Activation of the PDK-1/Akt/eNOS pathway involved in aortic endothelial function differs between hyperinsulinemic and insulin-deficient diabetic rats

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Kobayashi T, Taguchi K, Nemoto S, Nogami T, Matsumoto T, Kamata K. Activation of the PDK-1/Akt/eNOS pathway involved in aortic endothelial function differs between hyperinsulinemic and insulin-deficient diabetic rats. Am J Physiol Heart Circ Physiol 297: H1767–H1775, 2009. First published August 28, 2009; doi:10.1152/ajpheart.00536.2009.—In diabetic states, altered plasma insulin is likely to play key roles in 3-phosphoinositide-dependent protein kinase (PDK)/Akt pathway activation, in insulin resistance and in endothelial dysfunction. Since the molecular mechanism(s) remains unclear, we examined the relationship between the PDK/Akt/endothelial nitric oxide synthase (NOS) pathway and endothelial function in aortas from diabetic rats that were either insulin deficient or hyperinsulinemic. Untreated diabetic (diabetic) rats exhibited hyperglycemia and hypoinsulinemia, whereas high-insulin-treated diabetic (HI-diabetic) rats exhibited hyperinsulinemia. Aortas from the diabetic group displayed impaired endothelium-dependent relaxation in response to ACh, whereas the insulin-induced relaxation was increased. In HI-diabetic aortas, the ACh-induced relaxation was normal, but that induced by insulin was impaired. The insulin-induced relaxation was inhibited by treatment with an Akt inhibitor in control and diabetic aortas, but not in the HI-diabetic aorta. This inhibitory effect on insulin-induced relaxation was greater in diabetic aortas than in control aortas. In all groups, ACh-induced relaxation was unaffected by the above inhibitor. In the diabetic group, various insulin-stimulated levels (nitric oxide production, phosphorylation of endothelial NOS at Ser1177, of Akt at Thr308, and of PDK-1 at Ser241) were significantly increased, whereas, in the HI-diabetic group, these levels were all decreased (vs. control aortas). These results suggest that the plasma insulin level has a close relation to the level of aortic PDK-1/Akt (at Thr308)/NOS activities, and that reduced actions of the PDK-1/Akt (at Thr308) signal pathway may contribute to the impairments of insulin-induced endothelial functions seen in hyperinsulinemic diabetes.

hyperinsulinemia; diabetes; nitric oxide; 3-phosphoinositide-dependent protein kinase

Numerous epidemiological studies have indicated that the hyperglycemia or hyperinsulinemia associated with diabetes makes important contributions to the development of hypertension and cardiovascular diseases (4, 8, 12, 41). Many of the vascular complications seen in type 1 and type 2 diabetes arise from a hyperglycemia that cannot be completely prevented using the methods of blood-glucose control available today (9, 34, 36). Indeed, systemic hyperinsulinemia is inevitable during the insulin treatment of type 1 diabetes mellitus, and it may play an important role in the progression of coronary artery disease (37, 40). One possibility raised by previous in vitro studies is that the hyperinsulinemia may result in hypertension, an increased sensitivity of blood vessels to vasoconstrictors such as catecholamines (18, 37, 40), and impaired endothelium-dependent vasodilation [which has been described both in humans and in animal models of the disease (8, 19, 20)].

In addition to the well-known Ca2+/calmodulin-dependent activation of endothelial nitric oxide (NO) synthase (eNOS), a variety of stimuli [including insulin, vascular endothelial growth factor (VEGF), α-agonists, β-agonists, thrombin, and shear-stress signals] can reportedly induce efficient NO production via eNOS phosphorylation through the Akt pathway (6, 16, 20, 32, 49). Activation of phosphoinositide 3-kinase (PI3K) leads to Akt phosphorylation at Thr308 and Ser473 (29, 43). Akt phosphorylation at Thr308 is mediated by phosphoinositide-dependent kinase (PDK)-1 (29, 43), whereas Akt phosphorylation at Ser473 is mediated by the rictor-mammalian target of rapamycin, namely PDK-2 (39). However, it is not known how (or whether) the regulation of PDK/Akt phosphorylation is altered in the state of endothelium dysfunction seen in diabetic models.

Since the major pathway for insulin signaling is the PI3K/PDK/Akt pathway, it is important to consider the possibility that the altered plasma insulin levels present in diabetic models might lead to changes in PI3K/PDK/Akt pathway activation and to insulin resistance (25, 46, 49). Indeed, the lack of insulin in experimental type 1 diabetes is associated with baseline reductions in cardiac Akt activity (7), whereas, in streptozotocin (STZ)-diabetic rats, systemic insulin administration results in an enhanced activity of most of the components of the PI3K/Akt signaling cascade in the myocardium, including an increase in Akt phosphorylation (27). Thus the lack of insulin seen in experimental type 1 diabetes may result in lower baseline PI3K/Akt activities, whereas, in diabetic states associated with hyperinsulinemia and insulin resistance, there is an apparent impairment of this action of insulin on the PI3K/Akt signal cascade. Indeed, there is evidence that insulin-induced vascular Akt phosphorylation is blunted, both in obese insulin-resistant rats and in type 2 diabetic patients (15, 33). Moreover, we observed a few years ago that, in aortic rings from a murine type 2 diabetic model, clomidine-induced relaxation, NO production, and Akt phosphorylation were all impaired (20, 45). Thus the plasma insulin levels seen in diabetic states would be expected to modulate Akt activation. All this led us to speculate that, in diabetic rats, the presence of altered plasma insulin levels and abnormal activity in the PDK/Akt/eNOS pathway system might be directly related to the impairments of the associated endothelial functions that are present in such animals.

Abnormal regulation of Akt/eNOS may be one of several factors contributing to the endothelial dysfunction seen in association with both hyperinsulinemia and insulin deficiency.
We decided to examine the rat aorta, a vessel that is mainly NO synthase (NOS) dependent in its endothelium-mediated relaxations. Our laboratory previously found that, in mouse aortas, the clonidine- and insulin-induced vasorelaxations, but not the acetylcholine (ACh)-induced one, are regulated by the PI3K/Akt signaling pathway (20). The aim of the present study was to investigate the molecular mechanisms underlying the endothelial dysfunctions mediated by alterations in PDK/Akt pathway activities and NOS activities in two groups of diabetic rats (high-insulin treated and untreated). We also asked whether, in the diabetic state, insulin deficiency and hyperinsulinemia might be associated with different PDK-1, Akt (phosphorylation at Thr308 and Ser773), and NOS expression profiles and activities, and whether such abnormalities might lead to impairments of endothelium-dependent relaxation via decreases in PDK/Akt pathway functions.

MATERIALS AND METHODS

Drugs and solutions. STZ, indomethacin, human insulin, and N\textsuperscript{N}-nitro-l-arginine (l-NNA) were purchased from Sigma Chemical (St. Louis, MO). Sodium nitroprusside dehydrate (SNP) was from Wako (Osaka). ACh chloride was from Daiichi Pharmaceuticals (St. Louis, MO). Ethyl ether and killed by decapitation. A section of the thoracic aorta adopted by the Committee on the Care and Use of Laboratory Animals of Hoshi University (which is accredited by the Ministry of Education, Culture, Sports, Science, and Technology, Japan).

The high-insulin-treated control (HI-control) and high-insulin-treated diabetic (HI-diabetic) rats received a subcutaneous dose of insulin (human crystalline insulin zinc, 5–30 U·kg\textsuperscript{-1}·day\textsuperscript{-1}; Novo Nordisk, Bagsværd, Denmark) for 2 wk (18). The high-insulin-treated control (HI-control) and high-insulin-treated diabetic (HI-diabetic) rats received a daily evening injection (5:00 PM) of insulin in doses (5–30 U·kg\textsuperscript{-1}·day\textsuperscript{-1}) individually adjusted to maintain urine sugar levels at under 250 mg/dl (measured using a urine sugar tester from Bayer Health Care). Thus we studied four groups: control, insulin-untreated diabetic (diabetic), HI-control, and HI-diabetic rats. Food and water were given ad libitum. This study was approved by the Hoshi University Animal Care and Use Committee, and all studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and the Guide for the Care and Use of Laboratory Animals adopted by the Committee on the Care and Use of Laboratory Animals of Hoshi University (which is accredited by the Ministry of Education, Culture, Sports, Science, and Technology, Japan).

Assessment of blood parameters and blood pressure. Plasma glucose and insulin levels and systolic blood pressure were measured, as described previously (18, 30). Briefly, plasma glucose was determined by the use of a commercially available enzyme kit (Wako Chemical, Osaka, Japan), while plasma insulin was measured by enzyme immunoassay (Shibayagi, Shibukawa, Japan). For blood pressure measurements, a given rat was kept in a constant-temperature hot box at 37°C for 15 min. Then its blood pressure was measured by the tail-cuff method using a blood pressure analyzer (BP-98A; Softron, Tokyo, Japan) at least 5 min after the rat had been put in a restrainer for the purpose of measuring.

Measurement of isometric force. Rats were anesthetized with di-ethyl ether and killed by decapitation. A section of the thoracic aorta from the region between the aortic arch and the diaphragm was removed and placed in oxygenated, modified Krebs-Henseleit solution (KHS). The aorta (cut into helical strips) was placed in a bath containing 10 ml of KHS, with one end of each strip connected to a tissue holder, and the other to a force-displacement transducer, as previously described (21, 22). For the relaxation studies, strips were precontracted with an equi-effective concentration of norepinephrine (NE, 5 \times 10^{-8}–3 \times 10^{-7} mol/l) (i.e., so that the tension developed in response to NE was similar among all groups). All tissues were equilibrated for 40 min in the presence of 10^{-5} mol/l indomethacin (to block cyclooxygenase) before administration of NE. When the NE-induced contraction had reached a plateau level, ACh (10^{-9}–10^{-5} mol/l), SNP (10^{-10}–10^{-5} mol/l), or insulin (10^{-8}–3 \times 10^{-6} mol/l) was added in a cumulative manner. When the effects of l-NNa (10^{-4} mol/l) or an Akt inhibitor (10^{-6} mol/l) on the responses to these relaxant agents were to be examined, one of these inhibitors was added to the bath 20 min before the application of NE.

Measurement of NOx. The concentration of NOx (nitrite + nitrate) in the effluent from each tissue sample was measured by the method described previously (EN020; Eicom, Kyoto) (21, 22). Each aorta was cut into transverse rings 10 mm in length. These were placed in 0.5 ml KHS at 37°C. Samples of effluent were collected on two occasions as follows: sample 1, for a 20-min period after application of 10^{-7} mol/l ACh or insulin (with or without Akt inhibitor); sample 2, for a 20-min period without either ACh or insulin stimulation. The amount of NOx was calculated as follows: agonist-stimulated NOx (10^{-7} mol·min\textsuperscript{-1}·g\textsuperscript{-1} = \{sample 1 − sample 2\}/20 min·g\textsuperscript{-1}=wt·g\textsuperscript{-1}·wt of the aorta. The concentration of nitrite plus nitrate in the KHS and the reliability of the reduction column were examined in each experiment.

Measurement of the protein expressions of eNOS and akt (by Western blotting). Aortas (two pooled vessels) were homogenized in ice-cold lysis buffer containing 50 mM Tris-HCl buffer (pH 7.5), 150 mM NaCl, 1% Triton X-100, and protease inhibitor cocktail, as previously described (18). Homogenates were centrifuged at 13,000 g for 5 min. The supernatant was sonicated at 4°C, and the proteins were solubilized in Laemmli’s buffer containing mercaptoethanol.

The protein concentration was determined by means of a bichinchoninic acid protein assay reagent kit (Pierce). Samples (24 μg/lane) were resolved by electrophoresis on 10% SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes. Briefly, after blocking the residual protein sites on the membrane, the membrane was incubated with anti-eNOS antibody (1:1,000 dilution; BD Bioscience, San Jose, CA), anti-Akt antibody (1:1,000 dilution, Cell Signaling Technology, Danvers, MA), or β-actin antibody (1:5,000 dilution; Sigma, St. Louis, MO) in blocking solution. To normalize the data, we used β-actin as a housekeeping protein.

Measurements of Ser\textsuperscript{1177} eNOS, Ser\textsuperscript{773} Akt, Thr\textsuperscript{308} Akt, and Ser\textsuperscript{241} PDK-1 phosphorylations. Phosphorylation of eNOS at Ser\textsuperscript{1177}, Akt at Ser\textsuperscript{773}, Akt at Thr\textsuperscript{308}, or PDK-1 at Ser\textsuperscript{241} is coincident with eNOS, Akt, or PDK-1 activation, respectively, in vivo. Therefore, in vitro, such phosphorylations have been used as markers for eNOS, Akt, or PDK-1 activity (with the aid of phospho-specific antibodies). Here, each phosphorylation was examined using Western blotting. First, each aorta was cut into transverse rings 10 mm in length. These were then set up under tension in an organ bath under the same conditions as those used for the measurement of isometric force, the bath containing 0.5 ml KHS at 37°C. Samples of effluent were collected on two occasions as follows: agonist-stimulation samples, for a 20-min period after application of 10^{-7} mol/l ACh or insulin (with or without Akt inhibitor); and basal samples, for a 20-min period without either ACh or insulin stimulation. The samples were homogenized in ice-cold lysis buffer, and Western blotting was performed. The membrane was incubated with one of the following antibodies: anti-phospho eNOS Ser\textsuperscript{1177} (1:1,000 dilution), anti-phospho Akt Ser\textsuperscript{773} (1:500 dilution), anti-phospho Akt Thr\textsuperscript{308} (1:500 dilution), or anti-phospho PDK-1 Ser\textsuperscript{241} (1:500 dilution) in blocking solution (all antibodies being from Cell Signaling Technology). To normalize the...
data, we used β-actin as a housekeeping protein. The optical densities of the bands on the film were quantified using densitometry, with correction for the optical density of the corresponding β-actin band. Ratios were calculated for the optical density of phosphorylated eNOS, Akt, or PDK-1 over that of the corresponding total protein band.

Statistical analysis. Each relaxation response is expressed as a percentage of the contraction induced by NE. Values are means ± SE. When appropriate, statistical differences were assessed by Dunnett’s test for multiple comparisons after a one- or two-way ANOVA, with P < 0.05 being regarded as significant. Statistical comparisons between concentration-response curves were made using a one-way ANOVA, with post hoc correction for multiple comparisons by Bonferroni’s test, with P < 0.05 again being considered significant.

RESULTS

Plasma glucose and insulin and systolic blood pressure. As indicated in Table 1, nonfasting plasma glucose levels were significantly elevated after STZ treatment, whereas treatment of diabetic rats with insulin (10–40 U·kg⁻¹·day⁻¹ for 2 wk) resulted in a plasma glucose concentration that was not different from that of the controls. In control rats, plasma glucose levels were lower in insulin-treated animals than in untreated ones. Plasma insulin levels were significantly lower in the insulin-untreated diabetics than in their controls, and significantly higher in each of the insulin-treated groups than in the control group. Systolic blood pressure was not different between the insulin-untreated diabetic group and the controls, but it was significantly higher in the HI-diabetic group than in the other three groups.

Relaxation responses to ACh, SNP, and insulin. When the NE-induced (5×10⁻⁵–3×10⁻⁷ mol/l) contraction had reached a plateau, ACh or insulin was added cumulatively (with indo-methacin being present to eliminate the effects of prostaglandins). The results are summarized in Fig. 1. In all groups, the ACh- and insulin-induced relaxation responses were abolished by preincubation with the NOS inhibitor L-NNA at 10⁻⁴ mol/l. The ACh-induced relaxation was significantly weaker in the diabetic group than in the controls. This impaired relaxation response was improved in the HI-diabetic group. The relaxation response to insulin was significantly stronger in strips from diabetic rats than in those from the control rats, but significantly weaker in the HI-diabetic group than in the insulin-untreated controls. Treating the controls with insulin had no significant effect on the relaxation induced by ACh. In contrast, the relaxation response to insulin was significantly weaker in the HI-control group than in the insulin-untreated controls (Fig. 1, A and B). The relaxation induced by SNP (NO donor; endothelium-independent relaxation) was not different among the various groups (Fig. 1C). There is an accumulating body of evidence to indicate that H₂O₂ causes relaxation and NO production in both the rat aorta (31) and rabbit aorta (48). To examine the possible involvement of H₂O₂ in the present ACh- and insulin-induced relaxations, aortas were incubated with catalase [H₂O₂ scavenger (1,000 U/ml)]. Neither of these relaxations was changed by such treatment in any group (data not shown). These results suggest that the above enhancements observed in the diabetic aorta are not due to increases in H₂O₂ production.

To investigate the contribution made by the PDK/Akt pathway to the ACh-induced and insulin-induced relaxations in the rat aorta, we added ACh or insulin cumulatively to aortas precontracted by NE in the presence of an Akt inhibitor. The ACh-induced relaxation was not changed by treatment with 10⁻⁶ mol/l Akt inhibitor in any group (Fig. 2). In contrast, the relaxation induced in control aortas by insulin was significantly weaker in aortas treated with Akt inhibitor (Fig. 3A). In the diabetic group, the insulin-induced relaxation was markedly weaker in aortas treated with Akt inhibitor (Fig. 3B). In the HI-diabetic group, however, this relaxation showed no change following preincubation with Akt inhibitor (Fig. 3C). In both control and diabetic aortic strips that had been pretreated with Akt inhibitor, the insulin-induced relaxation was decreased to a level comparable to that seen in the HI-diabetic group. It should be noted that the effects of the inhibitor on insulin-induced relaxation were greater in the diabetic group than in the controls.

Analysis of NOS activity by measurements of phosphorylated eNOS expression and NO production, and effects of ACh and insulin. In view of the published evidence that the eNOS/NO signaling pathway plays a major role in endothelium-dependent relaxation in the aorta, we performed an analysis of this pathway using ACh- and insulin-stimulated aortas from three groups of rats. Use of anti-phospho-eNOS (Ser¹¹⁷⁷) antibody allowed detection of an immunoreactive protein with a molecular mass of 140 kDa. We found that basal levels of phosphorylated eNOS were beneath the lower limit of detection; however, phosphorylated eNOS was detectable in the ACh-stimulated and insulin-stimulated conditions. The ACh-stimulated levels of NOx and eNOS phosphorylation in the aorta were unchanged in the diabetic group (vs. control), whereas the former, but not the latter, was significantly increased in the HI-diabetic group (vs. control) (Figs. 4A and 5B). In contrast, insulin-stimulation of the aorta increased both the NOx level and the eNOS phosphorylation level in the diabetic group, but both were significantly below control in the HI-diabetic group (Figs. 4B and 5C). Neither ACh-stimulated NOx production nor ACh-stimulated eNOS phosphorylation was altered by pretreatment with the Akt inhibitor in any group (Figs. 4A and

Table 1. Plasma glucose and insulin levels and systolic blood pressure in age-matched controls, untreated diabetic, and high-insulin-treated rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Age-matched Control</th>
<th>Untreated Diabetic</th>
<th>High-insulin-treated Control</th>
<th>High-insulin-treated Diabetic</th>
</tr>
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<tbody>
<tr>
<td>n</td>
<td>10</td>
<td>10</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>535±14</td>
<td>274±12*</td>
<td>542±23</td>
<td>403±19†‡</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>122±16</td>
<td>540±24*</td>
<td>62±28*</td>
<td>108±16†‡</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>4.9±0.7</td>
<td>0.3±0.2*</td>
<td>15.9±2.7*</td>
<td>16.7±1.8*‡</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>111±2</td>
<td>108±2</td>
<td>119±5</td>
<td>128±4†‡</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of determinations. P < 0.05 vs. *age-matched control, †high-insulin-treated control, and ‡untreated diabetic.
In contrast, the insulin-stimulated NOx production and eNOS phosphorylation in the controls and insulin-untreated diabetics were markedly decreased by this inhibitor (Figs. 4B and 5C). These results suggest that, in the rat aorta, in diabetes, both ACh-stimulated NO production and eNOS activity are unchanged, whereas insulin-stimulated NO production and eNOS activity are increased; in HI-diabetic subjects, ACh-stimulated NO production is increased, whereas the stimulatory effects of insulin on NO production and eNOS activity via the Akt pathway are decreased.

Expressions of total eNOS and Akt proteins. Next, we used Western blot analysis to examine whether the protein expressions of total eNOS and total Akt might be altered in diabetic 5B). In contrast, the insulin-stimulated NOx production and eNOS phosphorylation in the controls and insulin-untreated diabetics were markedly decreased by this inhibitor (Figs. 4B and 5C). These results suggest that, in the rat aorta, 1) in diabetes, both ACh-stimulated NO production and eNOS activity are unchanged, whereas insulin-stimulated NO production and eNOS activity are increased; 2) in HI-diabetic subjects, ACh-stimulated NO production is increased, whereas the stimulatory effects of insulin on NO production and eNOS activity via the Akt pathway are decreased.

Fig. 1. Concentration-response curves for acetylcholine (ACh)-induced, sodium nitroprusside (SNP)-induced, and insulin-induced relaxations of aortic strips from control, diabetic, high-insulin (HI)-control, and HI-diabetic rats, together with the modulating effects of NG-nitro-L-arginine (L-NNA). ACh-induced relaxation was weaker in the diabetic group than in the controls (A), whereas insulin-induced relaxation was weaker in the insulin-treated groups than in the controls (B). C: SNP-induced relaxation was not different among the various groups. Both the ACh- and insulin-induced relaxation responses were absent following preincubation with the nitric oxide synthase (NOS) inhibitor L-NNA at 10^{-4} mol/l. Values are means ± SE from 8–10 experiments; SE is shown only when it is larger than the symbol. P < 0.05, *controls vs. diabetic, #control vs. HI-control, †control vs. HI-diabetic, and &diabetic vs. HI-diabetic.

Fig. 2. Effect of Akt inhibitor on ACh-induced relaxations of aortic strips from controls (A), diabetic rats (B), and HI-diabetic rats (C). The ACh-induced relaxation was not altered by treatment with Akt inhibitor (10^{-6} mol/l) in any group. Values are means ± SE from 8–10 experiments; SE is shown only when it is larger than the symbol. The particular “Akt inhibitor” we used was manufactured by Calbiochem (Darmstadt, Germany).
and HI-diabetic rats. Use of anti-eNOS antibody allowed detection of an immunoreactive protein with a molecular mass of 60 kDa. In the unstimulated (basal) condition, the Ser<sup>473</sup> and Thr<sup>308</sup> Akt phosphorylation levels were not different among the three groups. In aortas stimulated with insulin (Fig. 6C), the expression of phosphorylated Ser<sup>473</sup> Akt was significantly decreased in both the diabetic and HI-diabetic groups (vs. the controls). In insulin-stimulated aortas, Akt phosphorylation at Thr<sup>308</sup> was significantly greater in the diabetic group than in the controls, but lower in the HI-diabetic group than in the controls (Fig. 6D). Although pretreatment with the Akt inhibitor did not alter ACh-stimulated Akt phosphorylations (at Ser<sup>473</sup> or Thr<sup>308</sup>) in any group (data not shown), it markedly decreased the insulin-stimulated phosphorylation of each of these Akt phosphorylations in all groups (Fig. 6).

We next evaluated the insulin-stimulated PDK-1 phosphorylation levels. Use of anti-phospho-PDK-1 antibody allowed detection of immunoreactive proteins with molecular masses of 58 and 68 kDa. As shown in Fig. 7, the insulin-stimulated aortic phospho-PDK-1 level was 1) significantly increased in the diabetic group (vs. the controls), but 2) significantly decreased in the HI-diabetic group (vs. both the controls and the diabetics). The insulin-stimulated PDK-1 phosphorylation level was not changed in any group by pretreatment with the Akt inhibitor (Fig. 7).

Thus the results obtained for the phosphorylation levels of PDK-1/Akt (Thr<sup>308</sup>) were similar to those described above for eNOS activity/NO production in diabetic and HI-diabetic aortas.

**DISCUSSION**

The most important observations made in the present study, on rat aortas, suggest that: 1) in untreated diabetic rats, which exhibited insulin deficiency, neither ACh-induced NOS activity nor ACh-induced Akt phosphorylation differed from the levels seen in the nondiabetic controls (although ACh-induced relaxation was impaired), whereas both the NOS activity and PDK-1/Thr<sup>308</sup> Akt phosphorylation induced by insulin were...
greatly enhanced (as was the insulin-induced relaxation); and 2) HI-diabetic rats, which exhibited hyperinsulinemia, displayed impairments of both the aortic relaxation and NOS activity induced by insulin, possibly due to reductions in PDK-1/Thr308 Akt phosphorylations (since there were no decreases in total eNOS or total Akt protein expression) in the hyperinsulinemic diabetic state. An abnormally high level of plasma insulin may act directly to cause an impairment of insulin-stimulated PDK/Akt/NOS activity in the diabetic state, with a consequent decline in endothelial function.

The mechanisms underlying the hypertension observed in hyperinsulinemia may be related to changes in endothelial function (26, 42) or in skeletal muscle blood flow and relative skeletal muscle fiber type (28), or to increased renal sodium reabsorption (5), or to increased activity within the sympathetic nervous system (38). In the diabetic model used in our experiments, the untreated diabetic rats had a raised plasma glucose level, whereas the HI-diabetic rats had 1) a markedly raised plasma insulin; and 2) normalized plasma glucose levels, suggesting that STZ-diabetic rats treated with high insulin are insulin resistant. Furthermore, our observations might seem to suggest that an increased plasma insulin level causes an increase in resting blood pressure, but only when there is a diabetic state (as seen in the present insulin-treated diabetic group).
ACh-induced Ser1177 eNOS phosphorylation was not different in diabetic rats (21). In fact, in the present study, we found that inactivation of NO may be responsible for the impairment of endothelium-dependent relaxation seen in diabetic rats (10, 16), leading to activation of eNOS via Ca$^{2+}$/calmodulin-dependent mechanisms. The present results suggest that, upon ACh stimulation, the PDK-1/Akt pathway may not be necessary for production of NO. Thus it is possible that the high insulin levels present in HI-diabetes increase NO production through a Ca$^{2+}$/calmodulin-dependent mechanism (as well as through other pathways), and that this improves ACh-induced aortic relaxation (vs. that seen in insulin-un-treated diabetes).

Many stimuli (including insulin, VEGF, α2-agonists, and shear-stress signals) regulate NO production by activating eNOS via phosphorylation through the Akt pathway (6, 11, 20, 46). When an Akt inhibitor was added to rat aortas in the present study, there was no significant effect on ACh-induced relaxation or on ACh-stimulated NO production in any group, whereas the insulin-induced responses in control aortas were reduced by the Akt inhibitor. These observations suggest that insulin-induced vasorelaxations are regulated by the Akt signal pathway.

The major finding in this study was that the mechanisms underlying endothelial dysfunction in the aorta in diabetic rats with hyperinsulinemia may include impairment of the PDK-1/Akt phosphorylation pathway. In our HI-diabetic model, both the aortic relaxation and NO production induced by insulin were attenuated, as was insulin-stimulated NOS phosphorylation. These results strongly suggested that the impairment of the insulin-induced relaxation response observed in the HI-diabetic group was due to decreases in NO release and NOS activity. We, therefore, hypothesized that the activities of PDK and/or Akt might be altered in such HI-diabetes. Interestingly, the insulin-induced phosphorylations of Akt at both Ser$^{473}$ and Thr$^{308}$ were decreased in our HI-diabetic group, whereas that of Akt at Thr$^{308}$ was increased in the diabetic group. Thus the results obtained for the phosphorylation levels of Akt at Thr$^{308}$ were similar to those described above for eNOS activity/NO production in diabetic and HI-diabetic aortas. These results suggest 1) that Akt phosphorylation at Thr$^{308}$ is closely related to the aortic relaxation response induced by insulin; and 2) that the impaired insulin-induced relaxation response seen in the HI-diabetic aorta may be the result of a decreased phosphorylation of Akt at Thr$^{308}$. There is emerging evidence that, in several cultured cell types, activation of Thr$^{308}$ Akt is mediated by PDK-1 (29). Here, we found that the PDK-1 phosphorylation induced by insulin was decreased in our HI-diabetic group. These data are consistent with the observation of a decreased insulin-induced relaxation in the HI-diabetic aorta, and directly suggest that the relaxation response, NO production, and Ser$^{1177}$ eNOS phosphorylation mediated via the PDK/Akt pathway are altered in our HI-diabetic rat model, possibly due to a high-insulin level and an established diabetic state may need to coexist (17, 18, 19). In our control model, the high-insulin-treated rats had markedly raised plasma insulin and reduced plasma glucose levels (and thus hypoglycemia). Our recent studies led us to suspect that, for diabetes-related hypertension to develop, a high-insulin level and an established diabetic state may need to coexist (17, 18). If so, high insulin alone would not be sufficient to cause increased blood pressure in the rat. Indeed, we now suspect that a high-insulin level, a diabetic state, and other factors (including raised plasma angiotensin II and catecholamine levels) may need to exist together to cause an increased resting blood pressure (by an as yet unknown mechanisms).

A considerable body of evidence now suggests that the impairment of endothelium-dependent relaxation seen in diabetes involves inactivation of NO by superoxide, which may, in turn, be due to excessive elevations in plasma glucose (13, 14, 21, 35, 47). Indeed, our laboratory previously reported that NO is metabolized by superoxide, and that the resulting rapid inactivation of NO may be responsible for the impairment of ACh-induced relaxation seen in aortic strips from STZ-induced diabetic rats (21). In fact, in the present study, we found that ACh-induced Ser$^{1177}$ eNOS phosphorylation was not different between control and diabetic rats. Furthermore, NO production in the aorta was not different between control and diabetic rats. Thus our results are consistent with the idea (21) that the impairment of the ACh-induced relaxation seen in aortic strips obtained from diabetic rats may be due to the presence of an abnormal oxidative metabolism of NO, rather than to decreases in eNOS activity and NO production.

In the present study, the aortas obtained from HI-diabetic rats displayed restored ACh-induced aortic relaxation, apparently via increases in total eNOS expression and NO production, as also reported by Kobayashi and Kamata (21, 22). It was noteworthy that the HI-diabetic aorta exhibited enhanced ACh-induced NO production, but not enhanced ACh-induced phosphorylations of PDK-1 and Akt (which are not mediated via PDK-1/Akt signal pathways). These observations suggest that other pathways, not involving PDK-1/Akt signaling, enhance ACh-induced NO production in the aorta in HI-diabetes. ACh is known to cause an intracellular release of Ca$^{2+}$ from its stores and an influx of extracellular Ca$^{2+}$ into endothelial cells (10, 16), leading to activation of eNOS via Ca$^{2+}$/calmodulin-dependent mechanisms. The present results suggest that, upon ACh stimulation, the PDK-1/Akt pathway may not be necessary for production of NO. Thus it is possible that the high insulin levels present in HI-diabetes increase NO production through a Ca$^{2+}$/calmodulin-dependent mechanism (as well as through other pathways), and that this improves ACh-induced aortic relaxation (vs. that seen in insulin-un-treated diabetes).
to the observed decreases in the phosphorylations of Akt at Thr^{308} and of PDK-1 at Ser^{241}. Coordinated phosphorylation of Thr^{308} and Ser^{373} is required for full activation of Akt (2), and indeed dissociated phosphorylation of Thr^{308} and Ser^{373} has been shown to result in impaired Akt activity (24). In the internal mammary artery of diabetic patients and in adipose cells with experimental insulin resistance, Akt phosphorylation at the Thr^{308} site is reportedly impaired, whereas neither total Akt protein expression nor its phosphorylation at the Ser^{373} site is affected (23, 33). Interestingly, mice with a PDK-1 mutation, which are incapable of binding phosphoinositides, are insulin resistant and hyperinsulinemic and exhibit reduced phosphorylation of Akt at Thr^{308} (3). Thus decreased activity in the PDK-1/Akt (at Thr^{308}) pathway would be expected to be closely related to insulin resistance and hence to the adverse effect on endothelial function seen in diabetes with hyperinsulinemia.

Since the major pathway for insulin signaling is the PDK/Akt pathway, an important consideration in diabetic models is whether the dose of insulin administered might lead to PDK/Akt activation and insulin resistance. Indeed, the impairment of insulin-induced PDK/Akt activity observed in the present HI-diabetic group may have been secondary to the rats’ high-plasma insulin levels. Of the two models used in our experiments: 1) one, the diabetic rats, exhibited markedly decreased plasma insulin levels, but increased insulin-stimulated PDK-1/Akt (at Thr^{308})/NOS activities, while 2) the HI-diabetic rats exhibited markedly increased plasma insulin levels, but decreased PDK-1/Akt (at Thr^{308})/NOS activities. Thus the plasma insulin level would appear to be closely related to the level of the associated PDK-1/Akt (at Thr^{308})/NOS activities and hence to the magnitude of any endothelial dysfunction, in diabetic states. The present results strongly suggest that the high-plasma insulin level present in our HI-diabetic group led to an impairment of the PDK-1/Akt (at Thr^{308}) pathway and in turn to a decrease in eNOS phosphorylation within aortic endothelial cells, and that this resulted in the associated endothelial functions being impaired. There are three isoforms of Akt (Akt1, Akt2, and Akt3), and blood vessels express primarily Akt1 and Akt2. A few years ago, it was reported that Akt1, but not Akt2, is important for basal and VEGF-stimulated eNOS activity/NO release (1). However, a very recent study by Symons et al. (44) identified an important role for Akt2 in insulin-stimulated eNOS phosphorylation. Our data suggest that insulin-induced aortic NO production and relaxation are mediated by total Akt (including Akt1 and Akt2) phosphorylation, but it remains to be established in future studies whether Akt1 or Akt2 levels are directly altered in our two diabetic models.

In conclusion, the present study on two diabetic models (diabetic rats exhibiting either insulin deficiency or hyperinsulinemia) has revealed changes in endothelium-dependent relaxation that occur via two pathways, with the direction of the change being dependent on the rat’s insulin level and the alterations in the PDK/Akt pathways that occur as a result of its disease. Specifically, our data reveal that, in diabetic rats with insulin deficiency and hyperglycemia, the aorta exhibits enhancements of insulin-induced NO production and eNOS phosphorylation, effects that may be mediated by elevated activity levels in the PDK-1 and Akt (at Thr^{308}) pathways. In contrast, in the hyperinsulinemic state seen in high-insulin-treated diabetic rats, there was an impairment of insulin-induced aortic relaxation and an increased systolic blood pressure, effects that appeared to be due to decreases in both NO production and NOS phosphorylation, mediated by decreased activity levels in the PDK-1 and the Akt (at Thr^{308}) pathways, but no impairment of ACh-induced relaxation. This latter sequence may represent a major cause of endothelial dysfunction and hypertension in hyperinsulinemic diabetes.

GRANTS
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