Increased Insulin Receptor Substrate 1 Serine Phosphorylation and Stress-Activated Protein Kinase/c-Jun N-Terminal Kinase Activation Associated With Vascular Insulin Resistance in Spontaneously Hypertensive Rats

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Abstract—Insulin resistance is associated with cardiovascular disease. Impaired insulin receptor substrate (IRS)–mediated signal transduction is a major contributor to insulin resistance. Recently, IRS-1 phosphorylation at serine 307 by stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) has been highlighted as a molecular event that causes insulin resistance. We investigated IRS-1–mediated insulin signaling, IRS-1 phosphorylation at serine 307, and SAPK/JNK activation status in the aorta of spontaneously hypertensive rats (SHR) by immunoprecipitation and immunoblotting. Insulin-stimulated tyrosine phosphorylation of insulin receptor and IRS-1 in SHR was decreased to 55% \( (P<0.01) \) and 40% \( (P<0.01) \) of the levels in Wistar-Kyoto rats (WKY), respectively. Insulin-stimulated IRS-1–associated phosphatidylinositol 3-kinase activation in SHR was reduced to 28% of the level in WKY \( (P<0.0001) \). Immunoblot analysis revealed that phosphorylated IRS-1 at serine 307 in SHR was increased to 261% \( (P<0.001) \) of the level in WKY. Phosphorylated (activated) SAPK/JNK in SHR was increased to 223% of the level in WKY \( (P<0.01) \). Serine-phosphorylated IRS-1 that was immunoprecipitated from the aorta of SHR was capable of inhibiting in vitro tyrosine phosphorylation by recombinant insulin receptor compared with WKY-derived IRS-1. These findings demonstrate that insulin resistance in the aorta of SHR was associated with elevated IRS-1 phosphorylation at serine 307 and increased SAPK/JNK activation. The present study suggests that increased SAPK/JNK activation may play an important role in the pathogenesis of vascular insulin resistance via inhibitory serine phosphorylation of IRS-1. \( (Hypertension. 2004;44:484-489.) \)

Key Words: insulin resistance ■ rats, spontaneously hypertensive ■ aorta ■ phosphorylation ■ kinase ■ signal transduction

Insulin resistance is associated with increased risk for cardiovascular diseases, including incidental hypertension,1 atherosclerosis,2-5 ischemic heart disease,6,7 and stroke.8 Binding of insulin to its receptor results in an activation of insulin receptor (IR) tyrosine kinase, which, in turn, phosphorylates tyrosine residues of IR substrates (IRSs). IRSs are adaptor proteins that transduce signals from IR to downstream signaling cascades including phosphatidylinositol 3 (PI3)-kinase–Akt/protein kinase B (PKB) pathway. A body of work indicates that impaired IRS-mediated signal transduction is a major contributor to insulin resistance.9 Gene disruption of IRS-1 or IRS-2 causes insulin resistance in mice.10,11 In animal models of and patients with insulin resistance and type 2 diabetes, insulin-stimulated tyrosine phosphorylation of IRS-1 and IRS-2 is impaired in skeletal muscle, adipose tissue, and liver.12-14 Moreover, the previous study revealed that IRS-mediated signaling in response to ex vivo exposure to insulin was attenuated in the aorta of genetically obese, diabetic Zucker fatty (fa/fa) rats.15

In vasculature, insulin induces vasodilation by upregulating endothelial NO synthase (eNOS) activity.16 Insulin increases eNOS activity via PI3-kinase–Akt/PKB pathway, not only by upregulation of its protein expression17 but also by Akt/PKB-mediated eNOS phosphorylation.18,19 In fact, the recent study demonstrated that endothelial cell-specific disruption of IR reduced eNOS expression in mice.16 Moreover, accumulated evidence indicates that vascular insulin resistance has a pathogenic role in endothelial dysfunction.20 Of note, IRS-1 has been shown to be required for insulin-stimulated NO production in endothelial cells,21 and polymorphism in IRS-1 gene has been proposed as a risk for cardiovascular disease22-24 as well as for type 2 diabetes. Collectively, these findings suggest that impaired IRS-1–mediated signal transduction may play an important role in endothelial dysfunction and the development of cardiovascular diseases.

Despite the intense investigation for a number of years, the molecular mechanisms responsible for insulin resistance still...
remain to be determined. However, recently, a contributory role for IRS-1 phosphorylation at serine 307 was highlighted.\textsuperscript{25,26} Stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) was shown to phosphorylate IRS-1 at serine 307 in vitro and in intact cells.\textsuperscript{25,26} In disease states with insulin resistance and type 2 diabetes, inflammatory cytokines such as tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), free fatty acid and oxidative stress, all of which activate SAPK/JNK, are upregulated. SAPK/JNK is activated via phosphorylation by SAPK kinase-1/mitogen-activated protein kinase kinase-4 (SEK1/MKK4). IRS-1 phosphorylation at serine 307 has been shown to be essential for impairment in IRS-1-mediated signaling induced by TNF-\(\alpha\), insulin, or okadaic acid in cultured cells.\textsuperscript{25,26} Furthermore, IRS-1 phosphorylation at serine 307 was elevated in skeletal muscle of genetically obese, diabetic (ob/ob) mice compared with wild-type mice.\textsuperscript{27}

Recently, an involvement of serine phosphorylation of IRS-1 by SAPK/JNK was corroborated in vivo by the study using SAPK1c/JNK1-deficient mice. Gene disruption of SAPK1c/JNK1, protected from leptin deficiency-induced obesity and insulin resistance, and the protection by SAPK1c/JNK1 deficiency were associated with concomitant reversal of elevated IRS-1 phosphorylation at serine 307.\textsuperscript{27}

Thus, inhibitory serine phosphorylation of IRS-1 by SAPK/JNK has been proposed to be a key molecular event in the pathogenesis of insulin resistance. However, serine phosphorylation of IRS-1 in the vasculature has not yet been investigated. Therefore, we investigated a possible involvement of IRS-1 phosphorylation at serine 307 and activation status of SAPK/JNK in vascular insulin resistance using aortae of spontaneously hypertensive rats (SHR). We chose SHR for the present study because it is a well-established rodent model of metabolic syndrome in which insulin resistance has been characterized extensively.

Methods
An expanded Methods section can be found in an online supplement available at http://www.hypertensionaha.org.

Animals
Male SHR and Wistar-Kyoto rats (WKY; Charles River Breeding Laboratories) at 23 weeks of age were used. The Institutional Animal Care Committee approved the study protocol.

Insulin Injection
After overnight fasting, rats were anesthetized with pentobarbital sodium (50 mg/kg IP). Next, insulin (10 IU/kg; Humulin R; Eli Lilly) diluted with saline or saline alone was injected into the portal vein, as described previously.\textsuperscript{28} Five minutes after insulin or saline injection, the thoracic aorta was removed and frozen in liquid nitrogen.

Immunoprecipitation and Immunoblotting
Tissue samples were homogenized as described previously.\textsuperscript{29} Immunoprecipitation was performed by incubating the lysates with anti-IR\(\beta\) or IRS-1 (Upstate) for 5 hours at 4°C, as described previously.\textsuperscript{30} Immunoblot analysis was done as described previously.\textsuperscript{30}

PI3-Kinase Assay
PI3-kinase activity in the immunoprecipitates with anti–IRS-1 antibody was measured by in vitro phosphorylation assay using phosphatidylinositol (Sigma) as a substrate, as described previously.\textsuperscript{26}

Statistical Analysis
Data were compared using 1-way ANOVA followed by Fisher protected least significant difference test. A value of \(P<0.05\) was considered statistically significant. All values are expressed as mean±SEM.

Results

Impaired IRS-1–Mediated Insulin Signaling in SHR
Male SHR and WKY received the insulin injection via the portal vein after overnight fasting. There was no difference in body weight between SHR and WKY (357.4±5.6 and 349.0±3.6 g, respectively). Insulin injection led to a marked tyrosine phosphorylation of IR in WKY aorta. However, insulin-stimulated tyrosine phosphorylation of IR in the aorta was significantly reduced in SHR compared with WKY. Phosphorylated IR was expressed as a percentage normalized to the average level observed in insulin-stimulated WKY. Number of animals: WKY, insulin (−); SHR, insulin (+) 11; SHR, insulin (−) 3; SHR, insulin (+) 11. There was no difference in protein expression of IR between SHR and WKY. Number of animals: WKY 7; SHR 7.

Figure 1. Impaired insulin-stimulated tyrosine phosphorylation of IR in SHR aorta. SHR and WKY received insulin injection after overnight fasting. Insulin-stimulated tyrosine phosphorylation of IR in the aorta was assessed by immunoprecipitation (IP) with anti-IR antibody followed by immunoblotting (IB) with anti-phosphotyrosine (PY) and IR antibodies. A, Insulin-stimulated tyrosine phosphorylation of IR in the aorta was significantly reduced in SHR compared with WKY. Phosphorylated IR was expressed as a percentage normalized to the average level observed in insulin-stimulated WKY. Number of animals: WKY, insulin (−) 3; WKY, insulin (+) 11; SHR, insulin (−) 3; SHR, insulin (+) 11. B, There was no difference in protein expression of IR between SHR and WKY. Number of animals: WKY 7; SHR 7.

In Vitro Phosphorylation Assay
In vitro phosphorylation by recombinant IR was performed as described previously,\textsuperscript{30} with minor modifications.

Increased IRS-1 Serine Phosphorylation and SAPK/JNK Activation in SHR
We examined the serine phosphorylation status of IRS-1. Immunoblot analysis revealed that phosphorylated IRS-1 at
serine 307 in SHR was increased to 261% of that in WKY (P < 0.001; Figure 4).

In consonance with increased serine phosphorylation of IRS-1 in SHR, phosphorylated (activated) SAPK/JNK in SHR was elevated to 223% of that in WKY (P < 0.01; Figure 5A). On the other hand, the protein expression of SAPK1c/JNK1 (p46) in SHR decreased to 57% of that in WKY (P < 0.005), whereas the expression of SAPK1a/JNK2 (p54) did not differ between WKY and SHR (Figure I, available online at http://www.hypertensionaha.org). Elevated SAPK/JNK activation was further confirmed by the phosphorylation status of c-Jun, an endogenous substrate of SAPK/JNK. Phosphorylation of c-Jun in SHR increased to 182% of that in WKY (P < 0.05; Figure 5B). We also examined the activation status of SEK1/MKK4, an upstream activator of SAPK/JNK. Phosphorylated (activated) SEK1/MKK4 in SHR increased to 197% of that in WKY (P < 0.01; Figure 5C). However, the expression of c-Jun and SEK1/MKK4 did not differ significantly between SHR and WKY (Figure II, available online at http://www.hypertensionaha.org).

Figure 2. Impaired insulin-stimulated tyrosine phosphorylation of IRS-1 in SHR aorta. Insulin-stimulated tyrosine phosphorylation of IRS-1 in the aorta was assessed by immunoprecipitation (IP) with anti-IRS-1 antibody followed by immunoblotting (IB) with anti-phosphotyrosine (PY) and IRS-1 antibodies. A, Insulin-stimulated tyrosine phosphorylation of IRS-1 was significantly reduced in the SHR aorta, whereas insulin induced a marked tyrosine phosphorylation of IRS-1 in WKY. Phosphorylated IRS-1 was expressed as a percentage normalized to the average level observed in insulin-stimulated WKY. Number of animals: WKY, insulin (-) 3; WKY, insulin (+) 11; SHR, insulin (-) 3; SHR, insulin (+) 11. B, There was no difference in protein expression of IRS-1 between SHR and WKY. Number of animals: WKY 14; SHR 14.

Figure 3. Impaired insulin-stimulated PI3-kinase activation in SHR. PI3-kinase activity in the aorta was examined 5 minutes after insulin injection. PI3-kinase activity in the immunoprecipitates with anti-IRS-1 antibody was assessed by in vitro phosphorylation of phosphatidylinositol to phosphatidylinositol phosphate (PIP). Insulin-stimulated IRS-1-associated PI3-kinase activation was impaired in SHR compared with WKY. PI3-kinase activity was expressed as a percentage normalized to the average level in insulin-stimulated WKY. Number of animals: WKY, insulin (-) 3; WKY, insulin (+) 10; SHR, insulin (-) 3; SHR, insulin (+) 10.

Figure 4. Increased serine phosphorylation of IRS-1 in SHR. IRS-1 phosphorylation at serine 307 in the aorta was analyzed by immunoblotting (IB) with anti-p-IRS-1 (Ser 307) antibody. Phosphorylated IRS-1 at serine 307 was significantly increased in SHR compared with WKY. Number of animals: WKY 14; SHR 14.

Figure 5. Increased activation of SAPK/JNK pathway in SHR. Activation status of SAPK/JNK pathway was assessed by SAPK/JNK phosphorylation (A), c-Jun, an endogenous substrate for SAPK/JNK (B), and SEK1/MKK4, an upstream activator of SAPK/JNK (C). Immunoblot analysis (IB) with phosphospecific antibodies revealed that phosphorylation of SAPK/JNK (A), c-Jun (B), and SEK1/MKK4 (C) was significantly increased in the aorta of SHR compared with WKY. Number of animals: WKY 14; SHR 14 for A; WKY 11; SHR 11 for B and C.
Reduced In Vitro Tyrosine Phosphorylation of IRS-1 in SHR

To investigate further a role of IRS-1 phosphorylation at serine 307, we performed in vitro tyrosine phosphorylation assay by incubating recombinant active IR with IRS-1 that was immunoprecipitated from SHR and WKY aortae. The incubation with recombinant IR increased tyrosine phosphorylation of WKY-derived IRS-1. However, tyrosine phosphorylation by recombinant IR of SHR-derived IRS-1 was significantly reduced compared with WKY (Figure 6). Of note, the preincubation with alkaline phosphatase reversed reduced tyrosine phosphorylation of IRS-1 from SHR in parallel with dephosphorylation of serine 307 of IRS-1.

In vitro tyrosine phosphorylation of IR was also significantly decreased when phosphorylation reaction was done with SHRD-derived IRS-1 compared with IR incubated with WKY-derived IRS-1. However, when the immunoprecipitated IRS-1 was pretreated with alkaline phosphatase, there was no difference in autophosphorylation of IR between those incubated with SHRD- and WKY-derived IRS-1.

Next, we asked whether in vitro IRS-1 phosphorylation at serine 307 by SAPK/JNK inhibits in vitro tyrosine phosphorylation of recombinant IR and immunoprecipitated IRS-1. When WKY-derived IRS-1 was pretreated with recombinant active SAPK/JNK, IRS-1 was phosphorylated at serine 307. In parallel with serine IRS-1 phosphorylation, tyrosine phosphorylation of IR and IRS-1 was reduced by the pretreatment with recombinant SAPK/JNK (Figure III, available online at http://www.hypertensionaha.org).

To examine whether phosphorylation of serine 307 is required for the effects of SAPK/JNK, we transfected COS-7 cells with wild-type IRS-1 and mutated IRS-1 (S307A), in which serine 307 residue was replaced by alanine. Wild-type and mutated IRS-1 was immunoprecipitated and preincubated with or without recombinant SAPK/JNK. Then, immunoprecipitates were incubated with recombinant IR after being washed 3X. When wild-type IRS-1 was preincubated with SAPK/JNK, SAPK/JNK attenuated tyrosine phosphorylation of IR and IRS-1. In contrast, when mutated IRS-1 (S307A) was preincubated with SAPK/JNK, neither tyrosine phosphorylation of mutated IRS-1 (S307A) by IR nor autophosphorylation of IR was reduced by SAPK/JNK preincubation (Figure IV, available online at http://www.hypertensionaha.org).

To address whether IRS-1 is required for inhibitory SAPK/JNK effects on tyrosine kinase IR activity, the immunoprecipitates with rat normal IgG from COS-7 cells, which were transfected with wild-type IRS-1, were preincubated with SAPK/JNK. SAPK/JNK failed to reduce IR autophosphorylation in the absence of IRS-1, indicating that IRS-1 is required for SAPK/JNK treatment to inhibit IR tyrosine kinase activity in vitro.

Discussion

Our results clearly demonstrate that insulin-stimulated tyrosine phosphorylation of IR and IRS-1 and insulin-stimulated IRS-1-associated PI3- kinase activation were impaired in the aorta of SHR compared with WKY (Figures 1 through 3). In addition, we found that IRS-1 phosphorylation at serine 307 was significantly increased in the aorta of SHR compared with WKY (Figure 4). Increased serine phosphorylation of IRS-1 was accompanied by elevated activation of SAPK/JNK in SHR (Figure 5), which was further corroborated by increased phosphorylation of its endogenous substrate, c-Jun, and its upstream activator, SEK1/MKK4. These results suggest that serine IRS-1 phosphorylation may be involved in vascular insulin resistance in SHR and that increased IRS-1 serine phosphorylation may be attributed to elevated SAPK/JNK activation in SHR.

IRS-1 immunoprecipitated from the aorta of SHR was resistant to in vitro tyrosine phosphorylation by recombinant IR compared with WKY-derived IRS-1. Of interest, IR autophosphorylation was also significantly reduced when preincubated with SHR-derived IRS-1 (Figure 6). Moreover, in vitro incubation with recombinant SAPK/JNK resulted in the inhibition of in vitro tyrosine phosphorylation of IRS-1 and IR, in parallel with phosphorylation of serine 307 of IRS-1. Importantly, the reversal of serine 307 phosphorylation in SHR-derived IRS-1 by alkaline phosphatase restored attenuated in vitro tyrosine phosphorylation of IR and IRS-1 to the level observed in WKY-derived IRS-1 (Figure 6). Therefore, phosphorylation and dephosphorylation of serine 307 of IRS-1 correlated quite well with attenuation and its restoration of tyrosine phosphorylation of IRS-1 by IR and also autophosphorylation of IR. These findings suggest that phosphorylation at serine 307 may be capable of inhibiting not only tyrosine phosphorylation of IRS-1 by IR but also tyrosine kinase activity of IR. Furthermore, preincubation with SAPK/JNK failed to impair IR-mediated tyrosine phosphorylation when incubated with mutated IRS-1 (S307A). These data indicate that serine 307 phosphorylation is required for the inhibitory effects of SAPK/JNK in vitro.
The present results are consistent with the previous study showing that serine phosphorylation of IRS-1 by TNF-α attenuated insulin-stimulated tyrosine phosphorylation of IR and IRS-1 in cultured adipocytes. The authors proposed that serine phosphorylation of IRS-1 converts IRS-1 into an inhibitor of IR kinase activity and showed that the inhibitory form of IRS-1 was observed in muscle and fat of obese, diabetic rats. SAPK/JNK was shown to mediate TNF-α–induced IRS-1 phosphorylation at serine 307. Together, our results suggest that serine phosphorylation of IRS-1 might also contribute to reduced tyrosine phosphorylation of IR as well as IRS-1 in SHR. However, further studies will be required to clarify a precise role of serine phosphorylation of IRS-1 in the regulation of IR kinase activity in vasculature.

SHR exhibit whole-body insulin resistance, although they are not diabetic. Impaired IR–IRS-1–mediated insulin signaling in the aorta in the present study is consistent with the results in the recent study in SHR. Therefore, the salient novel findings of our study are that vascular insulin resistance was accompanied by increased phosphorylation of IRS-1 at serine 307 and by activation of SAPK/JNK pathway, as reflected by increased phosphorylation of SEK1/MKK4, SAPK/JNK, and c-Jun in the aorta of SHR compared with WKY and that serine phosphorylation of SHR-derived IRS-1 was associated with reduced IR-mediated in vitro tyrosine phosphorylation of IRS-1 and IR.

Our finding of elevated SAPK/JNK activation in the aorta of SHR is in accordance with previous studies showing increased activity of SAPK/JNK in heart and renal proximal tubule of SHR compared with WKY. Notably, several lines of evidence indicate that SAPK/JNK plays an important role in cardiovascular diseases including hypertension and atherosclerosis. Acute hypertension and volume overload activate SAPK/JNK in the arterial wall. Angiotensin II, an inducer of hypertension and a proatherogenic agent, is a potent activator of SAPK/JNK. Moreover, increased SAPK/JNK activation has been observed in atherosclerotic lesions in cholesterol-fed rabbits, and the gene transfer of dominant-negative mutant of SAPK/JNK prevented intimal thickness induced in balloon-injured arteries. SAPK/JNK activation was also shown to play a pivotal role in hypertension–induced cardiac hypertrophy. Collectively, the present study suggests that augmented SAPK/JNK activation in vasculature may contribute to development of cardiovascular diseases, in part, by promoting insulin resistance via inhibitory serine phosphorylation of IRS-1.

In contrast to the increased SAPK/JNK activation in SHR, the protein expression of SAPKc/JNK1 in WKY was significantly reduced in SHR compared with WKY, and SAPK1a/JNK2 expression was not altered. These results suggest that increased SAPK/JNK activation was not attributable to upregulated SAPK/JNK expression but perhaps to elevated activation of the upstream signal transduction cascade that activates SAPK/JNK. This assumption was supported by the increased phosphorylation (activation) of SEK1/MKK4, an upstream activator of SAPK/JNK.

Insulin resistance syndrome (also named syndrome X or metabolic syndrome) is a cluster of metabolic and cardiovascular abnormalities, including dyslipidemia and hypertension, and increases the risk for cardiovascular disease. The common denominator of this syndrome is presumed to be insulin resistance. Oxidative stress is considered to be involved in insulin resistance syndrome. Previous studies have shown that increased oxidative stress is involved in hypertension and endothelial dysfunction in SHR and that reactive oxygen species are sufficient and required for SAPK/JNK activation in many cell types, including endothelial cells and vascular smooth muscle cells. Together with these previous findings, our study suggests that SAPK/JNK activation, possibly associated with increased oxidative stress or proinflammatory state, may play an important role in insulin resistance syndrome in SHR.

Perspectives

Impaired IRS-1–mediated insulin signaling was accompanied by increased IRS-1 phosphorylation at serine 307 and elevated SAPK/JNK activation in the aorta of SHR compared with WKY. We found that IRS-1 phosphorylation at serine 307 and its dephosphorylation were associated with impairment and its restoration of in vitro tyrosine phosphorylation of IRS-1 and IR. Our results suggest that SAPK/JNK-mediated IRS-1 phosphorylation at serine 307 may play an important role in vascular insulin resistance. Therefore, our study highlights SAPK/JNK and upstream signals that activate SAPK/JNK as potential molecular targets to prevent or treat vascular insulin resistance and hence metabolic syndrome–involved cardiovascular diseases including atherosclerosis.

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