flux through the mitochondrial respiratory chain (69) to generate reactive oxygen species that further increases tissue oxidative stress. On the other hand, oxidative stress can precedes formation of some AGE, such as pentosidine and CML, and activation of the DAG-PKC pathway (74).

Although multiple studies using vascular cell in culture or diabetic animals have all supported that oxidative stress play an important role in vascular complications of diabetes. However, clinical studies have not yet provided conclusive results. The Heart Outcomes Prevention Evaluation Study (HOPE) has shown that treatment with vitamin E at a dose of 400 IU per day for a mean of 4.5 years has no apparent effect on cardiovascular outcomes in patients who had CVD or diabetes in addition to one other risk factor (75). Similarly, the MICRO-HOPE study also yielded negative results showing



Table 4  
Summary of DAG Levels and PKC Activities in Cultured Cells Exposed High Glucose Condition and Tissues Isolated From Diabetic Animals

	<i>Diacylglycerol</i>	<i>Protein kinase C</i>
Cultured cells		
Retinal endothelial cells	↑	↑
Aortic endothelial cells	↑	↑
Aortic smooth muscle cells	↑	↑
Renal mesangial cells	↑	↑
Tissues		
Retina (diabetic rats and dogs)	↑	↑
Heart (diabetic rats)	↑	↑
Aorta (diabetic rats and dogs)	↑	↑
Renal glomeruli (diabetic rats)	↑	↑
Brain (diabetic rats)	→	→
Peripheral nerve (diabetic rats)	→	→

that 400 IU per day of vitamin E failed to show difference in cardiovascular outcomes and diabetic nephropathy (76). However, we have reported that oral vitamin E treatment at a dose as high as 1800 IU per day appears to be effective in normalizing retinal hemodynamic abnormalities and improving renal function in type 1 diabetic patients of short disease duration without inducing a significant changes in glycemic control (77). At this dose, vitamin E is capable of inhibiting PKC activity (74). These results suggest that high-dose vitamin E supplementation may reduce the risks of diabetic vascular complications by antioxidant-dependent and -independent pathways. These largely inconclusive clinical results have suggested that oxidative stress play a supporting rather than central role in the pathogenesis of diabetic vascular complications.

*Activation of the DAG–PKC Pathway*

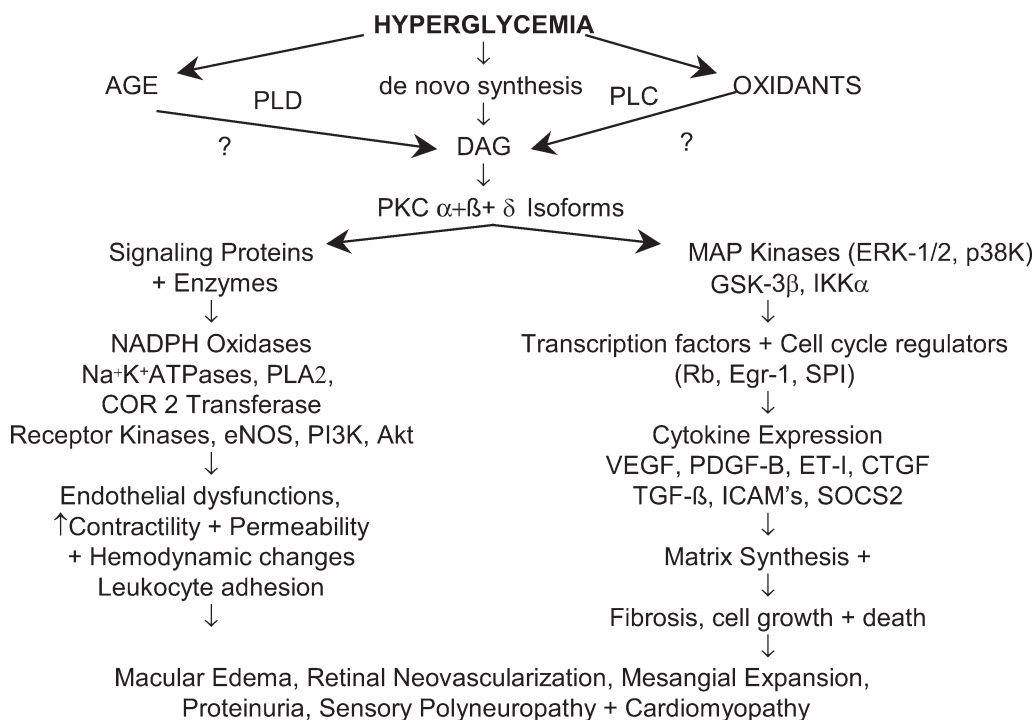
One major advance in the understanding of diabetic vascular disease is the unraveling of changes in signal transduction pathways in diabetic states. One of the best-characterized signaling changes is the activation of DAG–PKC pathway. Such activation appears to be related to elevation of DAG, a physiological activator of PKC. Increases in total DAG contents have been demonstrated in a variety of tissues associated with diabetic vascular complications, including retina (78), aorta, heart (79), renal glomeruli (80), and nonvascular tissues as in the liver (81), but not in the brain and peripheral nerves of diabetic animals and patients (Table 4). Increasing glucose levels from 5 to 22 mol/L in the media elevated the cellular DAG contents in aortic endothelial cells and SMCs (79), retinal endothelial cells (78), and renal mesangial cells (82,83). The increase in DAG–PKC reaches maximum in 3–5 days after elevating glucose levels and remain chronically elevated for many years. In fact, we have already shown that euglycemic control by islet cell transplant after 3 weeks was not able to reverse the increases in DAG or PKC levels in the aorta of diabetic rats (79). These data suggest that the activation of DAG–PKC could be sustained chronically and is difficult to reverse, similar to pathways of diabetic complications.

DAG can be generated from multiple pathways. Agonist-induced formation of DAG depends mainly on hydrolysis of phosphatidylinositol by phospholipase C (84). However, this mechanism is most likely minimally involved in diabetes, because inositol phosphate products were not found to be increased by hyperglycemia in aortic cells and glomerular mesangial cells (85,86). When the fatty acids in DAG were analyzed (87), DAG induced by high-glucose condition has predominantly palmitate- and oleic- acid-enriched composition, whereas DAG generated from hydrolysis of phosphatidylinositol has the composition of 1-stearoyl-2-arachidonyl-*SN*-glycerol (88). In labeling studies using [6-<sup>3</sup>H]- or [U-<sup>14</sup>C]- glucose, we have shown that elevated glucose increase the incorporation of glucose into the glycerol backbone of DAG in aortic endothelial cells (87), aortic SMCs (89), and renal glomeruli (90). These facts indicate that the increased DAG levels in high-glucose condition are mainly derived from the *de novo* pathway (Fig. 2).

It is also possible that DAG is produced through the metabolism of phosphatidylcholine as a result of the activation of phospholipase D (91). One potential pathway for the increase in DAG is the result of glyco-oxidation inducing activation of the DAG pathway because oxidants such as H<sub>2</sub>O<sub>2</sub> are known to activate DAG-PKC pathway (Fig. 3) (92). We have reported that vitamin E, a well-studied antioxidant, has the additional interesting property of inhibiting the activation of DAG-PKC in vascular tissues and cultured vascular cells exposed to high glucose levels (74). We have confirmed that vitamin E can inhibit PKC activation probably by decreasing DAG levels rather than inhibiting PKC, because the direct addition of vitamin E to purified PKC- $\alpha$  or - $\beta$  isoforms in vitro has no inhibitory effect (93).

PKC belongs to a family of serine-threonine kinases and plays a key role in intracellular signal transduction for hormones and cytokines. There are at least 11 isoforms of PKC and are classified as conventional PKCs ( $\alpha$ ,  $\beta$ 1,  $\beta$ 2,  $\gamma$ ); novel PKCs ( $\delta$ ,  $\epsilon$ ,  $\eta$ ,  $\theta$ ,  $\mu$ ); and atypical PKCs ( $\zeta$ ,  $\lambda$ ) (94,95). Multiple isoforms of PKC including  $\alpha$ ,  $\beta$ 1,  $\beta$ 2,  $\delta$ ,  $\epsilon$ , and  $\zeta$  are all expressed in endothelial cells (79,96). Activation of PKC has been suggested to play key roles in the development of diabetic cardiovascular complications (97).

The activation of PKC by hyperglycemia appears to be tissue-selective, because it has been noted in the retina, aorta, heart, and glomeruli but not in the brain and peripheral nerves in diabetic animals (Table 4). Among the various PKC isoforms, PKC- $\beta$  and - $\delta$  appear to be preferentially activated in the aorta and heart of diabetic rats (79) and in cultured aortic SMCs exposed to high levels of glucose (74). However, increases in multiple PKC isoforms were observed in some vascular tissues, such as PKC- $\alpha$ , - $\beta$ 2, and - $\epsilon$  in the retina and PKC- $\alpha$ ,  $\beta$ 1, and  $\delta$  in the glomeruli in the glomeruli of diabetic rats (98). Recently, we and others have shown that a number of in vivo abnormalities such as renal mesangial expansion, basement membrane thickening, blood flow, and monocyte activation in diabetic rats can be prevented or normalized using an orally effective specific inhibitor for PKC- $\beta$  isoform LY333531 (90). One of the early changes in the vasculature in diabetic states is the reduced bioavailability of endothelium-derived NO, which further aggravates endothelial dysfunctions. This process is apparently at least partly caused by the activation of PKC- $\beta$  by hyperglycemia. Beckman and colleagues applied forearm hyperglycemic clamps on fourteen healthy subjects to mimic the effects and demonstrated that endothelium-dependent vasodilation in response to methacholine chloride is decreased in hyperglycemia as compared to that in euglycemic conditions (99). The reduction of vasodilation can be normalized by oral treatment of PKC- $\beta$ -selective inhibi-



**Fig. 3.** Schematic diagram of pathways utilized by hyperglycemia to induce pathological changes in the vasculature. Hyperglycemia stimulates de novo synthesis of DAG that further activates multiple isoforms of PKC. Activation of the  $\alpha$ ,  $\beta$ , and  $\delta$  isoforms have all been reported. This will in turn affect the activity of other intracellular signaling pathways such as the Ras/MEK/MAPK, p38 MAPK and PI3K/Akt pathways. Alteration of key enzymes determining cellular homeostasis, i.e., NADPH oxidase, Na<sup>+</sup>/K<sup>+</sup>-ATPase; eNOS, COR 2 transferase has also been documented. All these changes can have profound impact on the regulation of vascular cell biology including cell cycle progression, gene expression, endothelial cell dysfunctions and hemodynamic change that constitute the cellular basis of diabetic vascular complications. PLC; phospholipase D, PLC; Phospholipase C, eNOS; endothelial nitric oxide synthase, Rb, retinoblastoma; Egr-1, early growth response-1, GSK-3 $\beta$ ; Glycogen synthase kinase-3 $\beta$ , IKK $\alpha$ ; I $\kappa$ B kinase $\alpha$ , VEGF; vascular endothelial growth factor, ANP; atrial natriuretic peptide; PDGF, platelet-derived growth factor; ET-1; endothelin-1, CTGF, connective tissue growth factor; TGF- $\beta$ , transforming growth factor- $\beta$ ; ICAM, intercellular adhesion molecules; SOCS2, suppressor of cytokine signaling.

tor LY333531 (32 mg per day) (99). These data support that the activation of PKC- $\beta$  isoform is involved in the development of some aspects of diabetic vascular complications.

For a hyperglycemia-induced change to be credible as a causal factor of diabetic complications, it has to be shown to be chronically altered, to be difficult to reverse, to cause similar vascular changes when activated without diabetes, and to be able to prevent complications when it is inhibited. So far, we have presented evidence on the DAG-PKC activation that fulfills at least three of these criteria. Clinical studies using a PKC- $\beta$  inhibitor are now in a phase II/III clinical trial to determine its usefulness in diabetic retinopathy (100) and neuropathy (101).

## DYSLIPIDEMIA

In more than half of all diabetic patients, especially those with type 2 diabetes and insulin resistance, decreases in high-density lipoprotein (HDL) cholesterol and hypertriglycemia have been reported (*102*). Increases in low-density lipoprotein (LDL) cholesterol levels are also frequently observed in diabetic patients, but such increases are more frequently in those with poor glycemic control or in parallel with hypertriglycemia. Additionally, LDLs can be modified in diabetes, as in the formation of glycated or oxidized LDLs (*103,104*), which have a decreased metabolism or are atherogenic.

Recent findings have shown that small, dense LDLs, and excess triglyceride-rich remnants, which are highly atherogenic, are increased in the insulin-resistant states (*105*). Hyperinsulinemia and central obesity, which are commonly accompanied by insulin resistance and type 2 diabetes can lead to an overproduction of very low-density lipoproteins (VLDLs) (*106*). VLDL particles contain a number of apolipoproteins and triglycerides. Increased free fatty acid and glucose levels can increase VLDL output from the liver, and elevated triglyceride levels can inhibit apoB degradation, resulting in increased secretion of VLDL. Lipoprotein lipase (LPL) activity is decreased in diabetic patients because insulin is a major regulator of LPL activity. Because LPL is necessary for the breakdown of chylomicrons and triglycerides and results in decreased clearance of VLDL, decreases in LPL activity are one of the causes for the increase in VLDLs. A decrease in LDL levels results in more triglyceride-rich particles, fewer HDL particles, and much smaller, dense LDL particles in type 2 diabetic patients. Increased VLDL levels can accelerate the atherosclerotic process in several ways: VLDLs could be toxic for the metabolism and growth of endothelial cells (*107*). Another possibility is that VLDLs from diabetic animals deposit more lipids in macrophages, which are precursors of foam cells in the arterial walls (*102*).

HDLs, which are decreased in diabetic states, reduce the inhibitory effect of LDL on endothelium-mediated vasodilation (*108*). Hypercholesterolemia increases the expression of endothelial adhesion molecules and platelets aggregability and adhesion (*109–112*), and augmenting vasoconstriction.

Small, dense LDLs, which are known to be a potent risk factor for coronary heart disease, oxidize easily and are rapidly taken up by macrophages (*113*), subsequently interacting with the endothelial cells, releasing vasoactive factors and becoming foam cells. Experimental and clinical data suggest that elevated serum levels of total and LDL cholesterol are associated with impaired endothelial functions (*114–116*). Modified (mostly oxidized) LDLs impair endothelial function more than native LDLs at similar doses, based on in vitro vasodilator responses (*116,117*). The levels of oxidized LDLs correlate better with impairment in endothelial function than cholesterol levels. Modified/oxidized LDL can affect gene expression (i.e., a decrease in eNOS expression and increase in endothelin-gene expression and production), which will promote vasoconstriction and hypertension.

Several studies have suggested that a key detrimental effect of hypercholesterolemia is to decrease NO availability (*113*). Administration of the NO precursor L-arginine restores endothelial dysfunction induced by oxidized LDLs, suggesting an impairment in NO synthesis or decreased L-arginine availability (*115,116*). In clinical studies, infusion of L-arginine can improve impaired endothelium-dependent vasodilation, including that as a result of hypercholesterolemia (*115,118*).

## CELLULAR AND FUNCTIONAL ALTERATIONS IN VASCULAR ENDOTHELIAL CELLS INDUCED BY DIABETIC STATE

### *Vascular Contractility and Blood Flow*

Hemodynamic abnormalities such as blood flow and vascular contractility have been reported in many organs of diabetic animals or patients, including the kidney, retina, peripheral arteries, and microvessels of peripheral nerves. In the retina of diabetic patients and animals with a short duration and without clinical retinopathy, blood flow has been shown to be decreased (119–123). One possible explanation for the decreased retinal blood flow in early stage of diabetes is as a result of an increase in vascular resistance at the microcirculatory level induced by PKC activation. We have reported that the decreased retinal blood flow can be mimicked by intravitreal injection of phorbol esters, which are PKC activators (78). Furthermore, decreases in retinal blood flow in diabetic rats have been reported to be normalized by PKC inhibitors (90). In addition to the retina, decreases in blood flow have also been reported in the peripheral nerves of diabetic animals by most investigators; these were normalized by PKC inhibitor, an aldose reductase inhibitor, and antioxidants respectively.

One of the possible mechanisms by which PKC activation could be causing vasoconstriction in the retina is by increased expression of endothelin-1 (ET-1). We have reported that the expression of ET-1, potent vasoconstrictor, is increased in the retina of diabetic rats and that intravitreal injection of endothelin-A receptor antagonist BQ123 prevented the decrease in retinal blood flow in diabetic rats (124). The induction of ET-1 expression could also be normalized by LY333531, a PKC- $\beta$ -selective inhibitor (125). The decrease in blood flow to the retina could lead to local hypoxia, which is a potent inducer of vascular endothelial growth factor (VEGF); this factor can cause increases in permeability and microaneurysms, as observed in diabetic retina (126,127).

Abnormalities in hemodynamic have been documented to precede diabetic nephropathy. Elevated renal glomerular filtration rate and modest increases in renal blood flow are characteristic finding in IDDM patients and experimental diabetic animals with poor glycemic control (128–131). Diabetic glomerular filtration is likely to be the result of hyperglycemia-induced decreases in arteriolar resistance, especially at the level of afferent arteriole, resulting in an elevation of glomerular filtration pressure. This effect of hyperglycemia can be mimicked in vitro by incubating renal mesangial cells with elevated glucose levels that reduced cellular response to vasoconstriction. Several reports have suggested that the activation of PKC via the induction of prostaglandins may involve in this adverse effects of hyperglycemia (132,133).

Changes in NO could also alter vascular contractility and blood flow. In the resistant vessels isolated from diabetic patients and animals, the relaxation phase after acetylcholine stimulation appears to be delayed (134–137). These impaired vascular relaxation can be restored by PKC inhibitors and mimicked by phorbol ester in normal arteries (137). The inhibition of PKC increased mRNA expression of eNOS in aortic endothelial cells (138). We have observed reduced eNOS expression in microvasculature in Zucker fatty rats, which are the model of insulin resistance (33).

Oral administration of effective specific inhibitor for PKC $\beta$  isoform LY333531 to diabetic rats for 2 weeks from the onset of the disease can normalize the retinal blood flow and glomerular filtration rate in parallel with inhibition of PKC activity (90). Similarly, the renal albumin excretion rate can be improved after 8 weeks of such treatment. These



data support the idea that the activation of PKC $\beta$  isoform is involved in the development of some aspects of diabetic vascular complications and endothelial dysfunctions.

### ***Vascular Permeability and Neovascularization***

Increased vascular permeability is another characteristic vascular abnormality in diabetic patients and animals, in which increased permeability can occur at as early as 4–6 weeks' duration of diabetes, suggesting endothelial cell dysfunctions (139). Because the vascular barrier is formed by tight junctions between endothelial cells, the increase in permeability as a result of the abnormalities in the endothelial cells. The activation PKC can directly increase the permeability of albumin and other macromolecules through barriers formed by endothelial cells, probably by phosphorylating the cytoskeletal proteins forming the intercellular junctions (140–142). Recently, PKC- $\beta$ 1 overexpression in human dermal microvascular endothelial cells has been reported to enhance phorbol ester-induced increase in permeability to albumin (143). Thus, the actions of phorbol ester and hyperglycemia in endothelial-barrier functions are mediated in part through activation of PKC- $\beta$ 1 isoform.

PKC activation can also regulate vascular permeability and neovascularization via the expression of growth factors, such as VEGF/vascular permeability factor (VPF), which is increased in ocular fluids from diabetic patients and has been implicated in the neovascularization process of proliferative retinopathy (144). We have reported that both the mitogenic and permeability-induced actions of VEGF/VPF are partly as a result of the activation of PKC $\beta$  via the tyrosine phosphorylation of phospholipase- $\delta$  (145). The use of the PKC $\beta$  selective inhibitor LY333531 can decrease endothelial cell proliferation, angiogenesis, and permeability induced by VEGF (145,146).

### ***Na<sup>+</sup>-K<sup>+</sup>-ATPase***

Na<sup>+</sup>-K<sup>+</sup>-ATPase, an integral component of the sodium pump, is involved in the maintenance of cellular integrity and functions such as contractility, growth and differentiation (147). It is well established that Na<sup>+</sup>/K<sup>+</sup>-ATPase activity is generally decreased in the vascular and neuronal tissues of diabetic patients and experimental animals (41,43,147–149). However, the mechanism by which hyperglycemia inhibits Na<sup>+</sup>/K<sup>+</sup>-ATPase activity have provided some conflicting results regarding the role of PKC. Phorbol esters have shown to prevent the inhibitory effect of hyperglycemia on Na<sup>+</sup>/K<sup>+</sup>ATPase, which suggest that PKC activity might be decreased in the diabetic condition.

However, we have reported that elevated glucose levels increased PKC and cytosolic phospholipase A2 (cPLA2) activities, resulting in increases of arachidonic acid release and prostaglandin E2 (PGE2) production and decrease in Na<sup>+</sup>-K<sup>+</sup> ATPase activity (150). Inhibitors of PKC or PLA2 prevented hyperglycemia-induced reduction in Na<sup>+</sup>-K<sup>+</sup> ATPase activities in aortic smooth muscle cells and mesangial cells. The apparent paradoxical effects of phorbol ester and hyperglycemia in the enzymes of this cascade are probably as a result of the quantitative and qualitative differences of PKC stimulation induced by these stimuli. Phorbol ester, which is not a physiological activator, probably activated many PKC isoforms and increased PKC activity by 5–10 times, whereas hyperglycemia can only increase PKC activities by twofold, a physiologically relevant change that affected selective PKC isoforms. Thus, the results derived from the studies using phorbol esters are difficult to interpret with respect to their physiological significance.



### ***Basement Membrane Thickening and Extracellular Matrix Expansion***

Thickening of capillary basement membrane is one of the early structural abnormalities observed in almost all the tissues, including the vascular system in diabetes (151). Because basement membrane can affect numerous cellular functions, such as in structure support, vascular permeability, cell adhesion, proliferation, differentiation, and gene expression, alterations in its components may cause vascular dysfunctions.

Histologically, increases in type IV and VI collagen, fibronectin and laminin and decreases in proteoglycans are observed in the mesangium of diabetic patients with nephropathy and probably in the vascular endothelium in general (152,153). These effects can be replicated in mesangial cells incubated in increasing glucose levels that were prevented general PKC inhibitors (154–156). Additionally, increased expression of transforming growth factor (TGF)- $\beta$  has been implicated in the development of mesangial expansion and basement membrane thickening in diabetes. Because PKC activation can increase the production of ECM and TGF- $\beta$ , it is not surprising that several reports have shown that PKC inhibitors can also prevent hyperglycemia- or diabetes-induced increases in ECM and TGF- $\beta$  in mesangial cells or renal glomeruli (98).

### ***Thrombosis***

The abnormalities in coagulation and platelet biology in type 2 diabetes patients are well documented (157). The development of thrombosis within the vasculature depends on the balance between procoagulant and anti-thrombotic factors, which are shifted toward thrombosis in type 2 diabetes patients (158). Plasminogen activator inhibitor (PAI-1) is produced by liver and endothelial cells and binds to the active site of both tissue plasminogen activator and urokinase plasminogen activator and neutralizes their activity (159). Thus, increased expression of PAI-1 can lead to decreased fibrinolytic activity and predispose to thrombosis. Higher insulin concentration, similar to those seen in the plasma of diabetic patients, induced accumulation of PAI-1. It was also shown that using intact anesthetized rabbits with euglycemic-hyperinsulinemic or hyperproinsulinemic cramps, insulin or proinsulin could increase PAI-1 accumulation. Insulin alone dose not have a significant effect of PAI-I expression in normal subjects. However, elevated insulin levels with an environment of increased glucose and triglycerides, which is typical of type 2 diabetic patients, elicit an insulin-dependent increase in circulating PAI-1. The PAI-1 content in atherectomy specimens from type 2 diabetes patients also has been shown to increase in normal subject.

Abnormalities in renin–angiotensin system, which are seen in diabetic patients, are one of the inducer in PAI-1 accumulation. The contribution of the renin–angiotensin system to diabetic vascular complications has been attributed mainly to an increased responsiveness of vascular tissue to angiotensin II (160). We observed that angiotensin II-induced PAI-1 and -2 expression in vascular endothelial and smooth muscle cells, which is partially dependent of PK C (161). These data suggests that the therapy for decreasing insulin resistance and improvement of glycemic control can restore the fibrinolytic response.

## **CONCLUSION**

It is likely that insulin resistance and hyperglycemia are responsible, directly or indirectly, for the abnormality of vascular endothelial functions in diabetic patients. New studies on the adverse effects of hyperglycemia have suggested that alterations in the

signal transduction pathways induced by glycation products, oxidants, and redox potentials are important mechanisms in endothelial and vascular cell functions, because it may affect both antiatherogenic and atherogenic actions. Selective impairment of insulin-signaling through the PI 3K/Akt pathway causes the blunting of insulin's antiatherogenic actions. Hyperinsulinemia, when present concomitantly with insulin resistance, may enhance insulin's atherogenic actions. Agents that can improve insulin resistance in the endothelium and inhibit the adverse effects of hyperglycemia will ultimately prevent the microvascular and cardiovascular complications of diabetes.

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