

**Invited Review****The PI3-K/Akt pathway: roles related to alterations in vasomotor responses in diabetic models**Tsuneo KOBAYASHI<sup>1,\*</sup>, Takayuki MATSUMOTO<sup>1</sup> and Katsuo KAMATA<sup>1</sup><sup>1</sup>*Department of Physiology and Morphology, Institute of Medicinal Chemistry, Hoshi University, Shinagawa-ku, Tokyo 142-8501, Japan***Abstract**

Macro- and microvascular disease states currently represent the principal causes of morbidity and mortality in patients with type I or type II diabetes mellitus. Abnormal vasomotor responses and impaired endothelium-dependent vasodilation have been demonstrated in various beds in different animal models of diabetes and in humans with type I or type II diabetes. Several mechanisms leading to endothelial dysfunction have been reported, including changes in substrate availability, impaired release of NO, and increased destruction of NO. The principal mediators of diabetes-associated endothelial dysfunction are (a) increases in oxidized low density lipoprotein, endothelin-1, angiotensin II, oxidative stress, and (b) decreases in the actions of insulin or growth factors in endothelial cells. An accumulating body of evidence indicates that abnormal regulation of the phosphatidylinositol 3-kinase (PI3-K)/Akt pathway may be one of several factors contributing to vascular dysfunction in diabetes. The PI3-K pathway, which activates serine/threonine protein kinase Akt, enhances NO synthase phosphorylation and NO production. Several studies suggest that in diabetes the relative ineffectiveness of insulin and the hyperglycemia act together to reduce activity in the insulin-receptor substrates (IRS)/PI3-K/Akt pathway, resulting in impairments of both IRS/PI3-K/Akt-mediated endothelial function and NO production. This article summarizes the PI3-K/Akt pathway-mediated contraction and relaxation responses induced by various agents in the blood vessels of diabetic animals.

Key words: diabetic model, PI3-K/Akt pathway, endothelium-dependent relaxation, contraction, NO synthase

**Introduction**

Macro- and microvascular disease states currently represent the principal causes of morbidity and mortality in patients with type I or type II diabetes mellitus (Calver *et al.*, 1992; Cohen, 1995; Hall *et al.*, 1995; Feener and King, 1997; Eckel *et al.*, 2002). Both metabolic and hormonal imbalances contribute to the pathogenesis of diabetic vascular diseases, and loss of

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the modulatory role of the endothelium may be a critical and initiating factor in the development of such diseases.

A large number of factors with roles to play in vascular biology (in particular, endothelial biology) operate by activating phosphatidylinositol 3-kinase (PI3-K)/Akt signaling, illustrating the central role of this pathway in the control vascular function. Indeed, several reports have suggested that in response to a variety of stimuli, efficient NO production requires NOS (NO synthase) phosphorylation through the PI3-K/Akt pathway (Dimmeler *et al.*, 1999; Fulton *et al.*, 1999; Zeng *et al.*, 2000). PI3-K subsequently phosphorylates Akt, which in turn phosphorylates NOS, and thereby increases NO production. Concerning vascular smooth muscle cells, several groups have presented evidence to link PI3-K activity and phosphatidylinositol -3,4,5-trisphosphate (PIP3) production to the opening of voltage-gated and receptor-coupled calcium channels (Macrez *et al.*, 2001; Northcott *et al.*, 2002; Su *et al.*, 2004). Further, it has been suggested that norepinephrine (NE) and angiotensin II (Ang II), as activators of G-protein-coupled receptors, exhibit cross-talk with PI3-K activity in human vascular smooth muscle cells (Hu *et al.*, 1996; Hafizi *et al.*, 2004).

Now, evidence is accumulating to indicate that an abnormal regulation of PI3-K/Akt may be one of several factors contributing to vascular dysfunction in diabetes. In this review, we will focus on the alterations in the vascular functions of PI3-K/Akt signaling that are present in diabetes models.

### **Endothelium-dependent relaxation in diabetic models**

Abnormalities of the modulatory roles played by the endothelium and/or smooth muscle may be critical and initiating factors in the development of diabetic vascular disease. Although an accumulating body of evidence indicates that endothelium-dependent relaxation is weaker both in a type I diabetic model, namely the streptozotocin (STZ)-induced rat (Oyama *et al.*, 1986; Kamata *et al.*, 1989a, b, 1996a,b,c, 1997; Abiru *et al.*, 1990a, b; Miyata *et al.*, 1992a,b; Tomlinson *et al.*, 1992; Poston and Taylor, 1995; Pieper, 1998; De Vriese *et al.*, 2000; Makino *et al.*, 2000, 2002; Matsumoto *et al.*, 2003, 2004; Kobayashi *et al.*, 2004c), and in type II diabetic rats (Sakamoto *et al.*, 1998; Walker *et al.*, 1999; Kagota *et al.*, 2000; Sandu *et al.*, 2000; Kim *et al.*, 2002; Witte *et al.*, 2002; Matsumoto *et al.*, 2004; Kobayashi *et al.*, 2004c), we and others have noted an augmented or unaltered endothelium-dependent relaxation at an early stage in STZ-diabetes (Brands and Fitzgerald, 1998; Pieper, 1999; Kobayashi and Kamata, 1999a, Kobayashi *et al.*, 2005b). Moreover, there is some clinical and experimental evidence of augmented blood flow at early stages of diabetes (Jaap and Tooke, 1995; Cipolla *et al.*, 1996; Kobayashi *et al.*,

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Abbreviations: ACh, acetylcholine; Ang II, angiotensin II; L-Arg, L-arginine; BH<sub>4</sub>, tetrahydrobiopterin; DOCA, deoxycorticosterone acetate; eNOS, endothelial NO synthase; ET-1, endothelin-1; GSK, glycogen synthase kinase; HDL, high density lipoprotein; HSP, heat-shock protein; IGF-1, insulin-like growth factor 1; IP<sub>3</sub>, inositol 1,4,5-trisphosphate; IRS-1, insulin receptor substrate-1; LDL, low density lipoprotein; NE, norepinephrine; NO, nitric oxide; NOS, NO synthase; PDK, PI-dependent kinase; PI3-K, phosphatidylinositol 3-kinase; PIP<sub>3</sub>, phosphatidylinositol-3,4,5-trisphosphate; PTEN, phosphatase and tensin homolog; STZ, streptozotocin; VEGF, vascular endothelial growth factor.

2003). In the type I model mentioned above, the STZ-induced diabetic rat, Pieper demonstrated an early (for 1 day) increase in endothelium-dependent relaxation, followed by a reversion phase (for 1–2 weeks), in which relaxation is normal, and then a final phase (for 8 weeks; *i.e.*, until the end of the study period) of impaired relaxation (Pieper, 1999). In a type II model, the obese Zucker rat, the aorta has been reported to exhibit either increased endothelium-dependent relaxation (Sexl *et al.*, 1995; Turner and White, 1996) or decreased reactivity (Walker *et al.*, 1999). We and others have demonstrated that both aortic endothelial dysfunction and hypertension are present in type II spontaneously diabetic (db/db) mice and in fructose-fed insulin-resistant mice (Pieper *et al.*, 1997; Kamata *et al.*, 2001). However, our recent observation that both endothelial function and NO production are impaired in aortic strips from spontaneously type II diabetic Goto-Kakizaki rats seemed to conflict with our finding that the expressions of the mRNA and protein for endothelial NO synthase (eNOS) were increased in such aortae (Kobayashi *et al.*, 2004a). Since endothelium-dependent relaxation seems to be attenuated in blood vessels in at least some diabetic models when the disease is well-established, it is possible that time-dependent changes in endothelial function may occur (*e.g.*, a two-phase influence of diabetes over endothelium-dependent relaxation).

### **Mechanisms underlying impaired endothelial function in diabetic models**

Impaired endothelium-dependent relaxation has been demonstrated in various vascular beds in different animal models of diabetes. It has been suggested that the excessive elevations in plasma glucose, insulin, low density lipoprotein (LDL) cholesterol, endothelin-1 (ET-1), Ang II, and reactive oxygen species that occur in diabetes are involved in the development of this dysfunction in several blood vessels. Indeed, a considerable body of evidence now suggests that in type I diabetes, the observed impairment of endothelial function may involve inactivation of NO by oxygen-derived free radicals (Meraji *et al.*, 1987; Hattori *et al.*, 1991; Pieper *et al.*, 1992; Tesfamariam, 1994; Keaney and Vita, 1995; Kamata *et al.*, 1996a; Ooboshi *et al.*, 1997; Kobayashi and Kamata, 1999a, 2001, 2002b). In experimental models of type I diabetes, an enhancement of NO production (which in such models is diminished by superoxide anion) and dismutation of free radicals has generally been found to improve impaired endothelium-dependent relaxation (Rubanyi and Vanhoutte, 1986; Marshall *et al.*, 1988; Kobayashi and Kamata, 2001). Convincing evidence indicates that the major enzymatic sources of vascular superoxide are NAD(P)H oxidase, xanthine oxidase, and uncoupled NOS (Landmesser and Harrison, 2001). Activation of NAD(P)H oxidase requires several steps, and the mechanisms by which Ang II stimulates this oxidase have been well studied (Cai *et al.*, 2003). Some reports suggest that a high vascular activity of angiotensin converting enzyme may promote a progressive deterioration of the cardiovascular system in STZ-induced diabetic rats (Piper *et al.*, 2000; Crespo *et al.*, 2003).

In addition, ET-1 (a vasoconstrictor peptide secreted from endothelial cell) is thought to play a pathological role in a number of vascular diseases (Goto *et al.*, 1996). Interestingly, it was recently reported that ET-1 can activate PI3-K in several cells (Ishibashi *et al.*, 2000; Kawanabe *et al.*, 2003) and that ET-1 activates NAD(P)H oxidase and induces superoxide production in cultured endothelial and smooth muscle cells (Duerschmidt *et al.*, 2000; Wedgwood *et al.*,

2001). We have shown both that the expression of the mRNA for the p22phox subunit of NAD(P)H oxidase is increased in STZ-induced diabetic aortae (Kanie and Kamata, 2002) and that this increase is normalized by the endothelin antagonist, J-104132, suggesting that ET-1 is involved in the increased formation of superoxide anions. Furthermore, we found that chronic administration of a relatively low dose of bezafibrate, which was insufficient to alter the levels of plasma lipids (including total cholesterol, LDL cholesterol, high density lipoprotein (HDL) cholesterol, and triglyceride), exerted an improvement effect on the endothelial dysfunction seen in the aorta in rats with established STZ-induced diabetes. This effect of bezafibrate may result from a reduction in the NAD(P)H oxidase p22phox subunit through an inhibitory effect on ET-1 synthesis (Kanie *et al.*, 2003).

Within the last few years, it has been suggested that depletion of L-arginine (L-Arg)/tetrahydrobiopterin (BH<sub>4</sub>) and high concentrations of glucose may reduce NO release by stimulating eNOS-dependent O<sub>2</sub><sup>-</sup> generation in the endothelium (Pieper, 1997, 1998; Hink *et al.*, 2001). Indeed, acute administration of L-Arg or BH<sub>4</sub>, as a co-factor for NO, normalizes the defective cyclic-GMP production and relaxation response to acetylcholine (ACh) seen in diabetes (Pieper, 1998). Thus, the impaired vasorelaxation seen in the diabetic state may be, at least in part, due to the formation of O<sub>2</sub><sup>-</sup> through NO synthase.

Oxidative modification of LDL cholesterol is thought to be an important step in the alteration of a variety of endothelial functions (Kugiyama *et al.*, 1990; Flavahan, 1992). Indeed, the elevation in the circulating modified LDL level seen in diabetes is one of the factors responsible for the endothelial dysfunction present in this disease (Clarkson *et al.*, 1996). Chronic administration of pravastatin produces an improvement in the endothelial dysfunction shown by aortae isolated from STZ-induced diabetic rats without lowering plasma cholesterol, an effect of pravastatin that may be due to decreased LDL oxidation (Kobayashi *et al.*, 2000). Further, we reported a few years ago that following incubation of aortae from control rats with LDL isolated from diabetic rats, (a) endothelium-dependent relaxation was impaired and (b) this inhibitory effect was prevented by superoxide dismutase, a scavenger of O<sub>2</sub><sup>-</sup> (Kobayashi *et al.*, 2000). These results suggest that the increased LDL level present in diabetic rats may cause a greater production of O<sub>2</sub><sup>-</sup>. Thus, it has been suggested that the excessive elevations in plasma glucose, LDL cholesterol, ET-1, and Ang II that occur in diabetic models may be involved in the development of this dysfunction in several blood vessels, possibly via a greater production of O<sub>2</sub><sup>-</sup>.

### **Alteration of vascular contractility in diabetes**

Abnormal functioning of the vascular smooth muscle cell has also been implicated as one of the mechanisms underlying vascular disease in diabetes. Many studies from different laboratories have demonstrated that vascular responsiveness to NE is altered in some way in experimental diabetes; however, the results have not always been consistent. For example, experimentally induced diabetes has been reported to depress (Pfaffman *et al.*, 1982; Cameron *et al.*, 1992), enhance (Kamata *et al.*, 1988; Taylor *et al.*, 1994), or have no effect (Mulhen and Docherty, 1989; Fulton *et al.*, 1991; Taylor *et al.*, 1994) on the  $\alpha_1$  agonist-induced contraction of rat arteries. The reasons for these discrepancies are not apparent, but may be attributable to

differences in the species used, the duration of the diabetes, the dose of diabetogen used, and the vascular preparation studied.

The elevation of the plasma insulin level in diabetes has long been thought to contribute to the pathogenesis of this condition (Reaven, 1991; Standly *et al.*, 1993; Hall *et al.*, 1995; Abe *et al.*, 1998). Since a high plasma concentration of insulin reportedly leads to an increased firing rate in sympathetic nerves and to enhanced NE release, an increase in blood pressure may result (Gans *et al.*, 1991; Tack *et al.*, 1996). One of the possibilities raised by *in vitro* studies is that in diabetes, the hyperinsulinemia may result in an increased sensitivity of blood vessels to vasoconstrictors such as Ang II or catecholamines (Gans *et al.*, 1991; Hall *et al.*, 1995; Kobayashi *et al.*, 1999b). Although both insulin and insulin-like growth factor 1 (IGF-1) reportedly increase  $\alpha_1$ -adrenoceptor expression via activated PI3-K/tyrosine protein kinase in rat vascular smooth muscle cells (Hu *et al.*, 1996), there is evidence that the high insulin levels found in patients with insulinomas do not cause hypertension or atherosclerosis (Hall *et al.*, 1995).

A few years ago, we showed that in aortae from rats with STZ-induced type I diabetes, insulin treatment can enhance NE-induced contractility (and presumably blood pressure) (Kobayashi *et al.*, 1999b). An enhancement of IGF-1-receptor expression within endothelial cells has been observed in diabetic models, and may be causally related to the potentiation of vascular contractility (Kobayashi *et al.*, 2003, 2005a). Many of the metabolic and vasomotor effects of insulin and IGF-1 are mediated by activation of PI3-K. A perturbation of the activity in the IGF-1 system, such as occurs in diabetes, may play an important role in the enhancement of vascular contractile function and hypertension observed in syndromes involving hyperinsulinemia.

### **PI3-K/Akt pathway in endothelium-dependent relaxation**

In endothelial cells, the main signal-transduction pathway for agonist-stimulated eNOS activation depends on  $\text{Ca}^{2+}$ /calmodulin/caveolin. At the cellular level, there is growing evidence that for some agonists, such as acetylcholine, histamine, and bradykinin (Peach, 1985; Moncada *et al.*, 1997; Kamata and Nakajima, 1998), a rise in intracellular  $\text{Ca}^{2+}$  is necessary for NO production. In contrast, with other forms of stimuli, such as fluid shear stress (Kuchan and Frangos, 1994), estrogen (Caubin-Glaser *et al.*, 1997), and insulin/IGF (Tsukahara *et al.*, 1994), a rise in  $\text{Ca}^{2+}$  is not required for NO production. Many stimuli [including insulin, vascular endothelial growth factor (VEGF),  $\beta$ -agonists, adrenomedullin and shear-stress signals] have been reported to regulate NO production by phosphorylation of eNOS, which facilitates association of the enzyme with calmodulin, thus reducing its inhibitory interaction with caveolin-1 (Dimmeler *et al.*, 1999; Fulton *et al.*, 1999; Zeng *et al.*, 2000; Luo *et al.*, 2000; Nishimatu *et al.*, 2001). The typical route of PI3-K/Akt/NOS activation involves mediation via tyrosine kinase receptors, such as receptors for insulin or growth factor. Upon stimulation, an agonist activates receptor kinase functions, thereby enhancing tyrosyl phosphorylation of some substrates, such as insulin receptor substrate 1 (IRS-1) (Sun *et al.*, 1991), IRS-2, and Shc proteins (Sun *et al.*, 1995). Tyrosyl phosphorylation of IRSs provides binding sites for specific proteins containing SH2 domains, including the 85kDa regulatory subunit of PI3-K (Burgering and Coffey, 1995).

Activation of PI3-K stimulates its lipid kinase activity via activation of the p110 catalytic subunit, resulting in the addition of phosphate in the D3 position of phosphatidylinositol (PI) and the production of PI3P. The pleckstrin homology domain of Akt kinases has an affinity for PI3P, and the binding of PI3P [including heat-shock protein (HSP) 90, and phosphatase and tensin homolog (PTEN)] triggers Akt translocation to the plasma membrane (Datta *et al.*, 1996; Bellacosa *et al.*, 1998; Stambolic *et al.*, 1998; Fontana *et al.*, 2002). Furthermore, the increasing levels of phosphoinositides function as intracellular second-messenger molecules leading to activation of PI-dependent kinase (PDK). PDK activates Akt, following its membrane translocation, by phosphorylation of threonine 308 and serine 473 (Alessi *et al.*, 1997; Bellacosa *et al.*, 1998). The activated Akt then activates eNOS by serine-1177/1179 phosphorylation (Luo *et al.*, 2000). Several studies have suggested that the vasorelaxation induced by insulin or IGF-1 is most likely mediated by the production of vascular NO via the PI3-K/Akt pathway (Walsh *et al.*, 1996; Kuboki *et al.*, 2000; Kobayashi *et al.*, 2004b). A recent study in our laboratory showed that addition of a PI3-K or Akt inhibitor did not significantly alter either ACh-induced relaxation or NOx/cyclic-GMP production in control aortae, whereas the clonidine- and insulin-induced responses were completely abolished by each inhibitor in such aortae (Kobayashi *et al.*, 2004b). A study by Isenovic and coworkers (Isenovic *et al.*, 2002) suggested that the wortmannin-sensitive p85 regulatory subunit of PI3-K is involved in the isoproterenol-mediated relaxation of aortic rings via stimulation of eNOS activity. Thus, the PI3-K/Akt pathway may play pivotal roles in the vascular endothelium, such as regulation of eNOS activities, NO production, and endothelium-dependent-relaxation.

### **PI3-K/Akt in vascular contractility**

Class I PI3-K is a multifunctional, heterodimer enzyme composed of a catalytic subunit (p85 $\alpha$ ,  $\beta$ , p55 $\gamma$ , or p101) and a regulatory subunit (p110 $\alpha$ ,  $\beta$ ,  $\delta$ , or  $\gamma$ ) (Kurosu *et al.*, 1997; Maier *et al.*, 1999; Foukas *et al.*, 2002). Most attention has focused on the pathways involved in cell growth and migration (Fruman *et al.*, 1998; Rameh and Cantley, 1999; Vanhaesebroeck *et al.*, 2001), but within the last few years PI3-K activity has been shown to be implicated in Ca<sup>2+</sup> signaling and vascular smooth muscle contractility. Indeed, PI3-K has been proposed to exert an indirect modulating effect on inositol 1,4,5,-triphosphate (IP3) receptors and Ca<sup>2+</sup> release-activated Ca<sup>2+</sup> channels in nonexcitable cells (Scharenberg and Kinet, 1998). A noncapacitative Ca<sup>2+</sup> influx is also regulated by PIP3 in RBL-2H3 cells (Ching *et al.*, 2001). In excitable cells, PI3-K stimulates voltage-gated Ca<sup>2+</sup> channels (Blair and Marshall, 1997; Viard *et al.*, 1999). Moreover, Macrez and coworkers have suggested that the lipid product of PI3-K, PIP3, is necessary and sufficient to transduce hormone-activated and PI3-K-dependent regulation of Ca<sup>2+</sup> entry through voltage-gated Ca<sup>2+</sup> channels (Macrez *et al.*, 2004).

Concerning contractile responses, several studies have demonstrated that an inhibition of PI3-K reduces force in vascular smooth muscle in response to either membrane depolarization or receptor activation (Komalavilas *et al.*, 2001; Northcott *et al.*, 2002; Su *et al.*, 2004). Indeed, Su and colleagues suggested that PI3-K performs at least two distinct roles in the regulation and/or modulation of force (Su *et al.*, 2004). The first is by a minor component, whereby PI3-K

increases the influx of  $\text{Ca}^{2+}$  through receptor-operated  $\text{Ca}^{2+}$  channels, resulting in an increase in MLC phosphorylation levels. The second major pathway is potentially protein kinase C-dependent, and enhances force independently of changes in either  $\text{Ca}^{2+}$  or myosin light chain (MLC) phosphorylation levels. PI3-K has also been implicated in the pathology of hypertension-induced vascular hypersensitivity and ethanol-induced cerebral contractions (Yang *et al.*, 2001; Northcott *et al.*, 2002). Watts and coworkers reported that an enhancement of PI3-K activation seems to underlie the increase in spontaneous vascular tone observed in both deoxycorticosterone acetate (DOCA)-salt and N-nitro-L-Arg hypertensive rats (Northcott *et al.*, 2002, 2004). They noted that a specific inhibitor of p110 $\delta$ , IC87114, inhibited the spontaneous aortic tone observed in DOCA-salt rats in a concentration-dependent manner. Moreover, PI3-K activity and PI3-K protein expression, specifically that of the p110 $\delta$  subunit, was upregulated in aortae from DOCA-salt hypertensive rats (compared with normotensive sham animals), suggesting that such upregulation may explain the PI-3K-mediated spontaneous tone that develops in that model.

Whether downstream kinases known to be regulated by PI3-K [specifically PDK1, Akt, glycogen synthase kinase (GSK)-3, and Rho A] are involved in setting vascular contractile tone remains. A serial connection between PI3-K and activation of Rho A activity in smooth muscle function has been suggested by Miao and coworkers, who demonstrated that PI3-K is involved in the activation of RhoA by ET-1 in the rabbit basilar artery (Miao *et al.*, 2002). In their study, PI-3K was suppressed by an inhibitor, LY294002, before ET-1 stimulation, and this inhibition resulted in decreased RhoA activation, suggesting that RhoA is a downstream effector of PI3-K. Wang and Bitar (1998) proposed a role for PI3-K in the activation of Rho and enhancement of force, but interestingly they suggested that the end-point might involve HSP27 and cytoskeletal remodeling. These findings are in contrast to work presented by Begum *et al.* (2000), who demonstrated that PI3-K-dependent inactivation of Rho kinases results in an increase in MLC phosphatase activity, and therefore in a decrease in MLC phosphorylation levels. Reif and colleagues (Reif *et al.*, 1996) analyzed the activation of Rho-related signaling associated with upstream activation of PI3-K, and concluded that PI3-K stimulation was not sufficient to activate Rho-mediated responses, such as stress-fiber and focal-adhesion formation. Interestingly, although PI3-K activity is increased in DOCA-salt hypertension, there is not an associated increase in the phosphorylation of Akt/GSK or in the activation of Rho kinase, indicating that another downstream effector of PI3-K is involved in this model (Loberg *et al.*, 2003; Wehrwein *et al.*, 2004).

The issue of the associations between PI3-K and other pathways is complex, because different tissues under different conditions yield seeming by contradictory results. However, it is possible that the PI3-K and other pathways converge further downstream to activate a common effector, such as a  $\text{Ca}^{2+}$  channel. Be that as it may, the mechanisms underlying the changes in the PI3-K pathway that are associated with the enhanced vascular contractility seen in diabetes are still not understood.

### PI3-K/Akt pathway and endothelial dysfunction in type II diabetes

Numerous epidemiological studies have indicated that the insulin resistance and hyperinsulinemia associated with type II diabetes make important contributions to the development of hypertension and cardiovascular diseases; and moreover impaired endothelium-dependent vasodilation has been described both in humans with type II diabetes and in animal models of the disease (Eckel *et al.*, 2002). In studies of insulin resistance using the euglycemic hyperinsulinemic clamp technique, skeletal muscle tissue accounts for the majority of whole-body glucose uptake (Sower, 2004). Normally, there is a close relationship between insulin-mediated glucose disposal and the incremental increase in blood flow in response to insulin. This normal response is lost in insulin-resistant states, suggesting a resistance to the action by which insulin induces vascular NO production (Steinberg *et al.*, 1996). Shinozaki and coworkers (Shinozaki *et al.*, 1999) have suggested that it may be insulin resistance, rather than the hyperinsulinemia itself, that is a pathogenic factor for decreased vascular relaxation in diabetes (through impaired eNOS activity and increased oxidative breakdown of NO due to an enhanced formation of superoxide, which is caused by a relative deficiency of BH<sub>4</sub> in vascular endothelial cells). IRS-1 has been identified as a major substrate of the tyrosine kinases of both the insulin receptor and the IGF-1 receptor (White *et al.*, 1985). Abe and coworkers (Abe *et al.*, 1998) previously showed that IRS-1-deficient mice exhibit insulin resistance, hypertension, and impaired endothelium-derived relaxation. Furthermore, a comparison of endothelium-dependent relaxation responses among IRS-1-deficient, IRS-2-deficient, and wild-type mice (Kubota *et al.*, 2003) revealed that relaxation was impaired in the IRS-2-deficient mice at 20 weeks, but not at 8 weeks. In addition, in the IRS-1-deficient mice there was a mild impairment of endothelium-dependent vascular relaxation at 20 weeks. These results suggest that insulin resistance plays an essential role in the endothelial dysfunction seen in mice lacking IRS-1/2.

To try to explain the modulation of the vasomotion on effects of insulin in type II diabetes, Jiang and colleagues studied aortae and microvessels from Zucker-lean and -obese diabetic rats (Jiang *et al.*, 1999). Jiang *et al.* (1999) found that both vascular insulin-induced phosphorylation and activation of the components of insulin signaling from the receptor level downstream to Akt were blunted in these obese insulin-resistant rats. Our recent observation that both endothelial function and NO production are impaired in aortic strips from spontaneously type II diabetic Goto-Kakizaki rats seemed to conflict with our finding that the expressions of the mRNA and protein for endothelial NO synthase (eNOS) were increased in such aortae (Kobayashi *et al.*, 2004a). However, a possible explanation may be that in the intact cells, NO synthesis is regulated independently of changes in eNOS enzyme activity. The most recent observations made in the our laboratory (Kobayashi *et al.*, 2004b) compared aortae from nicotinamide-STZ-induced type II diabetic mice with those from age-matched controls. Our findings were that (a) the diabetes altered neither ACh-induced relaxation nor ACh-induced NO/cyclic-GMP production (which are not mediated via the PI3-K/Akt signal pathways), while (b) the relaxations and NO/cyclic-GMP productions induced by clonidine or insulin were much weaker [passively, due to reductions in both Akt phosphorylation and Akt protein expression (but not to a decline in PI3-K activities)], in this type of diabetes. These results suggest that endothelium

derived relaxation responses and NO production mediated via the PI3-K/Akt pathway are decreased in this type II diabetic model. Chronically in type II diabetes, a lack of insulin sensitization of the endothelial component of Akt signaling may contribute to a decrease in endothelial function, and hence to the progress of hypertension.

The impairments of Akt activity and Akt expression observed in type II diabetic mice may be secondary to such insulin resistance. It is unclear, however, which missing action of insulin in endothelial cells might be responsible for decreasing the Akt expression level in the diabetic aorta, and indeed the effect might be initiated by changes in the plasma glucose level or in the level of any of several hormones, including insulin. Clinically, the high insulin levels found in patients with insulinomas apparently do not cause hypertension or atherosclerosis (Hall *et al.*, 1995). We have demonstrated, in fructose-fed insulin-resistant mouse models that exhibit normal glucose and high insulin levels in the plasma, that the nitric oxide formation mediated by endothelial  $\alpha_2$ -adrenoceptors is increased (Kamata *et al.*, 2001). Thus, hyperinsulinemia alone seems to be insufficient to cause insulin resistance in endothelial cells. Actually, in cultured bovine endothelial cells, high glucose levels inhibit eNOS phosphorylation at serine-1177, the Akt phosphorylation site (Du *et al.*, 2001). We suspect that the relative deficiency in the action of insulin and the hyperglycemia operate together to cause impairments of Akt activity and Akt expression in type II diabetic mice, resulting in impairments of endothelial function and NO production.

### **PI3-K/Akt pathway and endothelial dysfunction in type I diabetes**

Since the major pathway for insulin signaling in type I diabetes is the PI3-K/Akt pathway, as it is in type II diabetes, it is important to consider the possibility that the dose of insulin administered to type I diabetic models may lead to PI3-K/Akt activation and insulin resistance. The administration of various dose of exogenous insulin to treat the disease may lead to variable periods of hyperinsulinemia and variable levels of PI3-K/Akt activities. Indeed, systemic hyperinsulinemia is inevitable during the insulin treatment of type I diabetes mellitus, and it may play an important role in the progression of coronary artery disease (Reaven, 1991; Snell-Bergeon *et al.*, 2003). In contrast, in major experimental STZ-induced type I diabetic models, the absolute lack of insulin may result in lower basal Akt activities. We found that although the ACh-induced relaxation was impaired, the concentration-dependent relaxations induced by IGF-1 and insulin, which are also endothelium-dependent, were enhanced in such type I diabetic rats (Kobayashi and Kamata, 2002a). Further, the mRNA for the aortic IGF-1 receptor was increased in these diabetic rats and further increased in insulin-treated diabetic animals. This is supporting evidence for an increased PI3-K/Akt pathway in both insulin-deficient STZ-diabetes and insulin-treated diabetes. The above effects may be made possible as a result of the increases in IGF-1 receptors that occur in the aorta in both long-term insulin deficiency and systemic hyperinsulinemia.

However, the lack of insulin in experimental type I diabetes may result in lower baseline PI3-K/Akt activities. Indeed, STZ-induced diabetic rats demonstrate baseline reductions in cardiac Akt activity (Dobrzynski *et al.*, 2002). In the myocardium of STZ-diabetic rats, systemic

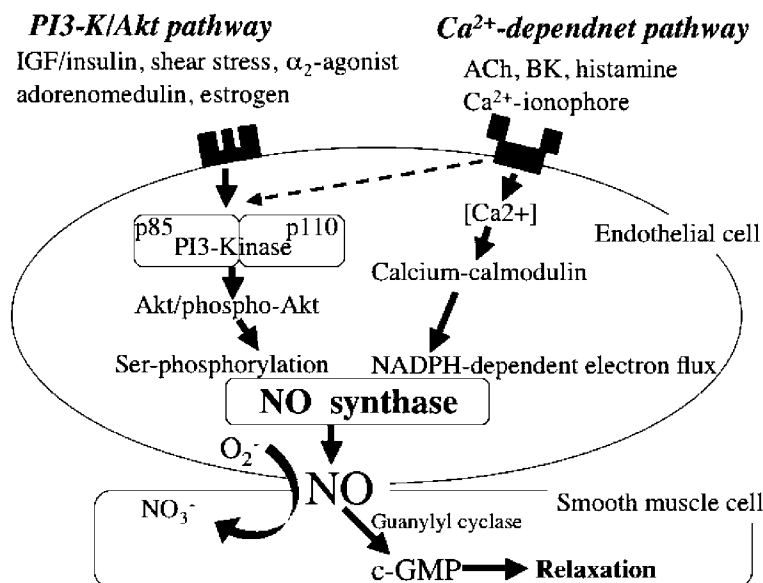
insulin administration results in an enhanced activity of most of the components of the PI3-K/Akt signaling cascade, including an increase in Akt phosphorylation (Laviola *et al.*, 2001). However, baseline Akt phosphorylation of serine-473, although not of threonine-308, and Akt activity (as determined by *in vitro* assay) showed reductions in STZ-diabetic rats (Laviola *et al.*, 2001). Gerhardinger and coworkers (Gerhardinger *et al.*, 2001) found that STZ-diabetic rats given low-dose insulin treatment tended to exhibit enhanced retinal Akt phosphorylation. We found that in STZ-diabetic rats, short-term insulin treatment can ameliorate endothelial dysfunction by inducing overexpressions of eNOS and/or VEGF mRNAs, possibly via IGF-1 receptors (Kobayashi and Kamata, 2002a). Actually, it had been reported previously that administration of VEGF results in improved endothelium-dependent responses in relatively large collateral vessels in normal rabbits (Bauters *et al.*, 1995). Thus, insulin treatment presumably leads to an increase in PI3-K/Akt activity, and is enhancement presumably increases NO production, thereby resulting in an amelioration of the endothelial dysfunction otherwise seen in type I diabetic models.

### Summary and Perspectives

There is persuasive evidence suggesting that defects in PI3-K/Akt signaling occur during the development of the insulin resistance seen in type II diabetes. Most attention has focused on the pathways involved in cell growth and migration, but within the last few years the PI3-K/Akt pathway has been shown to be implicated in vascular smooth muscle contractility, endothelium-derived relaxation, and the setting of blood pressure levels. Similar defects have been reported in the endothelium in several tissues in type II diabetic models, possibly contributing to the disturbances of NO production and endothelium-derived relaxation (Fig. 1). In vascular smooth muscle cells, establishing the role of diabetic alterations in PI3-K/Akt signaling remains a major future challenge for research in this area.

PI3-K activity is complex, because PI3-K comprises a p85 monomer, p85-p85 dimers, p85-p110 heterodimers etc. that are regulated in such a way as to alter PI3-K activity in response to an insulin signal (Kurosu *et al.*, 1997; Maier *et al.*, 1999). Mammalian genomes contain three Akt genes (Akt1, Akt2, and Akt3), and these encode three widely expressed isoforms of Akt kinase. PDKs activate Akt, and whereas phosphorylation of threonine 308 is necessary for Akt activation, serine 473 phosphorylation is required as well for maximal activity. It is unknown, however, whether complex abnormalities of the p85/p110 subunits and of the phosphorylation of Akt subunits contribute to the disturbances of both PI3-K/Akt activity and vascular responses observed in diabetic models. In recent years, evidence has been produced to suggest that an increase in the p85 monomer and a decrease in the p85-p110 heterodimer may play a role in negative PI3-K regulation as part of the insulin response (Anai *et al.*, 1998; Mauvais-Jarvis *et al.*, 2002). Since PI3-K-associated hyperactivity in the vascular wall might be responsible for alterations in the p85/p110 complex, we shall need to conduct further studies to examine the effects of the p85/p110 complex in diabetic models, focusing on the vascular dysfunctions seen in association with hyperinsulinemia and insulin deficiency.

Interestingly, it has been suggested that NE and Ang II etc., as activators of G-protein-



**Fig. 1.** Endothelium-dependent relaxation via PI3-K/Akt and Ca<sup>2+</sup>-calmodulin pathway. Relaxation responses induced by some agonists, such as acetylcholine, histamine and bradykinin need an increase in intracellular Ca<sup>2+</sup> for NO production. Contrast to this, relaxation responses induced by fluid shear stress, estrogen, adrenomedullin,  $\alpha_2$ -agonist and insulin/IGF do not need an increase in intracellular Ca<sup>2+</sup> for NO production, and are linked to PI3-K/Akt pathway. In response to various stimuli, PI3-K phosphorylates Akt, the phosphorylated Akt phosphorylates eNOS, resulting in NO production. It is likely that in type II diabetic model, the activity of PI3-K/Akt pathway is decreased, and thus endothelium-derived relaxation responses are impaired. ACh, acetylcholine; Akt, serine/threonine protein kinase; BK, bradykinin; IGF, insulin-like growth factor; PI3-K, phosphatidylinositol 3-kinase; p85, a catalytic subunit of PI3-K; p110, a regulatory subunit of PI3-K.

coupled receptors, exhibit cross-talk with PI3-K activity in human vascular smooth muscle cells (Hu *et al.*, 1996; Hafizi *et al.*, 2004). Cross-talk regulation of PI3-K activity by Ang II (although admittedly the biological actions of Ang II on PI3-K activities remain controversial) could have a important role to play in the development of insulin resistance (Henriksen *et al.*, 2001; Juan *et al.*, 2005). HMGCoA-inhibitors, the statins act via NO-dependent, proangiogenic, and antithrombotic mechanisms (Dimmerler *et al.*, 2001), and there is convincing evidence to implicate Akt in these processes. Thiazolidinediones act as agonists of peroxisome proliferators-activated receptors  $\gamma$  (PPAR $\gamma$ ), stimulation of which enhances Akt phosphorylation, as has been described in human skeletal muscle biopsies, and this is linked to the beneficial effects of these agents on insulin sensitivity (Jing *et al.*, 2002). In endothelial cells, prolonged treatment with thiazolidinediones increases NO production via Akt-mediated phosphorylation of NOS (Chao *et al.*, 2004). Future directions for research include addressing the mechanisms by which PI3-K/Akt activities might interact with isoforms of PI3-K and Akt, crosstalk between the PI3-K/Akt pathway and the other PI3-K/Akt activity systems, dimer enzyme composed of a PI3-K, phosphorylation site of Akt.

In conclusion, we believe that manipulation of PI3-K/Akt signaling activities within the vascular system may have considerable therapeutic potential. Undoubtedly, insights gained from basic research will lead to novel and pertinent clinical research targeted at the prevention and/or therapy of the vascular diseases associated with diabetes.

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