Losartan improves aortic endothelium-dependent relaxation via proline-rich tyrosine kinase 2/Src/Akt pathway in type 2 diabetic Goto-Kakizaki rats

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Nemoto S, Kobayashi T, Taguchi K, Matsumoto T, Kamata K. Losartan improves aortic endothelium-dependent relaxation via proline-rich tyrosine kinase 2/Src/Akt pathway in type 2 diabetic Goto-Kakizaki rats. Am J Physiol Heart Circ Physiol 301: H2383–H2394, 2011. First Published September 16, 2011; doi:10.1152/ajpheart.00178.2011.—In diabetic states, endothelial dysfunction is related to vascular complications. We hypothesized that insulin-induced relaxation and the associated proline-rich tyrosine kinase 2 (Pyk2)/Src/Akt pathway would be abnormal in aortas from the Goto-Kakizaki (GK) type 2 diabetic rat, which exhibits hyperglycemia/insulin resistance, and that losartan treatment of such rats (25 mg·kg⁻¹·day⁻¹ for 2 wk) would correct these abnormailities. Endothelium-dependent relaxation was measured by insulin-induced relaxation and by endothelial nitric oxide synthase (eNOS) signaling-pathway protein levels and activities were assessed mainly by Western blotting and partly by immunohistochemistry. In GK (vs. age-matched control) aortas, several insulin-stimulated levels (nitric oxide production and the phospho-Ser307 in IRS-1, which acts as a negative regulatory site, including IRS-1, and the phosphorilation of IRS-1 at Ser312, which is phosphoinositide-dependent kinase-1 (PDK1; Refs. 30, 41). Recently, our laboratory (22) found that in rat aortas the insulin-induced tyrosine phosphorylation of insulin receptor substrate (IRS)-1 is mediated through an action of the ANG II type 1 receptor (Ref. 31). The observed increase in phospho-IRS-1 (at Ser312) may result from increased angiostatin II activity.

The risk of cardiovascular disease is considerably increased in diabetic patients, and novel cardioprotective strategies are being sought that could target both diabetic and cardiovascular outcomes (33, 43). Endothelial dysfunction often precedes type 2 diabetes-associated cardiovascular complications, and many animal models have been used to gain more insight into the pathogenesis of the vasculopathy in type 2 diabetes. Unfortunately, many of these models exhibit features of the metabolic syndrome other than diabetes itself, such as hyperlipidemia, obesity, or hypertension. This makes it difficult to assess the pathogenetic relevance of these confounding factors to the development of diabetic vasculopathy in these models. However, the Goto-Kakizaki (GK) rat, a type 2 diabetic model, is a relatively unique strain in that it develops no obesity, hyperlipidemia, or hypertension (12). In the GK rat, the advent of moderate diabetes usually occurs at between 3 and 4 wk of age and is the result of several pathomechanisms, including impaired ontogenic development of islet cells, abnormal insulin release following a glucose load, insulin resistance, basal hyperinsulinemia, and abnormal glucose metabolism (6, 25, 36, 37, 45). This model provides our studies with a valuable tool for dissecting the pathogenesis of insulin resistance.

It is a well-established idea that important causative factors involved in endothelial dysfunction may be related to hyperinsulinemia and a high plasma angiotensin II (ANG II) levels. Indeed, an enhancement of the renin-angiotensin system (RAS) has been reported in diabetic subjects with vascular complications, in diabetic rats, and in insulin-infused hyperinsulinemic diabetic rats (15, 42). Moreover, ANG II interferes with insulin signaling in vascular cells, mainly by affecting the insulin-induced tyrosine phosphorylation of insulin receptor substrate (IRS)1 (9). Actually, several serine residues in IRS-1 have been identified as negative regulatory sites, including Ser112 in human (orthologous to Ser307 in rat IRS-1), which is phosphorylated by ANG II through ANG II type I receptor/β-catenin N-terminal kinase (JNK; Refs. 1, 2).

Losartan, an ANG II type 1 receptor blocker (ARB), also improves endothelial function in various vessels in subjects with type 2 diabetes (4, 5, 33, 34), suggesting that at least some of the beneficial effects achieved by modulating the effects of ANG II are mediated through an action of the ANG II type 1 receptor on the insulin signaling pathway. In addition to the well-known Ca⁡²⁺/calmodulin-dependent activation of endothelial nitric oxide (NO) synthase (eNOS), a variety of stimuli, including insulin, can reportedly induce efficient NO production via eNOS phosphorylation through the Akt pathway (7, 18, 22, 23, 35, 47). Insulin induces its effects by binding to insulin receptors and triggering downstream signaling pathways, of which the most important is the IRS-1/phosphatidylinositol 3-kinase 3-kinase (PI3K)/Akt pathway. Akt phosphorylation at Thr308 is mediated by 3-phosphoinositide-dependent kinase-1 (PDK1; Refs. 30, 41). Recently, our laboratory (22) found that in rat aortas the insulin-induced vasorelaxation is regulated by the PDK1/Akt eNOS signaling pathway. Furthermore, Ying et al. (46) demonstrated that in rats enhancements of Akt activation, eNOS phosphorylation, and NO production can be prevented by a Src inhibitor (PP2) or a proline-rich tyrosine kinase 2 (Pyk2) inhibitor (tyrophostin A, also called AG17) and moreover that NOS activation is regulated by increased activity of the Pyk2/Src/PI3K complex. Thus eNOS activation via the Akt pathway may be at least partly regulated by, or related to, activation of Pyk2 and/or Src. However, uncertainty surrounds the mechanisms by which, in the type 2 diabetic state(s), dysfunctions of
Pyk2, Src, and the PDK1/Akt/eNOS pathway might contribute to endothelial dysfunction.

In the present study, we hypothesized that perturbations of the activities and functions of Pyk2, Src, and the PDK1 pathway, upstream of Akt/eNOS, would be key events in the impairment of insulin-induced relaxation that occurs in diabetes. Furthermore, we postulated that an alteration of IRS-1 activity might be involved in mediating such diabetic abnormalities. The main aim of this study was to investigate the relation between the endothelial dysfunction seen in aortas from type 2 diabetic rats and any abnormalities of IRS-1, Pyk2, Src, and the PDK1/Akt/eNOS pathway. We also examined whether long-term ANG II type 1 receptor blockade (losartan treatment) might correct the impairments of insulin-induced relaxation and any perturbations in Pyk2/Src/PDK1/Akt/NOS activities/proteins that might occur in association with insulin resistance in type 2 diabetes.

MATERIALS AND METHODS

Animals and study design. GK and Wistar control rats were obtained at the age of 4 wk (Clea, Tokyo, Japan). From their arrival, all animals were allowed a standard laboratory diet (MF; Oriental Yeast Industry, Tokyo, Japan) and water ad libitum. Half of the GK and half of the Wistar cohort (selected randomly) were given losartan (25 mg·kg⁻¹·day⁻¹ po; Nolutan; Banyu, Tsukuba, Japan) for 2 wk (see below). The remaining rats formed the vehicle control groups. Thus we studied four groups: normal Wistar (control: n = 30), GK (diabetic: n = 30), losartan-treated normal (losartan-treated control: n = 30), and losartan-treated GK (losartan-treated diabetic: n = 30) rats. Food and water continued to be allowed ad libitum, as above. Losartan or vehicle treatment was started at 32, 33, 34, 35, or 36 wk old (6 control and 6 diabetic rats started at each time point). These groups of rats were killed by decapitation while they were under diethyl ether anesthesia at the end of 2-wk treatment (i.e., at 34, 35, 36, 37, or 38 wk old, respectively), and their aortas were excised. Before they were killed, 16 rats from each group provided blood-pressure and blood-parameter data, as described below. Aortas from the four animal groups were used for the nitrite analysis (NOx), the Western blot, the immunohistochemical staining (IHC), and isometric force experiments. The number of animals/aortas and details of the protocols are given in the appropriate experimental section.

Assessments of blood parameters and blood pressure. Plasma glucose and insulin levels and systolic blood pressure were measured as described previously (21, 32). The used number of plasma samples measurements of blood parameters (1 sample per animal) and the number of blood pressure experiments were each as follows: control (n = 16), diabetic (n = 16), losartan-treated control (n = 16), and losartan-treated diabetic (n = 16). For those purposes, animals and plasma samples were chosen randomly from the four groups at the end of the 2-wk losartan- or vehicle-treatment period. 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 systolic blood pressure was measured by the tail-cuff method using a blood pressure analyzer (BP-98A; Softron, Tokyo, Japan; see Refs. 22, 26) ±5 mm after the rat had been put in a restrainer for the purpose of measuring. The immununoreactivity (IR) index was estimated from the fasting plasma insulin and plasma insulin levels by means of the homeostasis model assessment (HOMA), as follows: HOMA-IR = fasting plasma insulin (in micro-units/ml) × fasting plasma glucose (in mM/l)/22.5 (16).

Measurements of aortic isometric force in vitro. Rats were anesthetized with diethyl ether and killed by decapitation. Hearts were removed for the calculation of the heart weight/body weight ratio (HW/BW) [number of animals providing HW/BW values (chosen randomly from the various groups) was as follows: control (n = 16), diabetic (n = 16), losartan-treated control (n = 16), and losartan-treated diabetic (n = 16)]. 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 was cut into helical strips 3 mm in width and 20 mm in length. A given strip was placed in a bath containing 10 ml of KHS, with one end of the strip connected to a tissue holder and the other to an isometric force-displacement transducer (Nihon Kohden, TB-611T), as previously described (19) [number of aortic strips (1–2 strip per animal used for isometric force experiments) was as follows: control (n = 8), diabetic (n = 8), losartan-treated control (n = 6), and losartan-treated diabetic (n = 8) regardless of whether the experiment was conducted with or without inhibitors]. For the relaxation studies, strips were precontracted with an equieffecttive concentration of norepinephrine (NE; 5 × 10⁻⁸ M to 10⁻⁷ M; i.e., so that the tension developed in response to NE was similar among all groups). Routinely, tissues were equilibrated for 40 min in the presence of 10⁻⁵ M indomethacin (to block cyclooxygenase) before administration of NE. When the NE-induced contraction had reached a plateau level, ace- toylcholine chloride (ACH; 10⁻⁶ M to 10⁻⁵ M), sodium nitroprusside (SNP; 10⁻⁸ M to 10⁻⁵ M), or insulin (10⁻⁸ M to 10⁻⁶ M) was added in a cumulative manner. When the effects of PP2 (Src inhibitor; 5 × 10⁻⁶ M) and/or AG17 (Pyk2 inhibitor; 10⁻⁸ M) or that of N⁶-nitro-l-arginine (l-NNA; 10⁻⁴ M) on the responses to these relaxant agents were to be examined, the appropriate inhibitor(s) was added to the bath 20 min before the application of NE. For the contraction studies, NE (10⁻¹⁰ M to 10⁻⁵ M) was added cumulatively to the bath until a maximal response was achieved. The appropriate inhibitor(s) was added to the bath 20 min before the cumulative addition of NE.

Indomethacin, human insulin, and l-NNA were purchased from Sigma Chemical (St. Louis, MO). SNP was from Wako and ACh was from Daiichi Pharmaceuticals (Tokyo, Japan), while PP2 and AG17 were from Calbiochem (Darmstadt, Germany). Drug solutions were made up using saline, with indomethacin being dissolved first in a small amount of 0.1 M Na₂CO₃ solution. All concentrations are expressed as the final molar concentration of the base in the organ bath.

Analysis of NOx production. The concentration of NOx in the effluent from each aortic ring (see below) was sampled and assayed by the method described previously (ENO20; Eicom, Kyoto, Japan; Ref. 19). For the NOx experiments, transverse rings 10 mm in length were cut [number of aortic rings (1–2 ring per animal) used for the NOx experiments was as follows: control (n = 10), diabetic (n = 10), losartan-treated control (n = 8), and losartan-treated diabetic (n = 10) regardless of whether the experiment was conducted with or without inhibitors]. 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 M ACh or 10⁻⁷ M insulin (with or without PP2 or AG17); and 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⁻¹·g⁻¹·ly⁻¹) = (sample 1 – sample 2)/20 (min) g (wet weight of the aorta). The concentration of NOx in the KHS and the reliability of the reduction column were examined in each experiment.

Analysis for markers of Pyk2/Src/Akt pathway (Western blot and IHC). Aortic strips (2 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 (21) [number of aortic strips (1–2 strips per animal for the Western blot experiments) was as follows: control (n = 6), diabetic (n = 8), and losartan-treated diabetic (n = 8)]. 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 BCA protein assay reagent kit (Pierce, Rockford, IL). Samples (24 μg/lane) were resolved by electrophoresis on 10% SDS-PAGE gels (wt/vol) and transferred onto PVDF membranes. Briefly, after the residual protein sites on the membrane were blocked, the membrane was incubated with anti-eNOS antibody (1:1,000 dilution; cat. no. 610296; BD Biosciences, San Jose, CA), anti-Akt antibody (1:1,000 dilution; cat. no. 9272; Cell Signaling Technology, Danvers, MA), anti-Pi3K p85 antibody (1:1,000 dilution; cat. no. 4292; Cell Signaling Technology), anti-Pi3K p110γ antibody (1:1,000 dilution; cat. no. 4252; Cell Signaling Technology), anti-Pi3K p105 antibody (1:1,000 dilution; cat. no. 611014; BD Biosciences), or β-actin antibody (1:5,000 dilution; cat. no. A5310; Sigma) in blocking solution before overnight incubation at 4°C. Membranes were washed and placed for 30 min in horseradish peroxidase-conjugated anti-mouse (cat. no. W402B) or anti-rabbit (cat. no. W401B) antibody [each purchased from Promega (Madison, WI) and used at 1:10,000 dilution in Tween PBS (T-PBS)]. They were then washed five times in T-PBS, and bands were detected using Luminol/Enhancer SuperSignal reagent (Pierce, Rockford, IL) for 5 min and visualized using Light-Captrix (AE-6981: ATTO, Tokyo, Japan). To normalize the data, we used β-actin as a housekeeping protein. Phosphorylation of eNOS at Ser1177, Akt at Thr308, PDK1 at Ser241, Src at Tyr416, or Pyk2 at Tyr579 is coincident with eNOS, Akt, PDK1, Src, or Pyk2 activation in vivo. Therefore, in vitro such phosphorylations have been examined as markers for eNOS, Akt, PDK1, Src, or Pyk2 activation in vivo. In fact, in vitro such phosphorylations have been examined as markers for eNOS, Akt, PDK1, Src, or Pyk2 activity (with the aid of phospho-specific antibodies). The membrane was incubated with one of the following antibodies: anti-phospho IRS-1 Ser307 (1:1,000 dilution; cat. no. 2381), anti-phospho eNOS Ser1177 (1:1,000 dilution; cat. no. 9571), anti-phospho Akt Thr308 (1:1,000 dilution; cat. no. 29275), anti-phospho PDK1 Ser241 (1:1,000 dilution; cat. no. 3061), anti-phospho Src Tyr416 (1:1,000 dilution; cat. no. 2101), or anti-phospho Pyk2 Tyr579 (1:1,000 dilution; cat. no. discontinued) in blocking solution. The last named of these was from Sigma, while the others were from Cell Signaling Technology. 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, PDK1, Src, or Pyk2 over that of the corresponding total-protein band (anti-IRS-1 at 1:1,000 dilution; cat. no. 2381; anti-PDK1 at 1:1,000 dilution; cat. no. 2382; anti-Pi3K at 1:1,000 dilution; cat. no. 3062; anti-Src at 1:1,000 dilution, cat. no. 9275; anti-Py2 at 1:1,000 dilution, cat. no. 3092). The last-named of these was from Sigma, while the others were from Cell Signaling Technology. Tyr579 Pyk2 or eNOS protein levels were visualized by IHC of control, diabetic, and losartan-treated diabetic aortic strips that had been frozen in optimum cutting temperature compound (Sakura, Torrance, CA) [number of aortic slides (each from a separate animal used for IHC experiments) was as follows: control (n = 5), diabetic (n = 6), and losartan-treated diabetic (n = 6)]. After incubation, aortic strips were embedded in optimum cutting temperature compound. After a washout of the compound, histologic slides bearing sections (10-μm thickness) of aorta were treated with 10 mM citric acid and then microwave heated (for 1 min) to recover antigenicity. Nonspecific binding was blocked with a drop of normal sheep serum in Immunoblock (Dainippon-Pharma, Osaka, Japan) for 20 min before overnight incubation at 4°C with polyclonal anti-eNOS antibody (1:50; BD Biosciences) or anti-phospho Pyk2 Tyr579 (1:100; Sigma) in Immunoblock. Cy3-conjugated secondary antibody (cat. no. C2306, anti-rabbit; or cat. no. C2181, anti-mouse; Vector Laboratories, Burlingame, CA) was used at a 1:100 dilution in T-PBS. Sections of aorta were enclosed in VECTASHIELD mounting medium (Vector Laboratories) after being washed three times in T-PBS. They were imaged using a Zeiss LSM 5 confocal laser microscope (Carl Zeiss Microscopy).

**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.

**Institutional Animal Care and Use Committee approval statement.** This study was approved by the Hoshi University Animal Care and Use Committee, and all experiments 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).

### RESULTS

#### General parameters.

As indicated in Table 1, body weight was lower, and the HW/BW was higher, in losartan-treated or -untreated diabetic rats than in their respective controls (viz. losartan-treated or -untreated control rats). Fasting plasma glucose levels were significantly elevated in diabetic rats. Losartan treatment (25 mg·kg⁻¹·day⁻¹ for 2 wk) did not alter the plasma glucose concentration in either the control or diabetic rats. Plasma insulin levels were significantly higher in the losartan-treated and -untreated diabetic groups than in the corresponding control group. Losartan treatment significantly decreased the plasma insulin level in diabetics but not in control rats. Accordingly, HOMA-IR, an insulin-resistance index, was significantly higher in losartan-treated and -untreated diabetic rats than in control rats.

**Table 1. Body weight, plasma glucose and insulin levels, systolic blood pressure, heart rate, HOMA-IR, and HW/BW in age-matched controls, untreated diabetic, and losartan-treated rats**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Age-Matched Control (16)</th>
<th>Untreated Diabetic (16)</th>
<th>Losartan-Treated Control (16)</th>
<th>Losartan-Treated Diabetic (16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>575.2 ± 7.8</td>
<td>399.7 ± 2.2</td>
<td>581.4 ± 7.8</td>
<td>401.4 ± 5.1*</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>151.6 ± 3.5</td>
<td>488.9 ± 5.4</td>
<td>152.1 ± 2.5</td>
<td>480.6 ± 18.6*</td>
</tr>
<tr>
<td>Insulin, mg/ml</td>
<td>2.6 ± 0.2</td>
<td>4.8 ± 0.2</td>
<td>2.5 ± 0.1</td>
<td>3.5 ± 0.3*</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>117.6 ± 2.8</td>
<td>110.7 ± 1.7</td>
<td>112.8 ± 1.9</td>
<td>110.3 ± 1.2</td>
</tr>
<tr>
<td>HW/BW, g g⁻¹·10⁻³</td>
<td>2.8 ± 0.1</td>
<td>4.1 ± 0.1</td>
<td>3.0 ± 0.3*</td>
<td></td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>331.3 ± 7.2</td>
<td>399.9 ± 24.7</td>
<td>381.4 ± 5.2*</td>
<td>434.8 ± 4.5*</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>2.7 ± 0.3</td>
<td>16.3 ± 0.7</td>
<td>2.7 ± 0.1</td>
<td>11.8 ± 0.9*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Number of determinations is shown in parenthesis. HW/BW: heart weight to body weight ratio; HOMA-IR: homeostasis model assessment-insulin resistance. *P* < 0.05, *P* < 0.01, *P* < 0.001 vs. age-matched control. *P* < 0.05, *P* < 0.001 vs. untreated diabetic. *P* < 0.05, *P* < 0.001 vs. losartan-treated control.
treated diabetic groups than in the corresponding control groups. Losartan treatment significantly decreased the HOMA-IR level in the diabetic rats. However, it should be noted that some previous studies (28, 38) have suggested that GK rats exhibit impaired insulin secretion rather than peripheral insulin resistance. To study insulin sensitivity, Ling et al. (28) examined insulin responses in islets from 8- to 12-wk-old GK rats, while Picarel-Blanchot et al. (38) studied diaphragm, liver, skeletal muscle (epitrochlearis, soleus, and extensor digitorum longus muscles), and white adipose tissues from 4-wk-old GK rats. In contrast, we studied GK rats at 34–38 wk of age. Therefore, we think that the diabetic stage may explain the differences in the results obtained. Systolic blood pressure was not different among the various groups, whereas heart rate was significantly higher in the diabetic group than in the control group. Further, 2-wk administration of losartan increased heart rate vs. the corresponding untreated group (Table 1).

Relaxation responses to SNP and insulin. When the NE (5 x 10^-9 to 3 x 10^-7 M)-induced contraction had reached a plateau, insulin or ACh (data not shown) was added cumulatively to induce relaxation. The results are summarized in Fig. 1. In each of the four groups, the insulin-induced relaxation response was absent (Fig. 1A) after preincubation with the NOS inhibitor L-NNa at 10^-4 mol/l and (Fig. 1B) after removal of the endothelium (data not shown). The relaxation response to insulin was significantly weaker in the diabetic group than in their controls. Likewise, the ACh-induced relaxation response was significantly weaker in the diabetic group than in their controls. Likewise, the ACh-induced relaxation response was significantly weaker in the diabetic group than in their controls.

Fig. 1. Concentration-response curves for sodium nitroprusside (SNP; B, G, H, and I), acetylcholine (ACh; D–F), and insulin (A)-induced relaxations, and norepinephrine (NE; C)-induced contraction of aortic strips from control, diabetic, losartan-treated control, and losartan-treated diabetic rats. A: insulin-induced relaxations were weaker in the losartan-untreated diabetic group than in either the losartan-treated diabetic group or the control group, and those relaxations were absent in all 4 groups following preincubation with the nitric oxide synthase (NOS) inhibitor N^G-nitro-L-arginine (L-NNa) at 10^-4 mol/l. B–I: according to one-way ANOVA, neither the SNP-induced relaxation nor the NE-induced contraction differed significantly among the 4 groups of rats (B and C), and the same was true for the ACh-induced relaxation (data not shown). The above 2 relaxations did not differ significantly between with and without either PP2 (Src kinase inhibitor; 5 x 10^-6 M) or AG17 [proline-rich tyrosine kinase 2 (Pyk2) inhibitor; 10^-8 M; D–I]. However, Table 2 shows that the maximal agonist-induced response (E_max) and -log (corresponding agonist) required to produce 50% of the maximal response (pEC50) values obtained for SNP in the presence of indomethacin were significantly higher for losartan-treated diabetic than for diabetic. Moreover, PP2 and AG17 treatments each significantly decreased the E_max and pEC50 values for the SNP-induced relaxation in the losartan-treated diabetic group (vs. untreated diabetic). Values are means ± SE from 6–8 experiments; SE is shown only when it is larger than the symbol. *P < 0.01, diabetic vs. controls. †P < 0.001, losartan-treated diabetic vs. diabetic.
than in their controls (data not shown). The impaired relaxation response to insulin was significantly improved in the losartan-treated diabetic group, and treating the controls with losartan had no significant effect on the relaxation induced by insulin (Fig. 1A). The NE-induced contractile responses were not significantly different among the four groups (Fig. 1C). The relaxation induced by SNP (NO donor; endothelium-independent relaxation) was not significantly different among the losartan-treated or -untreated control or diabetic groups (Fig. 1B). However, in aortas from the losartan-treated diabetics, the maximal agonist-induced response (E_{max}) and −log (corresponding agonist) required to produce 50% of the maximal response (pEC_{50}) values for the SNP-induced relaxation were increased (vs. untreated diabetics; Table 2). We regard the last-mentioned results as questionable because there was no such significant difference between untreated diabetics and untreated controls or between losartan-treated controls and losartan-treated diabetics. The above results suggest that the described attenuations in the diabetic aorta are each due to a decrease in endothelium-derived NO production, a reflection of endothelial dysfunction.

To investigate the contributions made by the Pyk2/Src pathway to the ACh-induced, SNP-induced, and insulin-induced relaxations in the rat aorta, we added ACh, SNP, or insulin cumulatively to aortas precontracted by NE in the presence of the Src inhibitor PP2 and/or the Pyk2 inhibitor AG17. The ACh-induced (Fig. 1, D–F) and SNP-induced (Fig. 1, G–I) relaxations were unchanged by treatment with 5 × 10^{-8} M PP2 or with 10^{-8} M AG17 in the control (Fig. 1, D and G), losartan-treated control (data not shown), diabetic (Fig. 1, E and H), and losartan-treated diabetic (Fig. 1, F and I) groups. However, the E_{max} and pEC_{50} values for the SNP-induced relaxation were lower in the presence of either inhibitor than in its absence (Table 2). Specially, Table 2 shows that the E_{max} and pEC_{50} values obtained for SNP in the presence of indomethacin were significantly higher for losartan-treated diabetic than for diabetic. Moreover, PP2 and AG17 treatments each significantly decreased the E_{max} and pEC_{50} values for the SNP-induced relaxation in the losartan-treated diabetic group (vs. untreated diabetic). We consider that the losartan-treated diabetic aorta having higher SNP sensitivity than the diabetic one is an effect of losartan-treatment per se, because it was also observed in the losartan-treated controls (data not shown). However, although we think that losartan-treatment per se may be closely related to this effect on the relaxation to SNP and the associated ANG II response, we cannot be sure.

Likewise, the NE-induced contraction was unchanged by the above inhibitors in each of the four groups (Table 2). In contrast, the insulin-induced relaxation was significantly reduced (almost abolished) in control, losartan-treated control, and losartan-treated diabetic aortas by preincubation with PP2 and/or AG17 (Fig. 2, A, C, and D). In the diabetic group, however, this (admittedly very weak) relaxation showed no change following preincubation with PP2 and/or AG17 (Fig. 2B). In each of the four groups of aortas, there was no significant difference in the insulin-induced relaxation among aortas incubated with PP2 alone, AG17 alone, or PP2 plus AG17 (Fig. 2).

Table 2. Maximal responses and EC_{50} values for ACh-, insulin-, and SNP-induced relaxations and NE-induced contraction of aortas from age-matched controls, untreated diabetic, and losartan-treated rats

<table>
<thead>
<tr>
<th>Agonist/Treatment</th>
<th>Age-Matched Control</th>
<th>Untreated Diabetic</th>
<th>Losartan-Treated Control</th>
<th>Losartan-Treated Diabetic</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>E_{max}</td>
<td>pEC_{50}</td>
<td>E_{max}</td>
<td>pEC_{50}</td>
</tr>
<tr>
<td>ACh (6)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Indomethacin (10^{-8} M)</td>
<td>97.2 ± 0.7d</td>
<td>7.6 ± 0.1d</td>
<td>60.3 ± 5.8</td>
<td>5.8 ± 0.4</td>
</tr>
<tr>
<td>–Indomethacin</td>
<td>97.5 ± 0.7</td>
<td>7.5 ± 0.1</td>
<td>66.0 ± 4.2</td>
<td>6.0 ± 0.1</td>
</tr>
<tr>
<td>+PP2 (5 × 10^{-8} M)</td>
<td>91.6 ± 2.2</td>
<td>7.0 ± 0.1</td>
<td>66.9 ± 6.7</td>
<td>6.1 ± 0.2</td>
</tr>
<tr>
<td>+AG17 (10^{-8} M)</td>
<td>96.0 ± 1.2</td>
<td>7.4 ± 0.2</td>
<td>73.8 ± 3.2</td>
<td>6.3 ± 0.1</td>
</tr>
<tr>
<td>Insulin (8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indomethacin (10^{-8} M)</td>
<td>49.1 ± 2.7</td>
<td>5.6 ± 0.1</td>
<td>7.0 ± 1.2e</td>
<td>ND</td>
</tr>
<tr>
<td>–Indomethacin</td>
<td>49.0 ± 3.1</td>
<td>5.5 ± 0.1</td>
<td>6.5 ± 2.0</td>
<td>ND</td>
</tr>
<tr>
<td>+PP2 (5 × 10^{-8} M)</td>
<td>5.6 ± 4.3b</td>
<td>ND</td>
<td>4.1 ± 2.2°</td>
<td>ND</td>
</tr>
<tr>
<td>+AG17 (10^{-8} M)</td>
<td>10.3 ± 2.8b</td>
<td>ND</td>
<td>7.8 ± 1.8°</td>
<td>ND</td>
</tr>
<tr>
<td>Sodium nitroprusside (8)</td>
<td>100.0 ± 0.0</td>
<td>8.4 ± 0.1</td>
<td>98.0 ± 1.5</td>
<td>8.1 ± 0.2</td>
</tr>
<tr>
<td>–Indomethacin</td>
<td>99.9 ± 0.5</td>
<td>8.2 ± 0.1</td>
<td>100.0 ± 1.3</td>
<td>8.2 ± 0.1</td>
</tr>
<tr>
<td>+PP2 (5 × 10^{-8} M)</td>
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<td>8.4 ± 0.1</td>
<td>102.1 ± 1.1</td>
<td>8.4 ± 0.1</td>
</tr>
<tr>
<td>+AG17 (10^{-8} M)</td>
<td>99.0 ± 0.0</td>
<td>8.2 ± 0.2</td>
<td>100.5 ± 0.6</td>
<td>8.1 ± 0.1</td>
</tr>
<tr>
<td>Norepinephrine (8)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Indomethacin (10^{-8} M)</td>
<td>100 ± 4.6</td>
<td>7.6 ± 0.1</td>
<td>95.2 ± 2.8</td>
<td>7.6 ± 0.1</td>
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<tr>
<td>–Indomethacin</td>
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<td>7.8 ± 0.1</td>
<td>107.5 ± 2.4</td>
<td>7.9 ± 0.0</td>
</tr>
<tr>
<td>+PP2 (5 × 10^{-8} M)</td>
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<td>7.7 ± 0.1</td>
<td>103.5 ± 4.5</td>
<td>7.9 ± 0.1</td>
</tr>
<tr>
<td>+AG17 (10^{-8} M)</td>
<td>108.9 ± 4.6</td>
<td>7.9 ± 0.1</td>
<td>101.1 ± 4.3</td>
<td>7.8 ± 0.1</td>
</tr>
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</table>

Values are means ± SE. Number of determinations is shown in parenthesis. SNP, sodium nitroprusside; NE, norepinephrine; ND, not determined; E_{max}, maximal agonist-induced response expressed as % tension/kg tissue; pEC_{50}, −log (corresponding agonist) required to produce 50% of the maximal response. °P < 0.01, †P < 0.001 vs. age-matched control (presence of indomethacin). ‡P < 0.01, §P < 0.001 vs. untreated diabetic (presence of indomethacin). *P < 0.01, ‡P < 0.001 vs. losartan-treated diabetic (presence of indomethacin).
Neither ACh-stimulated NOx production nor ACh-stimulated eNOS phosphorylation was altered by pretreatment with PP2 or AG17 in any of the three groups (Figs. 3A and 4C). Likewise, the insulin-stimulated NOx production and eNOS phosphorylation were decreased in the diabetic group (vs. the controls), and losartan treatment of diabetic rats significantly restored each response (Figs. 3B and 4B). However, each response to insulin was markedly decreased by pretreatment with PP2 and by pretreatment with AG17 in the controls and in the losartan-treated diabetics (Figs. 3B and 4B).

Protein levels of total eNOS, Akt, and PI3K subunits. Next, we used Western blot analysis to examine whether the protein expressions of total eNOS, total Akt, and total PI3K subunits (p85, p110γ, and p110δ) might be abnormal in aortas from diabetic and/or losartan-treated diabetic rats. The aortic expressions of total eNOS and total Akt were significantly increased in the diabetic group (vs. control; Fig. 4, A and D), but these increases were not present in the losartan-treated diabetic group. In contrast, the protein expressions of the total PI3K subunits (p85, p110γ, and p110δ) were not different among the three groups of aortas (Fig. 5A).

Fig. 3. Analysis of NO production, under acetylcholine (ACh; A) or insulin stimulation (B), in aortas from control, diabetic, and losartan-treated diabetic rats. Aortic strips were treated with vehicle (Basal), ACh (10⁻⁷ M), ACh (10⁻⁷ M) + PP2 (5 × 10⁻⁶ M), ACh (10⁻⁷ M) + AG17 (10⁻⁸ M), insulin (10⁻⁷ M), insulin (10⁻⁷ M) + PP2 (5 × 10⁻⁶ M), or insulin (10⁻⁷ M) + AG17 (10⁻⁸ M). Values are means ± SE of 8–10 determinations. ***P < 0.001 vs. ACh- or insulin-stimulated untreated controls. ###P < 0.001 vs. ACh- or insulin-stimulated diabetic. †††P < 0.001 vs. insulin-stimulated losartan-treated diabetic (without inhibitor).

Fig. 2. Effect of PP2 (Src inhibitor) and/or AG17 (Pyk2 inhibitor) on insulin-induced relaxations of aortic strips from controls (A), diabetic (B), losartan-treated control (C), and losartan-treated diabetic (D) rats. Insulin-induced relaxations were inhibited by treatment with PP2 (5 × 10⁻⁶ M) and/or AG17 (10⁻⁸ M) in control, losartan-treated control, and losartan-treated diabetic aortas. Values are means ± SE from 6–8 experiments; SE is shown only when it is larger than the symbol. *P < 0.001, &P < 0.01 vs. controls (without inhibitor). †P < 0.001, ††P < 0.01 vs. losartan-treated controls (without inhibitor). §P < 0.001, §§P < 0.01 vs. losartan-treated diabetic (without inhibitor).
Protein levels of phosphorylated Thr<sup>408</sup>Akt and Ser<sup>241</sup>PKD1 and effects of insulin. In the unstimulated (basal) condition, the Akt phosphorylation levels were not different among the three groups of aortas. In aortas stimulated with insulin, the level of phosphorylated Akt was significantly decreased in the diabetic group (vs. the controls), and losartan treatment restored its level (Fig. 4E). Although pretreatment with PP2 or AG17 did not alter ACh-stimulated Akt phosphorylation in any of the three groups of aortas (data not shown), each inhibitor markedly decreased the insulin-induced stimulation of the Akt phosphorylation in the controls (Fig. 4E). In the diabetic group, neither inhibitor altered the insulin-induced stimulation of Akt phosphorylation, but each inhibitor significantly decreased this response in the losartan-treated diabetic group (Fig. 4E).

As shown in Fig. 5B, the insulin-stimulated aortic phospho-PDK1 level was significantly decreased in the diabetic group (vs. the controls; Fig. 5A) but not different between the losartan-treated diabetic group and the controls (Fig. 5B). The effects of PP2 and AG17 on the insulin-stimulated PDK1 phosphorylation levels (Fig. 5B) were similar to those (see above) on the insulin-stimulated Akt phosphorylation levels across the three groups of aortas (viz. a significant decrease by each inhibitor in control and losartan-treated diabetic but no change with either inhibitor in the diabetic group).

Protein levels of phosphorylated Tyr<sup>416</sup>Src and Tyr<sup>579</sup>Pyk2 and effects of insulin. In the unstimulated (basal) condition, the Src phosphorylation levels did not differ among the three groups of aortas. In aortas stimulated with insulin, the level of phosphorylated Src was significantly decreased in the diabetic group (vs. the controls), and losartan treatment restored it (Fig. 5C). Pretreatment with neither PP2 nor AG17 altered the insulin-stimulated Src phosphorylation level in the diabetic group, but each inhibitor markedly decreased this response in the controls and in the losartan-treated diabetic group (Fig. 5C).

As shown in Fig. 5D, the insulin-stimulated aortic phospho-Pyk2 level was 1) significantly decreased in the diabetic group (vs. the controls; Fig. 5A), but was 2) not different between the losartan-treated diabetic group and the controls (Fig. 5B). Interestingly, the insulin-stimulated Pyk2 phosphorylation level was not changed by pretreatment with the Src inhibitor PP2 in either the controls or the losartan-treated diabetic group, whereas AG17 treatment markedly decreased it in both of those groups. Thus the results obtained for the insulin-stimulated phosphorylation levels of Pyk2/Src/PDK1/Akt were similar to those described above for insulin-stimulated eNOS activity/NO production in the diabetic and losartan-treated diabetic aortas. The pharmacological inhibition of Pyk2 is problematic, since no specific inhibitors have been demonstrated to date. AG17 is primarily known as a PDGF-receptor tyrosine kinase inhibitor. Although this substance has been shown to inhibit Pyk2 activation in a few settings (46), it is by no means certain that Pyk2 is a direct target of AG17. Therefore, we looked for evidence that might support the role of...
Pyk2 using a nonpharmacological approach, namely immunohistochemical technique (Fig. 6A). In the endothelial layer, the insulin-stimulated aortic phospho-Pyk2 level was apparently decreased in the diabetic group (vs. the controls; Fig. 6A) but not different between the losartan-treated diabetic group and the controls (Fig. 6B). These results were similar to those obtained by Western blotting.

Protein levels of phosphorylated Ser307 IRS-1 and effects of losartan treatment. In aortas stimulated with insulin, the level of phosphorylated IRS-1 was significantly increased in the diabetic group (vs. the controls), and losartan treatment normalized this level (Fig. 6B).

DISCUSSION

The most important observations made in the present study were that 1) in aortas isolated from untreated GK type 2 diabetic rats, which are animals exhibiting hyperinsulinemia and insulin resistance, both insulin-induced relaxation and NOS activity were greatly impaired, possibly due to the presence of reductions in Pyk2/Src/PDK1/Akt activities; and 2) after 2-wk losartan treatment of such diabetic rats, aortas displayed improvements of these abnormalities.

The mechanisms underlying the hypertension and vascular diseases observed in association with hyperinsulinemia may be related to changes in endothelial function (22, 23) and/or to increased activity within the sympathetic nervous system (39). In the diabetic model used in the present experiments, the untreated diabetic rats and the losartan-treated diabetic rats had markedly raised plasma glucose and plasma insulin levels (vs. the corresponding controls), but the insulin resistance index HOMA-IR was markedly restored by losartan treatment, suggesting that diabetic rats treated with losartan (an ARB) may display improved insulin sensitivity. Our laboratory’s recent studies (18, 20) led us to suspect that for diabetes-related endothelial dysfunction to develop, a high insulin level and an established diabetic state may need to coexist. If so, high insulin alone would not be sufficient to account for endothelial impairment, at least in the rat. Although our study found that both endothelial function and NO production are impaired in aortic strips from the present diabetic model, these results seem
to conflict with our findings that the eNOS and Akt protein levels are increased in diabetic aortas. A plausible explanation may be that in intact cells NO synthesis can be regulated independently of changes in eNOS/Akt enzymes. We previously reported increased eNOS protein levels, but not increased relaxation responses to ACh in 36-wk-old GK rats (20), and that in streptozotocin-induced diabetic rats with accompanying hyperinsulinemia, but not in control rats with hyperinsulinemia, insulin-stimulated Akt and eNOS protein levels were enhanced (22). We now suspect that a high insulin level, a diabetic state, and other factors (including a raised plasma ANG II) may need to exist together to cause an increased insulin resistance-induced endothelial dysfunction (e.g., reduced activities of Akt and eNOS).

Many stimuli (including insulin, VEGF, \(\alpha_2\)-agonists, and shear-stress signals) regulate NO production by activating eNOS via Ser\(^{1177}\) phosphorylation through the Akt pathway (7, 17, 12, 23, 35, 47). ACh is known to cause an intracellular release of \(\text{Ca}^{2+}\) from its stores and an influx of extracellular \(\text{Ca}^{2+}\) into endothelial cells (8, 17), leading to activation of eNOS via \(\text{Ca}^{2+}\)/calmodulin-dependent mechanisms (Fig. 7). In vitro, various techniques are used to study the arterial smooth muscle cell’s calcium dynamics and contraction/relaxation mechanisms. Most experimental studies use either an isometric or an isobaric setup. We chose the isometric setup, because Koenigsberger et al. (24) demonstrated that vasoconstrictor sensitivity is higher in isometric than in either isobaric or isotonic conditions, in agreement with other experimental observations. When a Src inhibitor (PP2) or Pyk2 inhibitor (AG17) was applied to rat aortas in the present study, there was no effect on ACh-induced relaxation or on ACh-stimulated NO production in any group, whereas the responses (relaxation, NO production, NOS phosphorylation, and Akt phosphorylation) induced by insulin in control aortas were markedly inhibited by PP2 and also by AG17. Our laboratory (22) previously published evidence that in rat aortas the insulin-induced vasorelaxation, but not the ACh-induced one, is regulated by the PDK1/Akt/eNOS signaling pathway. Collectively, the above observations suggest that at least in rat aorta insulin-induced vasorelaxation may be regulated by activation of the Pyk2/Src/PDK1/Akt signaling pathway, whereas upon ACh stimulation the Pyk2/Src/PDK1/Akt pathway may not be

Fig. 6. A: immunohistochemical staining for Pyk2 phosphorylation at Tyr\(^{579}\) or eNOS in aortic sections in slides of aortas obtained from control (a, d, and g), diabetic (b, e, and h), and losartan-treated diabetic (c, f, and i) rats. Aortas were examined for eNOS (a–c) or Tyr\(^{579}\) phospho-Pyk2 (d–i) protein levels, the latter under insulin-stimulation without (d–f) or with (g–i) AG17 (10\(^{-8}\) M). Positive staining is shown as red. Original magnification = \(\times 100\). Representative pictures from 5 to 6 independent experiments. Upper area is lumen. EC, endothelial cells. B: analysis of insulin receptor substrate-1 (IRS-1) phosphorylation at Ser\(^{307}\) under insulin (10\(^{-7}\) M) stimulation in aortic strips from control, diabetic, and losartan-treated diabetic rats. Top: representative Western blots. Bottom: several bands were quantified by scanning densitometry. Optical-density ratios were calculated for phosphorylated IRS-1 over IRS-1. Lanes were run on the same gel but were noncontiguous. **P < 0.01 vs. control. ###P < 0.01 vs. diabetic. Values are means ± SE of 6 to 8 determinations.

Fig. 7. Endothelium-dependent relaxation via the Pyk2/Src/PDK1/Akt and Ca\(^{2+}\)/calmodulin pathways. Relaxation responses induced by acetylcholine need an increase in intracellular Ca\(^{2+}\) for NO production. In contrast, relaxation responses induced by insulin do not need an increase in intracellular Ca\(^{2+}\), for NO production and are linked to the Akt pathway. In response to various stimuli, phosphorylated Pyk2/Src phosphorylates PDK1, the phosphorylated PDK1 phosphorylates Akt, and the phosphorylated Akt phosphorylates eNOS, resulting in NO production. It is likely that in the present type II diabetic model, the activity of the Pyk2/Src/PDK1/Akt pathway is decreased, and thus endothelium-derived relaxation responses are impaired. We hypothesize that angiotensin II (ANG II) causes impairment of the insulin-signaling pathway through phosphorylation of IRS-1 at Ser\(^{307}\). Furthermore, losartan antagonizes the ANG II -induced abnormalities present in the diabetic state. AT\(_1\)-R, angiotensin type 1 receptor; CaM, calmodulin; M\(_3\)-R, muscarinic receptor subtype 3.
necessary for either vasorelaxation or increased NO production (Fig. 7).

A major finding made in this study was that in aortas from type 2 diabetic rats with hyperinsulinemia, the mechanisms underlying endothelial dysfunction may include impairments in the activities of IRS-1 and the Pyk2/Src/PDK1/Akt pathway. We found evidence strongly suggesting that in our diabetic model the observed impairments of insulin-induced aortic relaxation and NO production are due to decreases in both IRS-1 and IRS-2. We therefore hypothesized that IRS-1/Pyk2/PDK1 activities might be altered in type 2 diabetes. Interestingly, the insulin-induced phosphorylations of Pyk2 and Src were decreased in the diabetic group (vs. the controls), whereas in the losartan-treated diabetic group each of these responses was increased (vs. diabetic) to a level not different from that in the controls. Thus in our insulin-stimulation experiments the above results obtained for the phosphorylation levels of Pyk2 and Src were similar to those described (in an earlier paragraph) for eNOS activity/NO production in diabetic and losartan-treated diabetic aorta. These results suggest that Pyk2 and Src phosphorylations are closely related to the aortic relaxation response to insulin, and 2) that the impaired insulin-induced relaxation response seen in the diabetic aorta may be the result of decreased phosphorylation of Pyk2 and/or Src. In a previous report (29), eNOS was evidently phosphorylated at the Tyr657 residue by ANG II through Pyk2 in human umbilical vein endothelial cells. Another report (43) suggested that ANG II acts against Pyk2 via both phosphorylation through Yes kinase (one of the Src kinase family) and dephosphorylation through SH2-containing inositol phosphatase (SHIP)-2 (tyrosine phosphatase) in rat pulmonary vein endothelial cells. Furthermore, in type 2 diabetic db/db mice, increased activation of SHP2 was associated with the progression of insulin resistance (14). These results lead us to speculate that Pyk2 is dephosphorylated by ANG II through SHP-2 and that this culminates in impairment of the Akt/eNOS pathway. Along with this phenomenon, Pyk2 may be phosphorylated at the above distinct tyrosine residue by ANG II through Yes kinase. The consequence may be enhanced tyrosine phosphorylation of eNOS. On the other hand, differential effects on Pyk2 may result from complicated regulation of phosphorylation sites and/or from Pyk2 forming a complex with certain other molecules (Src kinase, etc.).

Several studies (4, 13, 27) have demonstrated that inhibition of the RAS (e.g., by treatment with an ACEI or ARB) has beneficial effects on diabetic vasculopathy. Our results are consistent with a model of losartan treatment of diabetes in which signal enhancement increases insulin-stimulated NO production and Pyk2/Src/PDK1/Akt/eNOS pathway activity, with insulin-induced vasorelaxation being thereby restored following such treatments. The major pathway for insulin signaling is the Pyk2/Src/PDK1/Akt pathway, and conceivably the impairment of insulin-induced Pyk2/Src/PDK1/Akt activity observed in the present GK diabetic group could have been secondary to the rats’ high plasma insulin levels and/or ANG II levels. Actually, of the two models used in our experiments: 1) one, the GK diabetic rat, exhibited a markedly increased plasma insulin level but decreased insulin-stimulated Pyk2/Src/PDK1/Akt/IRS-1/NOS activities in the aorta, while 2) the other, the losartan-treated diabetic rat, exhibited marked decreases (vs. the losartan-untreated diabetic rat) in its plasma insulin level and in its HOMA-IR and also in its phosphorylation of IRS-1. Thus the level of insulin sensitivity would appear to be directly related to the level of the associated IRS-1/Pyk2/Src/PDK1/Akt/IRS-1/NOS activities, and in the diabetic state the magnitude of any decrease in insulin sensitivity would appear to be directly related to the magnitude of any endothelial dysfunction. The present results suggest that long-term (daily for 2 wk) administration of losartan can normalize diabetes-related impairments of insulin-induced relaxation and Pyk2/Src/PDK1/Akt/IRS-1/NOS activities, at least in the rat aorta, and that the effects of losartan that are related to the increase in insulin sensitivity this ARB induces may be mediated via decreased IRS-1 phosphorylation.

A recent study by Matsui and coworkers found that Pyk2 is bound to Src but not to the PI3K p85 subunit (31). Moreover, in the GK rat, IRS-1 tyrosine phosphorylation and PI3K activity were stimulated by insulin, and these effects of insulin were reduced by association of the p85 regulatory subunit of PI3K with IRS-1 (40). Furthermore, a reduction in PI3K activity may play a role in the pathophysiology of insulin-resistant diabetic states, such as that seen in the ob/ob mouse (10). The present data (Fig. 5A) suggest that insulin-stimulated expressions of total PI3K subunits did not differ among the three groups of aortas (control, diabetic and losartan-treated diabetic). We speculate that an important feature of the GK diabetic rat may be altered PI3K phosphorylation levels or alternatively the formation of heterodimer complex (es) involving PI3K subunits.

In conclusion, our data reveal that in rat aorta insulin induces endothelium-dependent relaxation, NO production, and eNOS activity levels in the IRS-1/Pyk2/Src pathway. Our GK type 2 diabetic model (diabetic rats exhibiting insulin resistance and hyperinsulinemia) exhibits impairments of endothelium-dependent relaxation and the Pyk2/Src/PDK1/Akt pathway that occur as a result of its disease and may be mediated by IRS-1 phosphorylation. In 2-wk losartan-treated GK diabetic rats, there was both a normalization of insulin-induced aortic relaxation and an enhanced insulin sensitivity. These effects of losartan appear to be due to increases in both NO production and NOS phosphorylation that are mediated by increased activity levels in the IRS-1/Pyk2/Src pathway (Fig. 7).

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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