Pravastatin normalizes ET-1-induced contraction in the aorta of type 2 diabetic OLETF rats by suppressing the KSR1/ERK complex

Nemoto S, Taguchi K, Matsumoto T, Kamata K, Kobayashi T. Pravastatin normalizes ET-1-induced contraction in the aorta of type 2 diabetic OLETF rats by suppressing the KSR1/ERK complex. Am J Physiol Heart Circ Physiol 303: H893–H902, 2012. First published August 10, 2012; doi:10.1152/ajpheart.01128.2011.—Endothelin (ET)-1 is a likely candidate for a key role in diabetic vascular complications. In the present study, we hypothesized that treatment with pravastatin (an inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase) would normalize the ET-1-induced contraction in aortas isolated from type 2 diabetic Otsuka Long-Evans Tokushima fatty (OLETF) rats. Contractile responses were examined by measuring isometric force in endothelium-denuded aortic helical strips from four groups: Long-Evans Tokushima Otsuka (LETO; genetic control), OLETF (type 2 diabetic), pravastatin-treated LETO, and pravastatin-treated OLETF rats. Both immunoblot analysis and immunoprecipitation assays were used to examine Src, protein phosphatase (PP)2A, kinase suppressor of Ras (KSR1), and ERK signaling pathway protein levels and activities. In endothelium-denuded aortas isolated from OLETF rats at the chronic stage of diabetes (56–60 wk) (vs. those from age-matched LETO rats), we found the following: 1) ET-1-induced contraction was enhanced, 2) ERK1/2 phosphorylation was increased, 3) phosphorylations of KSR1 and PP2A were reduced (i.e., enhancement of the kinase active state), 4) ERK1/2-KSR1 complexes were increased, and 5) Src tyrosine kinase activity was diminished. Endothelium-denuded aortas isolated from OLETF rats treated with pravastatin (10 mg/kg po, daily for 4 wk) exhibited normalized ET-1-induced contractions and suppressed ET-1-stimulated ERK phosphorylation, with the associated phosphorylated KSR1 and phosphorylated PP2A levels being increased toward normal levels. These results suggest that in type 2 diabetic rats, pravastatin normalizes ET-1-induced contraction in aortic smooth muscle via a suppression of PP2A/KSR1/ERK activities after an enhancement of Src kinase activity.

endothelin-1; extracellular signal-regulated kinase; mitogen-activated protein kinase; protein phosphatase 2A; Src tyrosine kinase; kinase suppressor of Ras-1; Otsuka Long-Evans Tokushima fatty rat

IN RECENT YEARS, the worldwide prevalence of type 2 diabetes has increased significantly, and this is popularly referred to as a “diabetes time bomb” since its consequences may be felt for many years. Type 2 diabetes is associated with a markedly enhanced incidence of cardiovascular diseases. Indeed, it is believed that an impaired ability to vasodilate and/or an enhanced sensitivity to vasoconstrictor agonists underlie the vascular dysfunction associated with diabetes (12, 15, 22). Endothelin (ET)-1 is a potent vasoconstrictor peptide (39, 40) that is considered to play important roles in the physiological control of blood pressure and cardiac function as well as in the genesis and development of cardiovascular diseases such as atherosclerosis, the cardiac remodeling accompanying chronic heart failure, and pulmonary hypertension (40). Interesting data from patients with type 2 diabetes have revealed increased circulating levels of ET-1 and a positive correlation between plasma ET-1 levels and vasculopathy (6). Collectively, these observations suggest a role for ET-1 in the pathogenesis of diabetic vascular complications (2, 3, 6, 45). Indeed, abnormal ET-1 signaling has been reported to be involved in diabetes-related vasculopathy (15, 19, 20, 26), and, moreover, both ET-converting enzyme/ET-1 expression and MAPK activity have been reported to be increased in the Otsuka Long-Evans Tokushima fatty (OLETF) rat, a model of type 2 diabetes (11). The MAPK family, which consists of three isoforms, performs central functions in the intracellular signal transduction initiated by extracellular stimuli, including growth factors and hormones (38). Two isoforms, ERK and MEK, are activated by ET-1, leading to smooth muscle contraction (14, 20, 30).

It is known that kinase suppressor of Ras (KSR1) is a conserved component of the Ras pathway that, in vitro, acts as a molecular scaffold to facilitate signal transmission through the MAPK cascade (1, 31, 36). Consistent with the idea of MAPK scaffolds, KSR1 interacts with the kinase components of the ERK cascade and facilitates signal transmission from Raf-1 to MEK and ERK (31). Moreover, recent studies using RNA interference to investigate Drosophila melanogaster and Caenorhabditis elegans KSR have confirmed that KSR is required for Ras-mediated ERK pathway activation (1, 36). Although the recruitment of KSR1 is required for its scaffolding function, the precise mechanism(s) involved in diabetic vasculopathy has not been fully elucidated. In 2003, Ory et al. (37) determined that KSR1 is a novel substrate of protein phosphatase (PP)2A and that inducible dephosphorylation of KSR1 occurs in response to Ras pathway activation in COS and NIH 3T3 cells. KSR1 and Raf-1 are localized to the cytoplasm, and they constitutively interact with 14-3-3 dimers and the core subunits of PP2A (A and C subunits) in quiescent cells. The 14-3-3 dimer binds to the Ser297 and Ser392 phosphorylation sites of KSR1 and to the Ser259 and Ser421 phosphorylation sites of Raf-1. In addition, MEK and Cdc25C-associated kinase 1 are constitutive components of the KSR1 complex. In contrast, Ras activation induces the binding of PP2A to KSR1 and Raf-1 complexes, increasing PP2A activity and resulting in the dephosphorylation of KSR1 at Ser392 and of Raf-1 at Ser259 in stimulated cells. These dephosphorylation events contribute to the plasma membrane targeting of both proteins. In the case of Raf-1, dephosphorylation at Ser259 and release of 14-3-3 have been reported to facilitate the Ras-Raf interaction mediated by the Ras-binding domain of Raf-1. In the case of KSR1, dephosphorylation at Ser392 and release of 14-3-3 appears to expose the C1 domain, which is required for

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the membrane localization of KSR1, as well as the FXFP MAPK-binding site. At the membrane, Raf-1 is activated, and KSR1 provides a platform for the phosphorylation/activation of associated MEK and ERK (37). An interesting hypothesis for the present study was that stimulation of PP2A/KSR1 is followed by the activation of the downstream kinase MAPK family and that this might be, in turn, linked to vascular hyperreactivity.

Pravastatin [a hydrophilic inhibitor of 3-hydroxy-3-methyl-glutaryl (HMG)-CoA reductase] exerts lipid-independent protective effects, and these have been ascribed improvements in rat mesangial cells under high glucose (49). High-glucose conditions increase MAPK activity in these cells (vs. low glucose), and pravastatin treatment suppresses this enhancement of MAPK activity (49). Moreover, pravastatin treatment reportedly prevented cardiomyocyte cell death after simulated hypoxia and reoxygenation, a preventive effect partly related to a decrease in myocyte ET-1 actions (46). Although pravastatin is used in hyperglycemic states to normalize MAPK signaling and the action of ET-1 independently of its lipid-lowering effects, the underlying mechanism remains poorly understood. The present experiments were designed to test the hypothesis that pravastatin has a therapeutic action against diabetic abnormalities in ET-1-induced vasoconstriction. In this study, we investigated 1) whether there were enhancements of ET-1-induced contraction and the MEK/ERK pathway in descending thoracic aortas (without endothelial cells) isolated from OLETF rats (56–60 wk old) at the chronic diabetic stage [vs. age-matched Long-Evans Tokushima Otsuka (LETO) genetic control rats], 2) whether PP2A/KSR1 signaling was abnormal in the OLETF group, and 3) whether pravastatin (10 mg/kg po, daily) would have beneficial effects on the aortic contractile response to ET-1 after its administration in vivo for 4 wk after hyperglycemia had developed.

MATERIALS AND METHODS

Antibodies and reagents. Cantharidic acid, PP inhibitor 2 human recombinant Escherichia coli (PP2), and PP2 (Src inhibitor) were from Calbiochem (La Jolla, CA). ET-1 and PGE2 were from Peprotech (Rocky Hill, NJ). Recombinant Escherichia coli (room temperature: 21–22°C, room humidity: 50–55%) until

Measurement of blood parameters and blood pressure. Plasma or serum parameters and blood pressure under nonfasted conditions were measured as previously described (16–18, 20–22, 32). Briefly, plasma glucose, total cholesterol, triglyceride, HDL-cholesterol, and serum nonesterified fatty acid (NEFA) levels were each determined using a commercially available enzyme kit (Wako Chemical, Osaka, Japan). Plasma insulin was measured using an enzyme immunoassay (Shibayagi, Gunma, Japan). After a given rat had been in a constant-temperature box at 37°C for a few minutes, its blood pressure was measured by the tail-cuff method using a blood pressure analyzer (BP-98A, Softron, Tokyo, Japan). Tail-cuff systolic blood pressures (SBPs) were compared among the above four groups (n = 31 or 32 rats/group). These were each randomly subdivided into four teams of n = 7 or 8 rats/team. Rats were acclimated for 7 days to the measurement device (30 min/exposure to the device, 1 exposure/day) and measured at 7 days and 2 days before a given animal was killed under anesthesia. The data reported here are SBP values measured on days before death.

Measurement of isometric force. Vascular isometric force was recorded as in our laboratory’s previous reports (16–18, 20–22, 32). At 56–60 wk of age, after rats had been killed, the thoracic aorta was rapidly removed and immersed in oxygenated, modified Krebs-Henseleit solution. This solution consisted of (in mM) 118.0 NaCl, 4.7 KCl, 25.0 NaHCO3, 1.8 CaCl2, 1.2 NaH2PO4, 1.2 MgSO4, and 11.0 dextrose. The artery was carefully cleaned of all fat and connective tissue, and the aortic strips 3 mm in width and 20 mm in length were suspended in a well-oxygenated (95% O2–5% CO2) bath containing 10 ml Krebs-Henseleit solution at 37°C. Strips were stretched until an optimal resting tension of 1.0 g was reached and then allowed to equilibrate for at least 60 min. Force generation was monitored by means of an isometric transducer (model TB-611T, Nihon Kohden, Tokyo, Japan).

For the contraction experiments, ET-1 (10–10–10–7 M) or PGE2 (10–9–10–5 M) solutions were added cumulatively to the bath until a maximal response was achieved. To investigate the effects of cantharidic acid (5 x 10–8 M, a PP2A inhibitor), DMSO [final concentration: 0.0025% (vol/vol) DMSO in the organ bath, the vehicle for cantharidic acid], or PP2 (10–9 M, a PP1 inhibitor) on the ET-1-induced contractile response, strips were incubated for 30 min in medium containing the appropriate inhibitor (or vehicle) before the cumulative addition of agonist.

For removal of the endothelium, the intimal surface was rubbed with a cotton swab, with successful removal being functionally confirmed by the absence of a relaxation to 10 μM Ach. The same procedure was used for the experiments described below (e.g., immunoblot sampling).

Immunoblot analysis. Immunoblot analysis was performed as previously described by our laboratory (16–18, 20–22, 32, 33). Aortic strips were incubated with 10 nM ET-1 for 20 min at 37°C. For inhibitor experiments (also at 37°C), tissues were pretreated with 50 nM cantharidic acid or with 5 μM Src inhibitor for 30 min before the addition of ET-1 (or the addition of vehicle, final concentrations: 0.0025% (vol/vol) DMSO) in siliconeized tubes. Tissues were then

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frozen in liquid N\textsubscript{2} before being physically crushed to a fine powder in liquid N\textsubscript{2} using a Cryo-Press (Microtech Nichion, Chiba, Japan). Aortic tissues were homogenized in ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS containing protease and phosphatase inhibitor cocktails (Complete Protease Inhibitor Cocktail and PhosSTOP, Roche Diagnostics, Indianapolis, IN). The lysate was cleared by centrifugation at 16,000 g for 10 min at 4°C. The supernatant was collected, and proteins were solubilized in Laemmli’s buffer containing mercaptoethanol. Protein concentrations were determined by means of a bicinchoninic acid protein assay reagent kit (Pierce, Rockford, IL). Samples (24 μg/lane) were resolved by electrophoresis on 10% SDS-PAGE gels and then transferred onto polyvinylidene difluoride membranes. Briefly, after residual protein sites on the membrane were blocked with Immunoblock (Dainippon Pharm, Osaka, Japan) or polyvinylidene difluoride blocking reagent (Millipore), the membrane was incubated with anti-ERK1/2 (1:1,000), anti-p-ERK1/2 (1:1,000), anti-KSR1 (1:1,000), anti-MEK1/2 (1:100), anti-p-MEK1/2, anti-PP2A (1:1,000), anti-p-PP2A (1:1,000), anti-Src (1:1,000) antibody in blocking solution. Horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody was used at a 1:10,000 dilution in Tween-PBS. Sections of the rat aorta enclosed in Vectashield Mounting Medium (Vector Laboratories) were washed three times in Tween-PBS. They were then imaged using a Zeiss LSM 5 confocal laser microscope (Carl Zeiss Microscopy).

Statistical analysis. The contractile force developed by helical strips of the aorta was expressed as a percentage of the response to 80 mM KCl. Data are expressed as means ± SE. Statistical differences were assessed using the Bonferroni test for multiple comparisons after one- or two-way ANOVA, with P values of <0.05 being regarded as significant. Two-way ANOVA was used for the comparison of concentration-response curves between groups (Graph Pad Prism 5.0, GraphPad Software, San Diego, CA). For comparison with the “control” ET-1 group, data were analyzed using ANOVA with post hoc Bonferroni testing. Immunoblot data were analyzed by ANOVA with post hoc Bonferroni testing. P values of <0.05 were considered statistically significant.

RESULTS

General parameters. It has been previously established by Kawano et al. (13) that OLETF rats display stable clinical and pathological features that resemble those of human type 2 diabetes; indeed, OLETF rats exhibit hypertension, hyperglycemia, and hyperlipidemia (13, 47). As shown in Table 1, the heart weight-to-body weight ratio, SBPs, and nonfasted blood glucose, total cholesterol, triglyceride, and NEFA concentrations were significantly higher in OLETF rats than in LETO control rats (also nonfasted). Treatment of OLETF rats with pravastatin improved some of the parameters (total cholesterol and NEFA) versus untreated OLETF rats, although LETO levels were not reached. In contrast, pravastatin treatment did not affect the above parameters in LETO nondiabetic rats.

Contraction response to ET-1. Cumulative administration of ET-1 (10⁻¹⁰–10⁻⁷ M; Fig. 1) induced a concentration-dependent contraction in endothelium-denuded aortic strips from}

### Table 1. Values of various parameters in pravastatin-treated and untreated LETO and OLETF rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>LETO Group</th>
<th>OLETF Group</th>
<th>Pravastatin-Treated LETO Group</th>
<th>Pravastatin-Treated OLETF Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Means ± SE</td>
<td>Number of determinations</td>
<td>Means ± SE</td>
<td>Number of determinations</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>570.7 ± 8.2</td>
<td>31</td>
<td>551.3 ± 17.0</td>
<td>32</td>
</tr>
<tr>
<td>Heart weight-to-body weight ratio</td>
<td>2.78 ± 0.04</td>
<td>30</td>
<td>3.20 ± 0.07*</td>
<td>30</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>116.3 ± 1.9</td>
<td>16</td>
<td>157.9 ± 1.6*</td>
<td>16</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>144.6 ± 3.3</td>
<td>16</td>
<td>554.7 ± 34.7*</td>
<td>16</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>2.7 ± 0.2</td>
<td>16</td>
<td>2.5 ± 0.2</td>
<td>16</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>126.7 ± 10.0</td>
<td>16</td>
<td>267.8 ± 18.9*</td>
<td>16</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>417.6 ± 9.0</td>
<td>16</td>
<td>470.5 ± 51.8*</td>
<td>16</td>
</tr>
<tr>
<td>HDL, mg/dl</td>
<td>92.5 ± 7.2</td>
<td>16</td>
<td>79.5 ± 4.8</td>
<td>16</td>
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<tr>
<td>Nonesterified fatty acids, meq/l</td>
<td>0.22 ± 0.01</td>
<td>16</td>
<td>0.55 ± 0.04*</td>
<td>16</td>
</tr>
</tbody>
</table>

LETO, Long-Evans Tokushima Otsuka rat; OLETF, Otsuka Long-Evans Tokushima fatty rat. *P < 0.001 vs. the LETO group; †P < 0.05 and ‡P < 0.001 vs. the OLETF group; §P < 0.05, ¶P < 0.01, and ††P < 0.001 vs. the pravastatin-treated LETO group.

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Pravastatin-treated or untreated LETO or OLETF rats. The ET-1-induced contractile response was significantly greater (3 × 10⁻⁸–10⁻⁷ M) in OLETF aortas than in LETO aortas (Fig. 1, A and B). After pravastatin treatment of OLETF rats but not of LETO rats, ET-1-induced contractions were significantly weaker at the above concentrations (Fig. 1, A and B). In contrast, PGE2-induced contractions did not differ among the four groups (data not shown).

To investigate the possible contribution made by PP2A signaling to ET-1-induced contraction in the endothelium-denuded rat aorta, we added ET-1 cumulatively to aortas in the presence of a PP2A inhibitor (cantharidic acid) or a PP1 inhibitor (PPI2). Cantharidic acid (5 × 10⁻⁸ M) treatment decreased the ET-1-induced contraction of endothelium-denuded aortic strips in the OLETF group (Fig. 2B) but not in the other three groups (Fig. 2, A and B). In contrast, such contractions were unchanged by treatment with 10⁻⁹ M PPI2 in any group (data not shown).

Effects of pravastatin treatment on ET-1-stimulated ERK and KSR1 activations. ET-1 modulates vascular tone through the activation of MAPK pathways, including the ERK pathway (20, 30). To investigate whether such ERK pathway activation was altered by pravastatin treatment, we measured ET-1-stimulated ERK phosphorylation (active state) in endothelium-denuded aortas. ET-1-stimulated ERK phosphorylation was significantly elevated in the OLETF group (vs. the LETO group; Fig. 3A, top bands). Moreover, we studied KSR1 expression in endothelium-denuded aortas (Fig. 4, A and B), its phosphorylation (at an inactivating site; Fig. 4C), and its interaction with ERK (Fig. 3B) using immunoprecipitation. KSR1 was expressed in rat aortic smooth muscle (Fig. 4, A and B), and ET-1-stimulated KSR1 phosphorylation was significantly decreased in the OLETF group (vs. the LETO group; Fig. 4C). In contrast, ET-1-stimulated ERK binding to KSR1 was greater in OLETF aortas than in LETO aortas, and pravastatin treatment normalized such binding in the OLETF group (Fig. 3B). We also investigated the effect of pretreatment with 5 × 10⁻⁸ M cantharidic acid (a PP2A inhibitor) on ET-1-induced ERK activation. In endothelium-denuded aortas from the OLETF group, but not in the other two groups, treatment with pravastatin normalized both ERK phosphorylation and ERK binding to KSR1 (Fig. 3, A and B). There was decreased KSR1 phosphorylation in aortas from the OLETF group compared with the other two groups, and this decreased level was normalized by cantharidic acid treatment (Fig. 4C).

Effects of pravastatin treatment on ET-1-stimulated MEK binding to KSR1. The above results led us to speculate that the enhancement of ET-1-induced vasoconstriction seen in the OLETF group might be caused by abnormal activity in the MEK/ERK classical pathway. In 2003, Ory et al. (37) reported that KSR1 binding to MEK/ERK is regulated by changes in PP2A activity. Thus, we investigated whether MEK binding to KSR1, and PP2A activity, might be altered by diabetes (i.e., OLETF) and normalized by pravastatin treatment. First, we
We then investigated the Src phosphorylation level and found it to be significantly greater in the OLETF group than in the other groups with or without ET-1 stimulation (Fig. 6B). Cantharidic acid treatment normalized both PP2A binding to KSR1 (Fig. 6A) and PP2A phosphorylation (Fig. 6B) in the OLETF group but had no effects in the LETO and pravastatin-treated OLETF groups (Fig. 6). Treatment with pravastatin normalized both the increased KSR1-PP2A complexes and decreased PP2A phosphorylation seen in the smooth muscle of ET-1-stimulated and unstimulated OLETF aortas (Fig. 6).

These results suggest that in endothelium-denuded aortas from OLETF rats, PP2A/KSR1 activation causes ERK binding to KSR1 and ET-1-induced ERK phosphorylation. Furthermore, they suggest that 1) PP2A increases KSR1-ERK complexes, but not KSR1-MEK binding, and 2) that the enhancement of ET-1-induced vasoconstriction seen in OLETF rat aortic smooth muscle occurs as a result of the ERK activation caused by the elevation in PP2A activity via KSR1 dephosphorylation (active state).

Effects of pravastatin treatment on diabetes-related alterations in Src tyrosine kinase. Finally, we examined Src tyrosine kinase because Chen et al. (4) previously reported that in response to EGF or insulin, PP2A was phosphorylated at Tyr1070 through Src tyrosine kinase, thereby causing PP2A inactivation. Src inhibitor pretreatment significantly suppressed PP2A activity in LETO and pravastatin-treated OLETF aortas (Fig. 6B). We then investigated the Src phosphorylation level and found it to be significantly lower in endothelium-denuded aortas from OLETF rats than in those from LETO rats with or without ET-1 stimulation (Fig. 7). Treatment of OLETF rats with pravastatin normal-
ized the p-Src level, whereas cantharidic acid treatment did not affect Src phosphorylation in any group (Fig. 7).

**DISCUSSION**

Our study is the first to demonstrate that the enhancement of ET-1-induced vasoconstriction seen in the OLETF rat aorta is caused by an increase in KSR1/ERK complexes after PP2A activation and that this may be attributable to diabetes inducing Src inactivation constitutively. We also showed that beneficial effects of pravastatin occurred in the OLETF rat without an antihypercholesterolemic effect. These conclusions are based on several observations related to the ET-1 signaling pathway. First, 4-wk pravastatin treatment decreased the elevated levels of total cholesterol and NEFAs seen in type 2 diabetic OLETF rats,
although these did not reach the levels seen in LETO control rats. Second, PP2A, KSR1, and ERK activities as well as KSR1/ERK complexes were increased in OLETF rats compared with LETO rats, and treatment with pravastatin normalized such kinase activities. Third, using the organ bath technique, we showed that there was increased ET-1-induced vasoconstriction in OLETF rats (in endothelium-denuded aortas) and that this was normalized by pravastatin treatment. These findings identify a novel signaling cascade for ET-1 in rat aortic smooth muscle and pleiotropic effects of pravastatin, which may be important for the long-term regulation of vascular homeostasis in type 2 diabetes with vascular complications.

In our OLETF model, the heart weight-to-body weight ratio, SBP, and nonfasted glucose, total cholesterol, triglyceride, and NEFA levels were all increased (vs. LETO rats). When we administered pravastatin for 4 wk to such established diabetic OLETF rats, the treatment did not alter (vs. pravastatin-untreated OLETF rats) SBP or nonfasted glucose, insulin, triglyceride, or HDL-cholesterol levels, although it did decrease total cholesterol and NEFA levels. Pravastatin has been reported to reduce the levels of hypercholesterolemia and/or hypertension if treatment were started at an earlier stage, this drug might not be effective if OLETF rats were treated with pravastatin for a long time, or if treatment were started at an earlier stage, this drug might reduce the levels of hypercholesterolemia and/or hypertension and significantly improve insulin sensitivity.

In the present study, ET-1-induced contraction was greater in OLETF than LETO aortic smooth muscle (Fig. 1). This enhancement was suppressed by pretreatment with a PP2A inhibitor (cantharidic acid; Fig. 2B) but not with a PP1 inhibitor (data not shown). These results are consistent with our analysis of ERK activity (Fig. 3, A and B). As shown in Fig. 3A, ERK phosphorylation (active state) levels were higher in OLETF rats than in LETO rats, and this was accompanied by increased KSR1-ERK complexes (Fig. 3B). Furthermore, pravastatin treatment of OLETF rats reduced both ERK phosphor-
LEC (basal) group; †

same conditions. Endothelium-denuded aortas were treated with ET-1 (10 cantharidic acid/ET-1-stimulated lanes were run on separate gels, but under the same conditions. Endothelium-denuded aortas were treated with ET-1 (10^-8 M) or with ET-1 (10^-8 M) + cantharidic acid (5×10^-8 M); basal means not so treated. Values are means ± SE from 6 experiments. *P < 0.05 vs. the LETO (basal) group; †P < 0.05 vs. the OLETF (basal) group; $P < 0.05 vs. the LETO (ET-1) group; #P < 0.05 vs. the OLETF (ET-1) group.

Fig. 7. IB analysis for p-Src expression in endothelium-denuded aortic strips obtained from LETO rats and pravastatin-treated or untreated OLETF rats. In the case of Src and Src phosphorylation at Tyr416, basal, ET-1-stimulated, and cantharidic acid/ET-1-stimulated lanes were run on separate gels, but under the same conditions. Endothelium-denuded aortas were treated with ET-1 (10^-8 M) or with ET-1 (10^-8 M) + cantharidic acid (5×10^-8 M); basal means not so treated. Values are means ± SE from 6 experiments. *P < 0.05 vs. the LETO (ET-1) group; †P < 0.05 vs. the OLETF (basal) group; $P < 0.05 vs. the LETO (ET-1) group.

Fig. 8. Schematic representation of the proposed cause of abnormal ET-1-induced vasoconstriction in the type 2 diabetic OLETF rat aorta. In the type 2 diabetic state, constitutively decreased Src tyrosine kinase activity leads to enhanced KSR1 activation by dephosphorylation of PP2A (28). Pretreatment with cantharidic acid significantly reduced not only the p-ERK level but also KSR1/ERK binding in OLETF aortas (Figs. 3A and 4B). These results indicate that PP2A regulates KSR1 activity and are consistent with the results of Ory et al. (37). The present data lead us to conclude that long-term exposure to a diabetic state may cause constitutive activation of PP2A.

PP2A (C subunit) is phosphorylated at Tyr307 by Src tyrosine kinase after the suppression of phosphatase activity (5). We detected PP2A (C subunit) phosphorylation at Tyr307 (a negative regulatory site), as shown in Fig. 6B, and found that OLETF aortas exhibited reduced PP2A phosphorylation levels (vs. their LETO genetic control). In contrast, KSR1-associated PP2A (examined using immunoprecipitation and immunoblot analysis) was enhanced in the OLETF group (Fig. 6A), and pravastatin treatment normalized the PP2A hyperactivity (expression of dephosphorylated PP2A and PP2A binding to KSR1) seen in the OLETF group (Fig. 6A). PP2A phosphorylation at Tyr307 (data are in line with previous demonstrations by Favre et al. (8) and Ory et al. (37) showing that PP2A inhibition suppressed KSR1/ERK binding and ERK phosphorylation levels. Those results indicate that KSR1 is a promotinal molecule for the excessive activity in the ERK pathway present in the aorta in the type 2 diabetic OLETF rat and also that pravastatin treatment can suppress the excessive ERK phosphorylation seen in OLETF rats after KSR1/ERK binding. In addition, we investigated the PP2A and KSR1/ERK interaction using a PP2A inhibitor (cantharidic acid) (28). Pretreatment with cantharidic acid significantly reduced not only the p-ERK level but also KSR1/ERK binding in OLETF aortas (Figs. 3A and 4B). These results indicate that PP2A regulates KSR1 activity and are consistent with the results of Ory et al. (37). The present data lead us to conclude that long-term exposure to a diabetic state may cause constitutive activation of PP2A.

PP2A (C subunit) is phosphorylated at Tyr307 by Src tyrosine kinase after the suppression of phosphatase activity (5). We detected PP2A (C subunit) phosphorylation at Tyr307 (a negative regulatory site), as shown in Fig. 6B, and found that OLETF aortas exhibited reduced PP2A phosphorylation levels (vs. their LETO genetic control). In contrast, KSR1-associated PP2A (examined using immunoprecipitation and immunoblot analysis) was enhanced in the OLETF group (Fig. 6A), and pravastatin treatment normalized the PP2A hyperactivity (expression of dephosphorylated PP2A and PP2A binding to KSR1) seen in the OLETF group (Fig. 6A). PP2A phosphorylation at Tyr307 is enhanced by the addition of phosphatase (5). Hence, the present results strongly suggest that ET-1-induced vasoconstriction is enhanced in the OLETF aorta due to increases in KSR1/ERK complexes and ERK activity after an elevation of PP2A activity. Recently, Hersch et al. (9) demonstrated that ET-1 activated PP2A via the ET-1 type B receptor, and our laboratory (20, 30) has previously indicated that both type A and type B receptors were increased in the diabetic rat macrovaso-
culature and microvasculature (20, 30). Those previous data led us to speculate that ET-1 may have a double action (inducing vasoconstriction through the ET-1 type A receptor/ERK pathway and KSR1 dephosphorylation through the ET-1 type B receptor/PP2A pathway). However, our data are now not consistent with the above idea. Indeed, the present data indicate that the PP2A/KSR1 pathway is constitutively activated because the data were not different between with and without ET-1-stimulation (Figs. 4C and 6). This issue will require further examination.

It has been reported that the nonreceptor tyrosine kinase c-Src regulates MAPK activity in various cell types and that 60-kDa c-Src, which is the most abundantly expressed isoform in vascular smooth muscle cells (VSMCs), is rapidly activated by G protein-coupled receptors (GPCRs) (7, 29, 35, 43). Other proximal regulators of MEK include the Ras-Raf pathway, which may not necessarily involve c-Src (24, 48). ANG II and ET-1 are vasoactive agents that mediate multiple vascular actions through the activation of distinct GPCRs (34, 44). The cellular mechanisms and signaling pathways that are involved in hypertensive vascular damage are currently the subjects of intensive investigation. However, it remains controversial whether c-Src is a mediator of ANG II- and/or ET-1-induced MAPK activation in mouse vascular smooth muscle and also suggest that the enhancement of the Src kinase activation, at least in OLETF aortic smooth muscle.

In conclusion, our study demonstrated that 4-wk pravastatin treatment of type 2 diabetic OLETF rats normalizes ET-1-induced contraction in the aorta via both a suppressive effect on the PP2A/KSR1/ERK pathway and an improvement in Src kinase activity (Fig. 8). We believe that the Src/PP2A/KSR1 pathway warrants further investigation as a potentially important therapeutic target for newly developed drugs aimed at diabetic vasculopathy.

GRANTS

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES


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