Impairment of PI3-K/Akt Pathway Underlies Attenuated Endothelial Function in Aorta of Type 2 Diabetic Mouse Model

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Abstract—The phosphatidylinositol 3-kinase (PI3-K) pathway, which activates serine/threonine protein kinase Akt, enhances endothelial nitric oxide synthase (eNOS) phosphorylation and nitric oxide (NO) production. We investigated the involvement of the PI3-K/Akt pathway in the relaxation responses to acetylcholine (ACh) and clonidine in a new type 2 diabetic model (streptozotocin plus nicotinamide-induced diabetic mice). Plasma glucose and insulin levels were significantly elevated in our model, and intravenous glucose tolerance tests revealed clear abnormalities in glucose tolerance and insulin responsiveness. Although in our model the ACh-induced relaxation and NO_x^- ($NO_2^- + NO_3^-$)/cGMP production were unchanged, the clonidine-induced and insulin-induced relaxations and NO_x^-/cGMP production were all greatly attenuated. In control mice, the clonidine-induced and insulin-induced relaxations were each abolished by LY294002 and by Wortmannin (inhibitors of PI3-K), and also by Akt-inhibitor treatment. The ACh-induced relaxation was unaffected by such treatments in either group of mice. The expression level of total Akt protein was significantly decreased in the diabetic mice aorta, but those for the p85 and p110γ subunits of PI3-K were not. The clonidine-induced Ser-473 phosphorylation of Akt through PI3-K was significantly decreased in our model; however, that induced by ACh was not. These results suggest that relaxation responses and NO production mediated via the PI3-K/Akt pathway are decreased in this type 2 diabetic model. This may be a major cause of endothelial dysfunction (and the resulting hypertension) in type 2 diabetes. (*Hypertension.* 2004; 44:956-962.)

Key Words: diabetes mellitus ■ hyperinsulinism ■ aorta ■ endothelium-derived relaxing factor ■ nitric oxide

Numerous epidemiological studies have indicated that the insulin resistance and hyperinsulinemia associated with type 2 diabetes make important contributions to the development of hypertension and cardiovascular diseases, and impaired endothelium-dependent vasodilation has been described in humans and in animal models of the disease.[1,2] We and others have demonstrated that both aortic endothelial dysfunction and hypertension are present in type 2 spontaneously diabetic (db/db$^{-/-}$) mice and in fructose-fed insulin-resistance mice.[3–6] Our recent observation that endothelial function and nitric oxide (NO) production are impaired in aortic strips from spontaneously type 2 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 aortas.[7] However, a possible explanation may be that in the intact cells, NO synthesis is regulated independently of changes in eNOS enzyme activity.

Recent reports have suggested that in response to a variety of stimuli, efficient NO production requires eNOS phosphorylation through the phosphatidylinositol 3-kinase (PI3-K)/Akt pathway.[8–10] PI3-K subsequently phosphorylates Akt, which in turn phosphorylates eNOS, and so enhances NO production. Some studies have suggested a role for PI3-K/Akt

in the induction of NO in the endothelium by insulin or β-agonists, because inhibition of such agonist-induced activations of PI3-K leads to impaired NO availability.[10,11] However, it is not known how (or whether) PI3-K/Akt/eNOS regulation is altered in the impaired endothelium-dependent relaxation seen in type 2 diabetic models.

In a fairly new experimental model (adult rats given streptozotocin [STZ] and partially protected with a suitable dose of nicotinamide),[12–15] the diabetic syndrome shares a number of features with human type 2 diabetes and is characterized by stable moderate hyperglycemia, glucose intolerance, altered but significant glucose-stimulated insulin secretion, altered in vivo and in vitro responsiveness to tolbutamide, and a reduction in pancreatic β-cell mass.[12,14] In mice, no previous study has determined a suitable dose of nicotinamide for the production of a similar type 2 diabetic model (with mild hyperglycemia and hyperinsulinemia). In the present study, we established such a mouse model after testing various dosages of nicotinamide.

Abnormal regulation of PI3-K/Akt may be one of several factors contributing to endothelial dysfunction in type 2 diabetes. We decided to examine the mouse aorta, a vessel that is mainly NOS-dependent in its endothelium-mediated relaxations. The aim of the present study was to investigate

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Plasma Glucose, Insulin, Body Weight, and Systolic Blood Pressure in Controls and in Mice Treated with 0.35 g/kg, 0.7 g/kg, or 1.5 g/kg Nicotinamide plus STZ

Parameters	Control (n=8)	0.35 g/kg Nicotinamide–STZ (n=8)	0.7 g/kg Nicotinamide–STZ (n=8)	1.5 g/kg Nicotinamide–STZ (n=12)
Glucose, mg/dL	100.9±15.8	754.2±7.8‡	492.7±23.1‡	229.4±38.2*
Insulin, pg/mL	2112±88	ND	658.5±67‡	3014±248*
Body weight, g	53.8±2.8	38.5±0.7‡	47.6±0.6*	52.7±0.7
Blood pressure, mm Hg	112.0±2.2	105.8±3.7	123.4±1.4†	124.8±1.8†

N of determinations is shown in parentheses.
ND indicates beneath lower limit of detection.
*$P<0.05$, †$P<0.01$, ‡$P<0.001$ vs control.

the relationship between the PI3-K/Akt signal system and endothelium-dependent relaxation in nicotinamide–STZ-induced type 2 diabetic mice. We also asked whether aortas from control and such type 2 diabetic mice might differ in their Akt and PI3-K expression profiles.

Methods

Animals and Experimental Design

Male Institute of Cancer Research (ICR; Tokyo Animal Laboratories, Tokyo, Japan) mice (5 weeks old) received an intraperitoneal injection of 0.35, 0.7, or 1.5g/kg body weight of nicotinamide dissolved in saline 15 minutes before an injection via the tail vein of STZ 200 mg/kg dissolved in a citrate buffer. After a given mouse had been in a constant temperature box at 37°C for a few minutes (at 6 or 10 weeks after nicotinamide–STZ administration), its systolic blood pressure was measured by the tail-cuff method using a blood pressure analyzer (BP-98A; Softron).[5] Finally, mice were anesthetized with diethyl ether and euthanized by decapitation 12 weeks after treatment with nicotinamide–STZ or buffer. This study was conducted in accordance with *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, Science, Sports, and Culture, Japan). Concerning the intravenous glucose tolerance test, measurement of isometric force, measurement of NO_2^- and NO_3^-, Western blotting, Akt phosphorylation, and the statistical analysis (please see http://www.hypertensionaha.org for an expanded Methods section).

Results

Plasma Glucose, Insulin, Systolic Blood Pressure, and Body Weight

As indicated in the Table, nonfasting plasma glucose levels were significantly elevated in all 3 dosage groups (0.35, 0.7, 1.5 g/kg nicotinamide) of nicotinamide–STZ-induced diabetic mice (versus controls). The plasma insulin level was significantly higher in the 1.5 g/kg dosage group than in the controls, whereas in the 0.35 and 0.7 g/kg dosage groups it was either significantly lower than in the controls or not detected. Systolic blood pressure was significantly higher than in the age-matched controls in both the 0.7 g/kg and 1.5 g/kg dosage groups at 10 weeks (see values in the Table). In mice given nicotinamide (1.5 g/kg) alone, none of these parameters showed any change (data not shown). In all subsequent experiments, the mice referred to as "diabetic" had received 1.5 g/kg of nicotinamide in addition to STZ 12 weeks before the experiment.

Intravenous Glucose Tolerance Test

After an intravenous glucose injection (2 g/kg) in control mice, postload plasma glucose and insulin levels returned to basal values at ≈60 minutes (Figure 1). In our diabetic mice, the postload glucose disappearance rate was slower than in the controls; plasma glucose reached 672±55 mg/dL at 30
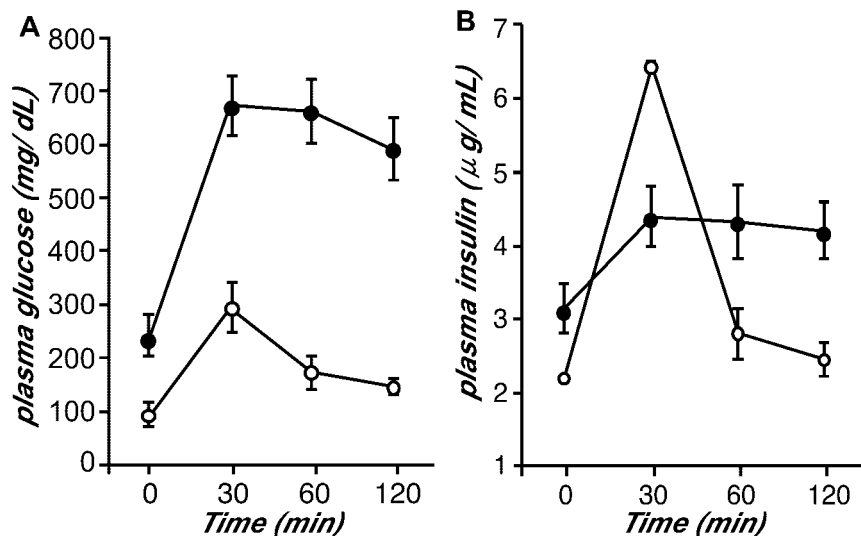

Figure 1. Plasma glucose (A) and insulin (B) levels during glucose tolerance test in age-matched control (○) and nicotinamide–STZ-induced diabetic mice (●). Glucose was administered at 2.0 g/kg. Values are each the mean±SEM of 8 determinations.
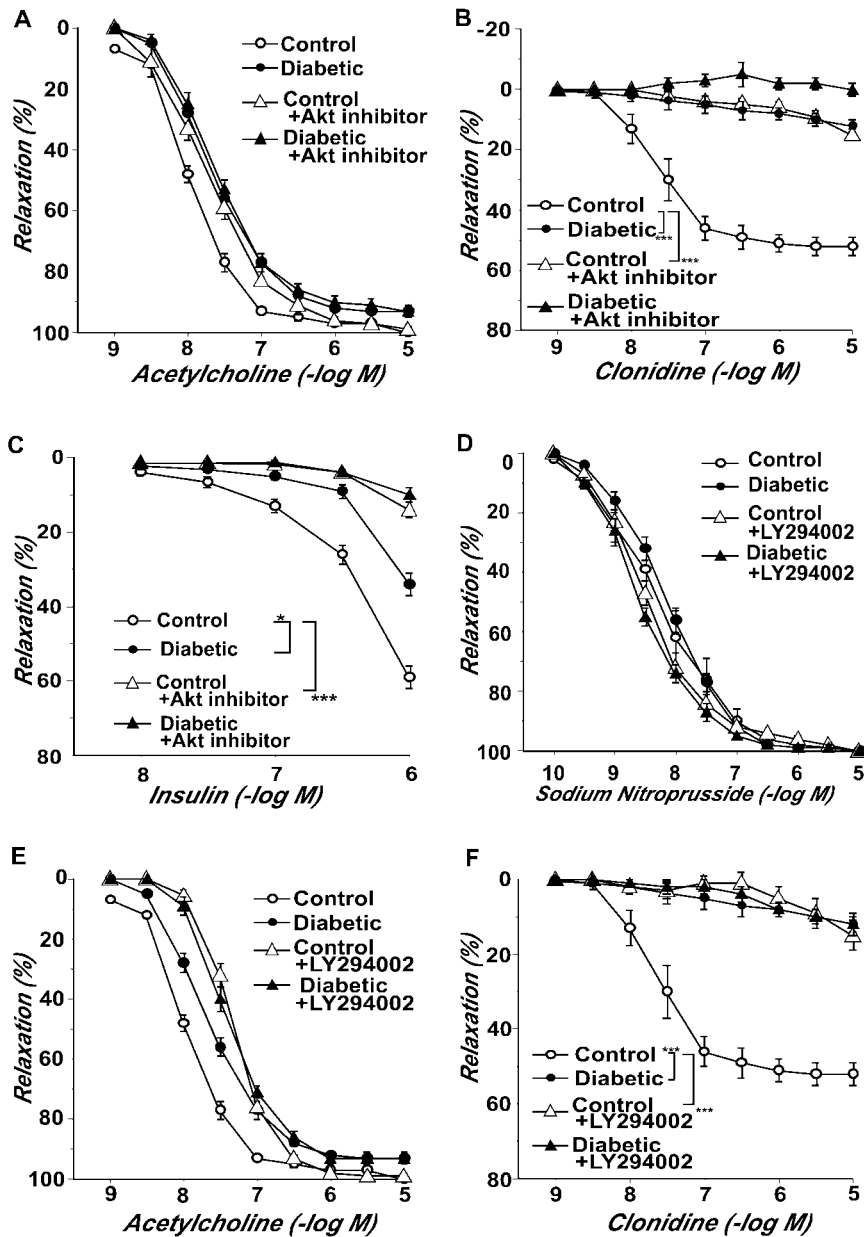
Figure 2. Concentration response curves for agonist-induced relaxations of aortic rings from controls (Control) and nicotinamide–STZ-induced diabetic mice (Diabetic), together with effects of Akt inhibitor (7×10^{-7} mol/L) (A, B, C) and LY294002 (3×10^{-6} mol/L) (D, E, F) on these relaxations (Control+inhibitors, Diabetic+inhibitors). Relaxations were ACh-induced (A, E), clonidine-induced (B, F), insulin-induced (C), and sodium nitroprusside-induced (D). Each data point represents the mean±SE for 6 to 8 experiments, with the SE being included only when it exceeds the dimension of the symbol used. *$P<0.05$, ***$P<0.001$.

minutes after the glucose injection, then slowly decreased (at 60 minutes, 660±59 mg/dL; at 120 minutes, 590±58 mg/dL) (Figure 1A). Glucose administration in our diabetic mice caused only a modest increase in the circulating insulin level, which was, however, still raised at 120 minutes (Figure 1B).

Vascular Reactivity in Aorta

The ACh-induced relaxation tended to be weaker in rings from diabetic mice, but not significantly (Figure 2A). In contrast, the relaxations to clonidine and insulin were very weak in rings from diabetic mice (Figure 2B and 2C). At 20 weeks after STZ–nicotinamide, both types of relaxation (ACh-induced and clonidine-induced) were significantly weaker in the diabetic group than in the control group (data not shown). Both of these relaxation responses were abolished by preincubation with the NOS inhibitor L-NNA at 7×10^{-7} mol/L (online Figure IA and IB), but not by prein-

cubation with indomethacin (data not shown). The relaxation induced by SNP did not differ significantly between the 2 groups of mice (Figure 2D). In mice given nicotinamide (1.5 g/kg) alone, the clonidine-induced relaxation showed no change (data not shown). The ACh-induced and SNP-induced relaxations were not changed by treatment with either 7×10^{-7} mol/L Akt inhibitor (Figure 2A) or 3×10^{-6} mol/L LY294002 (Figure 2D and 2E) (nor with 5×10^{-8} mol/L Wortmannin; data not shown) in either controls or diabetic mice. In contrast, the clonidine-induced and insulin-induced relaxations were almost completely abolished by each inhibitor in control mice, the residual response being similar to the very weak response seen in diabetic mice (Figure 2B, 2C, and 2F). The clonidine-induced relaxation was not changed by treatment with SOD (180 U/mL) in either controls or diabetic mice. In contrast, the ACh-induced relaxation tended to be enhanced in diabetic aortas treated with SOD, but not
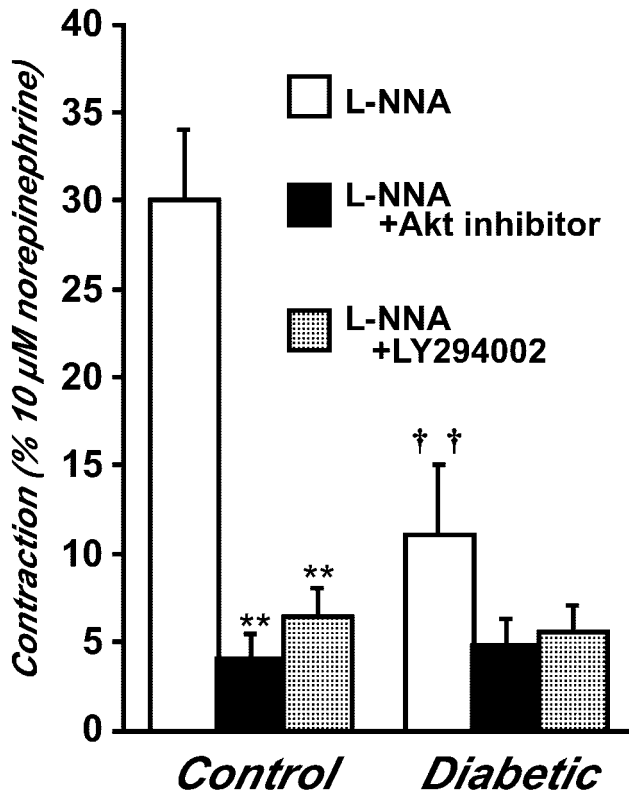
Figure 3. Contraction induced by the NOS inhibitor L-NNA as an index of basal NO formation in aortic strips from control (Control) and nicotinamide–STZ-induced diabetic mice (Diabetic); L-NNA (open column), L-NNA+LY294002 (closed column), and L-NNA+Akt inhibitor (hatched column) are shown. Contraction induced by L-NNA is shown as a percentage of the maximum norepinephrine-induced contraction. Each column represents the mean±SE for 6 animals. **$P<0.01$, ***$P<0.001$, vs L-NNA. ††$P<0.01$, diabetic vs control.

Figure 4. Effect of LY294002 (3×10^{-6} mol/L) or Akt inhibitor (7×10^{-7} mol/L) on acetylcholine (10^{-6} mol/L)-stimulated and clonidine (10^{-6} mol/L)-stimulated release of NO_2^- plus NO_3^- (NO_x^-) (A) and cGMP (B) in (untreated aortic rings) (open column), LY294002-treated rings (closed column), and Akt inhibitor-treated rings (hatched column). Each column represents the mean±SE for 5 animals. *$P<0.05$, **$P<0.01$, ***$P<0.001$, vs clonidine-stimulated untreated control.

significantly (Figure IC and ID). Neither of these relaxation responses was changed by preincubation with catalase (1000 U/mL) (data not shown). The NO synthase inhibitor L-NNA induced a significantly weaker contraction in diabetic aortas than in the controls (Figure 3), indicating a reduced basal NO formation in aortas from diabetic mice. The L-NNA–induced contraction in control aortas was significantly diminished by either Akt inhibitor or LY294002 (Figure 3).

Measurement of NO_x^- Production
ACh increased the NO_x^- level in the perfusate from aortic strips, the ACh-stimulated level was unchanged in diabetes (versus the controls). Pretreating aortic strips with LY294002 had no effect on this response in either group of mice. By contrast, the clonidine-stimulated NO_x^- level was significantly decreased in diabetes (versus the controls). When control aortic rings were pretreated with LY294002, the clonidine-stimulated NO_x^- level was significantly decreased to a level comparable to that seen in the diabetic group. In diabetic rings, the clonidine-stimulated NO_x^- level was not significantly affected by pretreatment with LY294002 (Figure 4A).

Measurement of cGMP Production
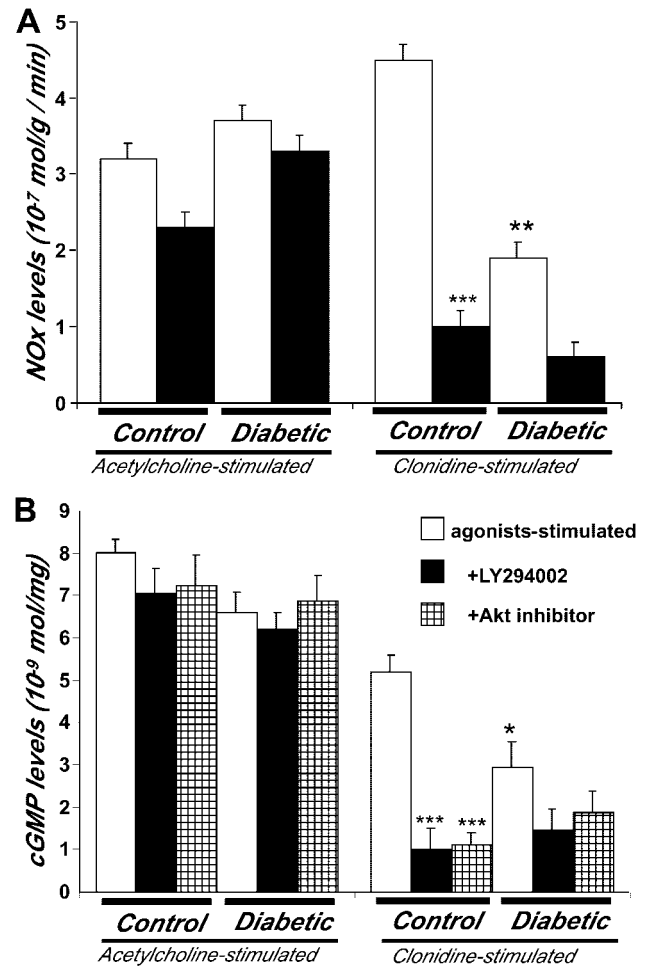ACh increased the cGMP level in aortic strips, and the ACh-stimulated level was unchanged in diabetes (versus the

controls). Pretreating aortic strips with LY294002 or Akt inhibitor had no effect on this response in either group of mice. By contrast, the clonidine-stimulated cGMP level was significantly decreased in diabetes (versus the controls). When control aortic rings were pretreated with LY294002 or Akt inhibitor, the clonidine-stimulated cGMP level was significantly decreased to a level comparable to that seen in the diabetic group. In diabetic rings, the clonidine-stimulated cGMP level was not significantly affected by pretreatment with LY294002 or Akt inhibitor (Figure 4B). Thus, these results were similar to those described for NO_x^- levels.

Expressions of the Proteins for the p85 and p110γ Subunits of PI3-K
In vivo, PI3-K is a heterodimer consisting of a p110 catalytic and a p85-type or p101-type noncatalytic subunit.[16] The p85-subunit binds to tyrosine-phosphorylated receptor tyrosine kinases, whereas the p110 subunit is required for G-protein–mediated activation of PI3-K.[16] Western blotting
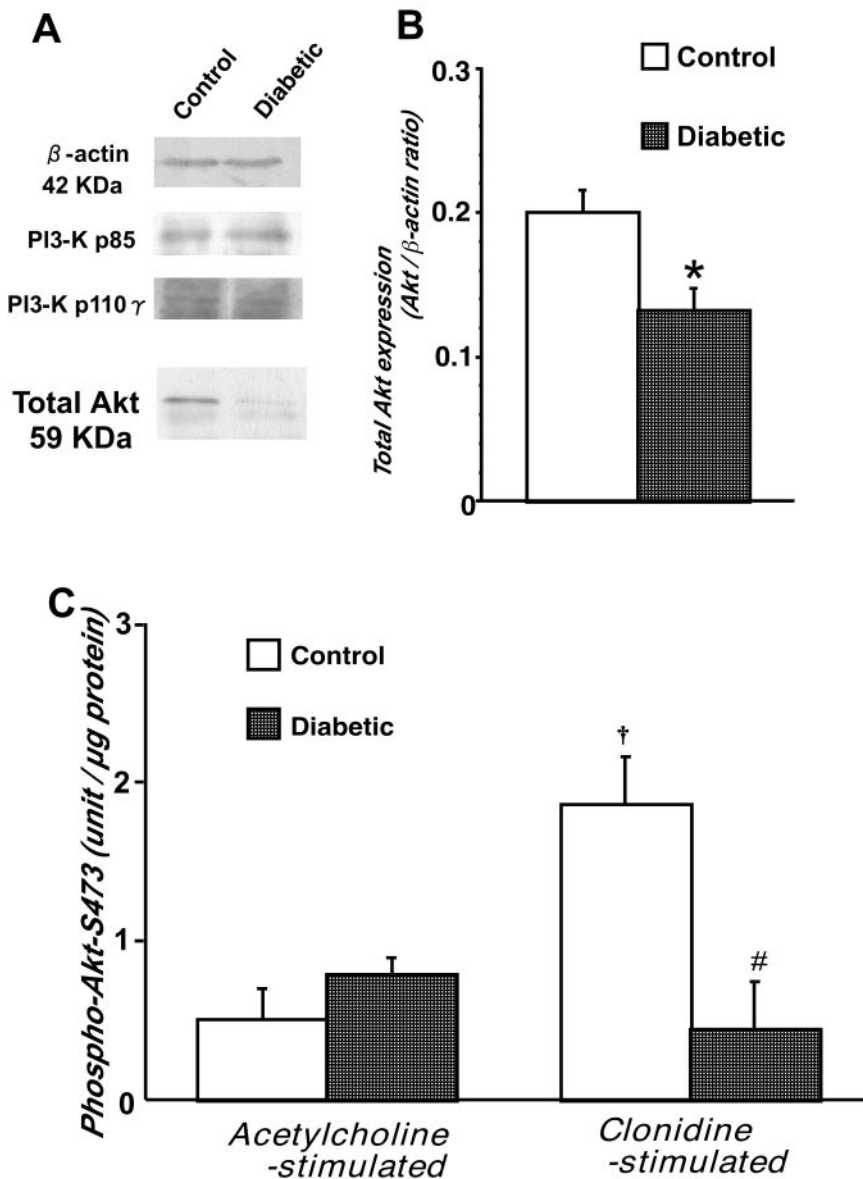
A

β-actin 42 KDa

PI3-K p85

PI3-K p110γ

Total Akt 59 KDa

B

Figure 5. Protein expression and phosphorylation of Akt in aortas from control and diabetic mice. A, Expressions of p85 and p110γ subunits and total Akt assayed by Western blotting. B, Quantitative analysis of total Akt expression by scanning densitometry. C, Phosphorylation of Akt-S473 by ACh (10^{-6} mol/L) or clonidine (10^{-6} mol/L) stimulation. Control mice (n=6, open column); nicotinamide–STZ-induced diabetic mice (n=6, closed column). Values are each the mean±SEM of 6 determinations. *$P<0.05$, diabetic vs control. †$P<0.05$, clonidine-stimulated control vs ACh-stimulated control. #$P<0.05$, clonidine-stimulated diabetic vs clonidine-stimulated control.

C

was performed on aortas obtained from our controls and diabetic mice. Use of anti-p85 and anti-p110γ subunit antibodies allowed detection of immunoreactive proteins with molecular weights of 85 kDa or 110 kDa (Figure 5A). Neither p85 nor the p110γ subunit protein level was different between diabetic mice and controls.

Expression of Akt Protein and Phosphorylation of Akt Through PI3-K

Use of anti-Akt antibody allowed detection of immunoreactive protein with a molecular weight of 59 kDa (Figure 5A, lower). The expression of such Akt protein was significantly decreased in aortas from diabetic mice (versus the controls) (Figure 5B). The enhancement of Akt phosphorylation induced by ACh or clonidine was attenuated in the presence of LY294002 (data not shown). ACh increased Akt phosphorylation through PI3-K in aortas from control mice (versus ACh-untreated aortas). The ACh-stimulated Akt phosphorylation through PI3-K was unchanged in our diabetic mice

(versus the controls) (Figure 5C). Clonidine increased Akt phosphorylation in aortas from control mice, and in the control group the clonidine-stimulated Akt phosphorylation level was significantly higher than the ACh-stimulated level (Figure 5C). Clonidine-stimulated Akt phosphorylation through PI3-K was significantly weaker in aortas from diabetic mice (versus the controls) (Figure 5C).

Discussion

The most important observations made in the present study, which compared aortas from nicotinamide–STZ-induced type 2 diabetic mice with those from age-matched controls, were that the diabetes did not alter ACh-induced relaxation or NO/cGMP production (which are not mediated via PI3-K/Akt signal pathways), whereas clonidine-induced and insulin-induced relaxations and NO/cGMP production were much weaker, passively because of reductions in both Akt phosphorylation and Akt protein expression (but not because of a decline in PI3-K activities) in this type of diabetes. These

reductions may underlie the vascular resistance to insulin associated with type 2 diabetes.

An approach similar to ours was taken by Masiello et al to produce a new animal model of human type 2 diabetic mellitus in adult rats given nicotinamide and STZ together.[12] Our mouse model differs in that we gave 1.5 g/kg nicotinamide before 200 mg/kg STZ treatment to achieve mild hyperglycemia and hyperinsulinemia. Our IGTT data indicate that our model had an impaired glucose tolerance and impaired insulin responsiveness (versus the controls), confirming its type 2 diabetic status.

The major finding in this study is that the mechanisms underlying endothelial dysfunction in type 2 diabetic mice may involve impairment of the PI3-K/Akt pathway. In our model, clonidine-induced relaxation and NO/cGMP production were greatly attenuated. Activation of α_2-adrenoceptors on endothelial cells reportedly stimulates the release of NO.[17,18] The present clonidine-induced relaxation was markedly diminished in control and diabetic aortas by treatment with L-NNA, an inhibitor of NOS, suggesting that the clonidine-induced response is regulated by NO released from the endothelium. Further, the clonidine-stimulated NO_x^-/cGMP level was decreased in our diabetic aortas. These results strongly suggest that the observed impairment of the clonidine-induced response in our diabetic mice is caused by a decrease in the NO release mediated through endothelial α_2-adrenoceptors. This is consistent with previous observations of endothelial dysfunction in type 2 diabetic models.[3–6] Interestingly, the clonidine-induced relaxation and NO production were impaired, whereas ACh-induced responses were unchanged in the present type 2 diabetic aorta (at 12 weeks after nicotinamide–STZ). Possibly, this may be attributable to one agonist being an α_2-adrenoceptor agonist, whereas the other is a muscarine-receptor agonist. Indeed, α_2-adrenoceptors are closely linked to GTP-binding protein,[19,20] whereas ACh receptors may not be linked to such a protein.[5,21]

Many stimuli (including insulin, vascular endothelial growth factor, β-agonists, and shear-stress signals) regulate NO production by activating eNOS via Ser-1177 phosphorylation through the PI3-kinase/Akt pathway.[10,11,22] When a PI3-K or Akt inhibitor was added in our study, there was no significant difference in the ACh-induced relaxation or in NO_x^-/cGMP production, whereas the clonidine-induced and insulin-induced responses were completely abolished by each inhibitor in control aortas. These observations suggest that clonidine-induced and insulin-induced vasorelaxations are regulated by the PI3-K/Akt signal pathway. Furthermore, our finding suggests that in aortic strips from our diabetic mice, eNOS activation through the PI3-K/Akt pathway (as induced by clonidine or insulin stimulation) is decreased, whereas eNOS activation that does not rely on that pathway (eg, that induced by ACh stimulation) is unchanged. We therefore wondered if the activities of PI3-K and/or Akt might be changed by the diabetes. In fact, the expression level of the total protein for Akt was decreased, but those for the p85 and p110γ subunits of PI3-K were unchanged in our diabetic mice. Moreover, the Akt phosphorylation induced by clonidine was decreased in our diabetic mice, whereas that

induced by ACh was not. These data are consistent with those relating to the magnitude of the ACh-induced and clonidine-induced relaxations in our diabetic mice and directly suggest that relaxation responses and NO production mediated via the PI3-K/Akt pathway are altered in our type 2 diabetic mouse model, possibly because of the observed decreases in the phosphorylation and expression of Akt.

In this study, we found that the contraction induced by the NO synthase inhibitor L-NNA was diminished in aortas from diabetic mice, indicating a reduced basal NO formation in those mice. When a PI3-K or Akt inhibitor was added, the basal NO formation was completely abolished by each inhibitor in control aortas. These observations suggest that basal NO formation is regulated by the PI3-K/Akt signal pathway.

There are known functional α_2-adrenoceptors and insulin receptors on the endothelium, and NO production via each receptor has been shown to inhibit the contractile effects of α-adrenergic agonists and catecholamines in vascular smooth muscle.[5,17,21,23] The $\alpha_{2A/D}$-adrenoceptor is expressed in the rat aorta and is coupled to endothelium-dependent NO-mediated relaxation.[18] Thus, the raised blood pressure seen in our diabetic mice may be secondary to a defect in α_2-adrenergic–induced and/or insulin-induced endothelial vasorelaxation (as evidenced by the impaired responses to clonidine and insulin). Chronically, in type 2 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.

Perspectives
The impairment of Akt activity and Akt expression observed in type 2 diabetic mice may be secondary to such insulin resistance. It is unclear at present, however, which missing action of insulin in endothelial cells might be responsible for decreasing the Akt expression level in the diabetic aorta. The effect might be initiated by changes in the plasma glucose level or in the level of any of several hormones, including insulin. Regarding hyperinsulinemia models, it has been reported that insulin-stimulated Akt phosphorylation is reduced in microvessels isolated from insulin-resistant obese Zucker rats.[24] However, the high insulin levels found in patients with insulinomas apparently do not cause hypertension or atherosclerosis.[25] Actually, in cultured bovine endothelial cells, high glucose levels inhibit eNOS phosphorylation at Ser 1177, the Akt phosphorylation site.[26] We suspect that the relative lack of action of insulin and the hyperglycemia act together to cause an impairment of Akt activity and Akt expression in type 2 diabetic mice, resulting in impairments of PI3-K/Akt-mediated endothelial function and NO production.

References

1. Eckel RH, Wassef M, Chait A, Sobel B, Barrett E, King G, Lopes-Virella M, Reusch J, Ruderman N, Steiner G, Vlassara H. Prevention Conference VI: Diabetes and Cardiovascular Disease: Writing Group II: pathogenesis of atherosclerosis in diabetes. *Circulation*. 2002;105:e138–e143.

2. Pieper GM. Review of alterations in endothelial nitric oxide production in diabetes: protective role of arginine on endothelial dysfunction. *Hypertension*. 1998;31:1047–1060.

3. Miyata N, Tsuchida K, Okuyama S, Otomo S, Kamata K, Kasuya Y. Age-related changes in endothelium-dependent relaxation in aorta from genetically diabetic rats. A study in WBN/Kob rats. *Am J Physiol*. 1992;262:H1104–H1109.

4. Pieper GM, Siebeneich W, Moore-Hilton G, Roza AM. Reversal by L-arginine of a dysfunctional arginine/nitric oxide pathway in the endothelium of the genetic diabetic BB rat. *Diabetologia*. 1997;40:910–915.

5. Kamata K, Kanie N, Inose A. Mechanisms underlying attenuated contractile response of aortic rings to noradrenaline in fructose-fed mice. *Eur J Pharmacol*. 2001;428:241–249.

6. Pannirselvam M, Verma S, Anderson TJ, Triggle CR. Cellular basis of endothelial dysfunction in small mesenteric arteries from spontaneously diabetic (db/db−/−) mice: role of decreased tetrahydrobiopterin bioavailability. *Br J Pharmacol*. 2002;136:255–263.

7. Kobayashi T, Matsumoto T, Ooishi K, Kamata K. Differential expression of α_{2D}-adrenoceptor and eNOS in aortas from early and later stages of diabetes in Goto-Kakizaki rats. *Am J Physiol Heart Circ Physiol*. 2004;287:H135–H143.

8. Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature*. 1999;399:601–605.

9. Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K, Franke TF, Papapetropoulos A, Sessa WC. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature*. 1999;399:597–601.

10. Zeng G, Nystrom FH, Ravichandran LV, Cong LN, Kirby M, Mostowski H, Quon MJ. Roles for insulin receptor, PI3-kinase, and Akt in insulin-signaling pathways related to production of nitric oxide in human vascular endothelial cells. *Circulation*. 2000;101:1539–1545.

11. Isenovic E, Muniyappa R, Milivojevic N, Rao Y, Sowers JR. Role of PI3-kinase in isoproterenol and IGF-1 induced ecNOS activity. *Biochem Biophys Res Commun*. 2001;285:954–958.

12. Masiello P, Broca C, Gross R, Roye M, Manteghetti M, Hillaire-Buys D, Novelli M, Ribes G. Experimental NIDDM: Development of a new model in adult rats administered streptozotocin and nicotinamide. *Diabetes*. 1998;47:224–229.

13. Kurup S, Bhonde RR. Combined effect of nicotinamide and streptozotocin on diabetic status in partially pancreatectomized adult BALB/c mice. *Horm Metab Res*. 2000;32:330–334.

14. Novelli M, Fabregat ME, Fernandez-Alvarez J, Gomis R, Masiello P. Metabolic and functional studies on isolated islets in a new rat model of type 2 diabetes. *Mol Cell Endocrinol*. 2001;175:57–66.

15. Matsumoto T, Wakabayashi K, Kobayashi T, Kamata K. Alterations in vascular endothelial function in the aorta and mesenteric artery in type II diabetic rats. *Can J Physiol Pharmacol*. 2004;82:175–182.

16. Katso R, Okkenhaug K, Ahmadi K, White S, Timms J, Waterfield MD. Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. *Annu Rev Cell Dev Biol*. 2001;17:615–675.

17. Angus JA, Cocks TM, Satoh K. The alpha adrenoceptors on endothelial cells. *Fed Proc*. 1986;45:2355–2359.

18. Bockman CS, Gonzalez-Cabrera I, Abel PW. Alpha-2 adrenoceptor subtype causing nitric oxide-mediated vascular relaxation in rats. *J Pharmacol Exp Ther*. 1996;278:1235–1243.

19. Flavahan NA, Shimokawa H, Vanhoute PM. Pertussis toxin inhibits endothelium-dependent relaxations to certain agonists in porcine coronary arteries. *J Physiol (London)*. 1989;408:549–560.

20. Boulanger CM, Vanhoutte PM. G proteins and endothelium-dependent relaxations. *J Vasc Res*. 1997;34:175–185.

21. Lembo G, Iaccarino G, Vecchione C, Barbato E, Morisco C, Monti F, Parrella L Trimarco B. Insulin enhances endothelial alpha2-adrenergic vasorelaxation by a pertussis toxin mechanism. *Hypertension*. 1997;30:1128–1134.

22. Hood JD, Meininger CJ, Ziche M, Granger HJ. VEGF upregulates ecNOS message, protein, and NO production in human endothelial cells. *Am J Physiol Heart Circ Physiol*. 1998;274:H1054–H1058.

23. Vanhoutte PM, Miller VM. Alpha 2-adrenoceptors and endothelium-derived relaxing factor. *Am J Med*. 1989;87:1S–5S.

24. Jiang ZY, Lin YW, Clemont A, Feener EP, Hein KD, Igarashi M, Yamauchi T, White MF, King GL. Characterization of selective resistance to insulin signaling in the vasculature of obese Zucker (fa/fa) rats. *J Clin Invest*. 1999;104:447–457.

25. Hall JE, Brands MW, Zappe DH, Alonso Galicia M. Insulin resistance, hyperinsulinaemia, and hypertension: causes, consequences, or merely correlations? *Proc Soc Exp Biol Med*. 1995;208:317–329.

26. Du XL, Edelstein D, Dimmeler S, Ju Q, Sui C, Brownlee M. Hyperglycemia inhibits endothelial nitric oxide synthase activity by posttranslational modification at the Akt site. *J Clin Invest*. 2001;108:1341–1348.