Angiotensin II Impairs the Insulin Signaling Pathway Promoting Production of Nitric Oxide by Inducing Phosphorylation of Insulin Receptor Substrate-1 on Ser$^{312}$ and Ser$^{616}$ in Human Umbilical Vein Endothelial Cells

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Abstract—It has been suggested that serine (Ser) phosphorylation of insulin receptor substrate-1 (IRS-1) decreases the ability of IRS-1 to be phosphorylated on tyrosine, thereby attenuating insulin signaling. There is evidence that angiotensin II (AII) may impair insulin signaling to the IRS-1/phosphatidylinositol 3-kinase (PI 3-kinase) pathway by enhancing Ser phosphorylation. Insulin stimulates NO production by a pathway involving IRS-1/PI3-kinase/Akt/endothelial NO synthase (eNOS). We addressed the question of whether AII affects insulin signaling involved in NO production in human umbilical vein endothelial cells and tested the hypothesis that the inhibitory effect of AII on insulin signaling was caused by increased site-specific Ser phosphorylation in IRS-1. Exposure of human umbilical vein endothelial cells to AII resulted in inhibition of insulin-stimulated production of NO. This event was associated with impaired IRS-1 phosphorylation at Tyr$^{612}$ and Tyr$^{632}$, two sites essential for engaging the p85 subunit of PI3-kinase, resulting in defective activation of PI 3-kinase, Akt, and eNOS. This inhibitory effect of AII was reversed by the type 1 receptor antagonist losartan. AII increased c-Jun N-terminal kinase (JNK) and extracellular signal–regulated kinase (ERK) 1/2 activity, which was associated with a concomitant increase in IRS-1 phosphorylation at Ser$^{312}$ and Ser$^{616}$, respectively. Inhibition of JNK and ERK1/2 activity reversed the negative effects of AII on insulin-stimulated NO production. Our data suggest that AII, acting via the type 1 receptor, increases IRS-1 phosphorylation at Ser$^{312}$ and Ser$^{616}$ via JNK and ERK1/2, respectively, thus impairing the vasodilator effects of insulin mediated by the IRS-1/PI 3-kinase/Akt/eNOS pathway. (Circ Res. 2004;94:1211-1218.)

Key Words: endothelium ■ angiotensin II ■ nitric oxide ■ insulin

Endothelial dysfunction is an early event in the pathogenesis of atherosclerosis and a feature of insulin-resistant conditions, including type 2 diabetes, obesity, and hypertension.1–4 Several preclinical and clinical studies have established the involvement of angiotensin II (AII) and its type 1 receptor (AT$_1$) in endothelial dysfunction.5–7 Insulin promotes vasodilatation by activation of the signaling pathway involving the insulin receptor/insulin receptor substrate-1 (IRS-1)/phosphatidylinositol 3-kinase (PI 3-kinase)/Akt that leads to activation of endothelial NO synthase (eNOS) in endothelium.8 Cross-talk between the renin-angiotensin system (RAS) and insulin signaling has been demonstrated.9 Inhibition of RAS by angiotensin-converting enzyme inhibitors or AT$_1$ antagonists has been shown to both increase insulin sensitivity and improve endothelial function.10–12 Evidence has been provided that AII interferes with insulin signaling in vascular cells mainly by affecting insulin-induced tyrosine phosphorylation of IRS-1 and impairing its interaction with the p85 regulatory subunit of PI 3-kinase.9 However, it is still unclear whether AII adversely affects the downstream signaling pathway involving Akt/eNOS that controls NO production in response to insulin. A hypothesis has emerged recently that serine phosphorylation of IRS proteins decreases their ability to be phosphorylated on tyrosine, thereby attenuating insulin signaling.13–17 Several serine residues in IRS-1 have been identified as negative regulatory sites, including Ser$^{312}$ (orthologous to Ser$^{307}$ in rat IRS-1), which is activated by c-Jun N-terminal kinase (JNK) and extracellular signal–regulated kinase (ERK) 1/2 activity, which was associated with a concomitant increase in IRS-1 phosphorylation at Ser$^{312}$ and Ser$^{616}$, respectively. Inhibition of JNK and ERK1/2 activity reversed the negative effects of AII on insulin-stimulated NO production. Our data suggest that AII, acting via the type 1 receptor, increases IRS-1 phosphorylation at Ser$^{312}$ and Ser$^{616}$ via JNK and ERK1/2, respectively, thus impairing the vasodilator effects of insulin mediated by the IRS-1/PI 3-kinase/Akt/eNOS pathway. (Circ Res. 2004;94:1211-1218.)

Original received October 17, 2003; revision received March 1, 2004; accepted March 15, 2004.
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Circulation Research is available at http://www.circresaha.org DOI: 10.1161/01.RES.0000126501.34994.96
on insulin signaling is caused by an increase in site-specific serine phosphorylation in IRS-1.

Materials and Methods

Materials

Monoclonal anti-phosphotyrosine antibodies and polyclonal antibodies against IRS-1, p85 subunit of PI 3-kinase, Akt, anti-IRS-1 antibody, and anti-Thr<sup>308</sup> Akt, ERK1/2, phospho-ERK1/2 (Thr<sup>202</sup>/Tyr<sup>204</sup>), JNK, and phospho-JNK (Thr<sup>183</sup>/Tyr<sup>185</sup>) were from Cell Signaling Technology (Beverly, Mass). Polyclonal antibodies against the insulin receptor (IR), Tyr<sup>1576/Tyr<sup>1577</sup>/Tyr<sup>1580</sup></sup>) in the active loop of the catalytic domain of the insulin receptor, Ser<sup>112</sup>-IRS-1, Ser<sup>616</sup>-IRS-1, Tyr<sup>632</sup>-IRS-1, and Tyr<sup>610</sup>-IRS-1 were purchased from Biosource (Camarillo, Calif). All other chemicals were from Sigma Chemical Co (St Louis, Mo).

Western Blotting

HUVECs were serum-starved for 18 hours and incubated in the presence or absence of the indicated hormones and inhibitors. The amount of NOS activity produced by HUVECs was assayed using an NOS Detection System (Sigma, Saint Louis, Mo) that measures the ability of NOS to convert L-<sup>14</sup>C-arginine (Amersham) to L-<sup>14</sup>C-citrulline, according to the manufacturer’s instructions. Data were normalized by the amount of protein and reaction time.

Statistical Analysis

All results are given as mean ± SE and were analyzed with the use of the Newman-Keuls test for ANOVA for multiple comparisons. P < 0.05 was considered statistically significant.

Results

Effects of AII on Site-Specific Serine Phosphorylation of IRS-1

Increased serine phosphorylation of IRS-1 has been shown to inhibit the ability of this substrate to be tyrosine phosphorylated by the insulin receptor and to bind and activate PI 3-kinase. Among the serine kinases that have been reported to phosphorylate IRS-1 at specific sites, it has been shown that JNK phosphorylates human IRS-1 at position Ser<sup>312</sup> and MAPK at position Ser<sup>632</sup>. Because AII activates upstream signaling pathways, leading to activation of both JNK and MAPK, we tested whether AII would induce serine phosphorylation of IRS-1 in human endothelial cells. As shown in Figures 1A and 1B, exposure of HUVECs to AII resulted in a time-dependent increase in IRS-1 phosphorylation at both Ser<sup>312</sup> and Ser<sup>616</sup>, respectively, with maximal effect occurring after 30 minutes of incubation. These stimulatory effects of AII were paralleled by a time-dependent increase in phosphorylation of JNK and the ERK1/2 members of the MAPK family, respectively (Figures 1C and 1D). The stimulatory effects of AII on IRS-1 phosphorylation at Ser<sup>312</sup> and Ser<sup>616</sup> were reversed by treatment with a cell-permeable JNK inhibitor and PD98059, a reversible MEK1 inhibitor, the enzyme that directly activates ERK1/2, respectively (Figures 2A and 2B). JNK inhibitor did not affect Ser<sup>632</sup> phosphorylation, and PD98059 did not affect Ser<sup>112</sup> phosphorylation, thus indicating that their inhibitory effects were specific (Figures 2A and 2B). The stimulatory effects of AII on phosphorylation of Ser<sup>112</sup> and Ser<sup>616</sup> were also reversed by losartan in parallel with inhibition of phosphorylation of both JNK and ERK-1/2, respectively (Figures 2A and 2B). Also, insulin was able to stimulate IRS-1 phosphorylation at both Ser<sup>312</sup> and Ser<sup>616</sup>, although to a less extent as compared with
AII (Figure 2C). These stimulatory effects of insulin were paralleled by phosphorylation of both JNK and ERK1/2, respectively (Figures 2D through 2F). The effects of insulin and those of AII on IRS-1 phosphorylation at either Ser312 or Ser616 were not additive. Losartan reversed the stimulatory effect of AII (Figures 2A and 2B) but not that of insulin (Figures 2C through 2F). Thus, these data indicate that AII induces IRS-1 phosphorylation at Ser312 and Ser616 by activation of JNK and ERK1/2, respectively, which can be effectively reversed by their respective inhibitors.

Effects of AII on Insulin-Stimulated Tyrosine Phosphorylation of IRS-1 and IRS-1/PI 3-Kinase Docking

Because prior studies in rat aortic smooth muscle cells have shown that treatment with AII (100 nmol/L) inhibited insulin-stimulated tyrosine phosphorylation of IRS-1 and its ability to engage PI 3-kinase,8 we tested whether AII would affect insulin signaling in human endothelial cells. As shown in Figure 3A, exposure of HUVECs to AII resulted in a time-dependent inhibition of insulin-stimulated tyrosine phosphorylation of IRS-1, with maximal effect occurring after 30 minutes of incubation. Therefore, subsequent experiments aimed at studying the inhibitory effect of AII on insulin signaling were performed with HUVECs exposed to AII for 30 minutes, a time at which AII exerted both its maximal stimulatory effect on IRS-1 phosphorylation at both Ser312 and Ser616 and its maximal inhibitory effect on insulin-stimulated tyrosine phosphorylation of IRS-1. Losartan reversed the inhibitory effect of AII in a dose-dependent manner, with maximal effect occurring at 200 nmol/L (Figure 3B). AII did not significantly alter basal tyrosine phosphorylation of IRS-1 or levels of expressed IRS-1 (Figures 3A and 4A). Nonimmune serum did not lead to immunoprecipitation of IRS-1, thus indicating that IRS-1 immunoprecipitation by anti-IRS-1 antibody was specific (Figure 4A). Because association of the p85 regulatory subunit of PI 3-kinase with tyrosine-phosphorylated IRS-1 is essential to promote downstream signaling, the effect of AII on IRS-1/p85 docking was examined by immunoprecipitation of IRS-1 from cell lysates followed by immunoblotting with anti-p85 antibody. Insulin stimulated by 1.7-fold the binding of IRS-1 to p85 subunit (P<0.01) (Figure 4B). AII treatment reduced by 30% insulin-stimulated binding of IRS-1 to p85 subunit (P<0.02). This inhibitory effect of AII on IRS-1/p85 association was reversed by losartan (Figure 4B). There is evidence that tyrosine residues in the YXXM motifs at positions 612 and 632 (Tyr612 and Tyr632) play a major role in engaging the tandem SH2 domains of p85 subunit of PI 3-kinase, thus allowing for its full activation.20 To test the possibility that impaired IRS-1/p85 association induced by AII was related to changes in phosphorylation states of these two YXXM motifs, IRS-1 was immunoprecipitated from cell lysates and immunoblotted with phosphospecific anti-Tyr612 or anti-Tyr632 IRS-1 antibody. Insulin stimulated by 3-fold phosphorylation of Tyr612 and Tyr632 on IRS-1 (P<0.01) (Figures 4C and 4D, respectively). AII treatment reduced by 40% insulin-stimulated phosphorylation of Tyr612 and Tyr632 (P<0.02). The inhibitory effect of AII on phosphorylation of Tyr612 and Tyr632 was reversed by losartan. Thus, these data indicate that AII inhibits tyrosine phosphorylation on IRS-1 sites necessary for p85 binding. Previous studies have shown that hyper-Ser phosphorylation of IRS-1 converts IRS-1 in an inhibitor of the intrinsic insulin receptor tyrosine kinase.17 To test the possibility that increased Ser phosphorylation of IRS-1 induced by AII was associated with impaired insulin-stimulated tyrosine phosphorylation of the receptor, the insulin receptor was immunoprecipitated from cell lysates and immunoblotted with anti-Tyr1152/Tyr1162/Tyr1163 phospho-
specific antibody directed to the active loop of the catalytic domain of the insulin receptor. Insulin stimulated by ≈4-fold tyrosine phosphorylation of the insulin receptor (P<0.002) (Figure 4E). All treatment reduced by 65% insulin-stimulated Tyr<sup>1158</sup>/Tyr<sup>1162</sup>/Tyr<sup>1163</sup> phosphorylation of the receptor (P<0.005). Nonimmune serum did not lead to immunoprecipitation of the insulin receptor, thus indicating that immunoprecipitation of the insulin receptor by anti-IR antibody was specific (Figure 4E). The inhibitory effect of AII on Tyr<sup>1158</sup>/Tyr<sup>1162</sup>/Tyr<sup>1163</sup> phosphorylation of the insulin receptor was reversed by losartan.

**Effects of AII on Insulin-Stimulated Activation of Akt and eNOS**

Evidence has been provided indicating that insulin regulates NO production via a pathway involving PI-3 kinase–dependent activation of Akt, which in turn leads to phosphorylation of eNOS on serine 1177. Therefore, we tested whether AII would affect insulin-stimulated Akt/eNOS activation. Insulin stimulated by 3-fold Ser<sup>472</sup> Akt phosphorylation (P<0.01) and by 2.5-fold Thr<sup>308</sup> Akt phosphorylation (P<0.01) (Figures 5A and 5B). All treatment reversed by 60% insulin-stimulated Ser<sup>472</sup> Akt activation (P<0.01) and by 85% Thr<sup>308</sup> activation. These inhibitory effects of AII were reversed by losartan (Figures 5A and 5B). AII did not affect Akt expression, as detected by immunoblotting (Figures 5A and 5B). Insulin increased by ≈3-fold phosphorylation of eNOS on Ser<sup>1177</sup> (P<0.01) (Figure 5C). AII treatment resulted in a significant decrease (45%) of insulin-stimulated Ser<sup>1177</sup> eNOS phosphorylation (P<0.01), whereas it did not affect eNOS expression (Figure 5C). The inhibitory effect of AII was reversed by losartan.

**Figure 2.** Effects of AII and insulin phosphorylation of IRS-1 at Ser<sup>312</sup> and Ser<sup>616</sup> and JNK and ERK1/2 phosphorylation in HUVECs. A, Ser<sup>312</sup> and Ser<sup>616</sup> phosphorylation of IRS-1 and JNK phosphorylation in response to AII in the presence or absence of losartan or JNK inhibitor. B, Ser<sup>312</sup> and Ser<sup>616</sup> phosphorylation of IRS-1 and ERK1/2 phosphorylation in response to AII in the presence or absence of losartan or PD98059. C, Ser<sup>312</sup> and Ser<sup>616</sup> phosphorylation of IRS-1 in response to AII, insulin, All plus insulin, or All plus insulin plus losartan. D, JNK phosphorylation in response to AII, insulin, All plus insulin, or All plus insulin plus losartan. E, ERK1/2 phosphorylation in response to AII, insulin, All plus insulin, or All plus insulin plus losartan. F, ERK1/2 activity assay in HUVECs treated with AII, insulin, All plus insulin, or All plus insulin plus losartan. To normalize the blots for protein levels, after being immunblotted with anti-phosphospecific antibodies, the blots were stripped and reprobed with anti-JNK, anti-ERK1/2, or anti-IRS-1 antibodies. Each bar represents the mean±SD of 3 independent experiments, and autoradiographs of a representative experiment are shown.
dose-response relationship was observed for AII-induced IRS-1 phosphorylation at both Ser\(^{312}\) and Ser\(^{616}\), respectively (Figure 6D). The inhibitory effect of AII on insulin-stimulated NO production was reversed by losartan (Figure 6C). Treatment of HUVECs with JNK inhibitor or PD98059 reversed the inhibitory effect of AII, causing an increase of up to 80% and 55%, respectively, of NO production stimulated by insulin in the absence of AII, whereas simultaneous incubation with both inhibitors completely restored the stimulatory effects of insulin (Figure 6C). These data are consistent with the idea that AII-induced inhibition of the stimulatory effects of insulin on NO production is mediated, at least in part, through IRS-1 phosphorylation at Ser\(^{312}\) and Ser\(^{616}\) induced by JNK and ERK1/2, respectively, which negatively affects the downstream signaling pathway involving PI 3-kinase/Akt/eNOS.

**Discussion**

AII has been reportedly involved in the development of both insulin resistance and endothelial dysfunction in patients with essential hypertension.\(^5\)-\(^7\) It has been suggested that crosstalk between AII- and insulin-signaling pathways may underlie AII-induced insulin resistance at a vascular level.\(^9\) Vasodilator effects of insulin are mediated by the signaling pathway involving IRS-1/PI-3 kinase/Akt/eNOS that leads to increased NO production by endothelium.\(^8\) In this study, we addressed the question of whether AII-induced alterations in insulin signaling contribute to impair endothelial insulin action. We demonstrate that exposure of HUVECs to AII resulted in inhibition of insulin-stimulated production of NO. This event was associated with impaired tyrosine phosphorylation of IRS-1 and its corresponding association with the SH2 domains of p85, Akt, or eNOS protein levels but rather to a reduced phosphorylation of IRS-1 at tyrosine 612 and 632, which play a major role in the binding of IRS-1 with the SH2 domains of the p85 regulatory subunit of PI 3-kinase. A growing body of evidence indicates that serine phosphorylation of IRS-1 induced by a variety of factors interferes with the ability of this substrate to be tyrosine phosphorylated on insulin stimulation and reduces its ability to engage endothelial insulin action. We found that HUVECs exposed to AII was not attributable to alterations in IRS-1, p85, Akt, or eNOS protein levels but rather to a reduced phosphorylation of IRS-1 at tyrosine 612 and 632, which play a major role in the binding of IRS-1 with the SH2 domains of the p85 regulatory subunit of PI 3-kinase. A growing body of evidence indicates that serine phosphorylation of IRS-1 induced by a variety of factors interferes with the ability of this substrate to be tyrosine phosphorylated on insulin stimulation and reduces its ability to engage the p85 subunit of PI 3-kinase. More recently, several specific serine phosphorylation sites in IRS-1 and the corresponding activating kinases have been identified as responsible for these inhibitory effects.\(^13\)-\(^17\) Activation of JNK has been shown to result in stimulation of Ser\(^{632}\) of IRS-1, whereas activation of ERK1/2 has been shown to result in an increased phosphorylation of Ser\(^{612}\). Because AII activates both ERK1/2 and JNK in cultured vascular smooth muscle cells as well as in intact arteries,\(^18\),\(^19\) we examined the possibility that AII-induced phosphorylation at Ser\(^{632}\) and Ser\(^{612}\) of IRS-1 mediated by JNK and ERK1/2, respectively, may account for the inhibitory effects of AII on insulin signaling pathway involved in NO production. We found that HUVECs exposed to AII exhibited increased JNK and ERK1/2 activity, which was associated with a concomitant increase in IRS-1 phosphorylation at both Ser\(^{312}\) and Ser\(^{616}\), respectively (Figure 6D). The inhibitory effect of AII on insulin-stimulated NO production was reversed by losartan (Figure 6C). Treatment of HUVECs with JNK inhibitor or PD98059 reversed the inhibitory effect of AII, causing an increase of up to 80% and 55%, respectively, of NO production stimulated by insulin in the absence of AII, whereas simultaneous incubation with both inhibitors completely restored the stimulatory effects of insulin (Figure 6C). These data are consistent with the idea that AII-induced inhibition of the stimulatory effects of insulin on NO production is mediated, at least in part, through IRS-1 phosphorylation at Ser\(^{312}\) and Ser\(^{616}\) induced by JNK and ERK1/2, respectively, which negatively affects the downstream signaling pathway involving PI 3-kinase/Akt/eNOS.
losartan inhibited the stimulatory effects of AII on JNK and ERK1/2 activity and reverted the enhanced Ser\textsuperscript{312} and Ser\textsuperscript{616} phosphorylation of IRS-1 stimulated by AII. We additionally demonstrated the cause-effect relationship between these two events by using inhibitors of JNK and MEK1. Indeed, we found that inhibition of JNK and MEK1 activity partly reversed the negative effects of AII on insulin-stimulated NO production, whereas the combined inhibition of JNK and MEK1 activity fully restored the stimulatory effects of insulin. Obviously we cannot exclude the possibility that other serine kinases may phosphorylate IRS-1 under the conditions used in the present study, leading to impairment in the activation of downstream events of insulin signaling pathway. Furthermore, it is possible that the inhibitory effects of AII on insulin-stimulated NO production is independent of IRS-1 serine phosphorylation and is partially related to an ERK1/2-dependent eNOS phosphorylation, leading to inhibition of the enzyme, as suggested by a recent study.\textsuperscript{29} Notwithstanding these possibilities, the present results suggest that AII-induced activation of JNK and ERK1/2 might be an important negative regulator for the insulin pathway involved in NO production.

In summary, we show that AII acting via the AT\textsubscript{1} receptor exerts an inhibitory effect on the insulin signaling pathway involved in NO production and, for the first time, correlate these changes with activation of JNK and ERK1/2. Our data suggest that the uncoupling of IRS-1 and PI 3-kinase may be linked to an increased phosphorylation at Ser\textsuperscript{312} and Ser\textsuperscript{616} of IRS-1 mediated by JNK and ERK1/2, respectively. These changes are associated with a concomitant reduction in phosphorylation of Tyr\textsuperscript{612} and Tyr\textsuperscript{632} in two YXXM motifs essential for engaging p85 regulatory subunit of PI 3-kinase, resulting in impairment in activation of IRS-1-associated PI 3-kinase and sequential activation of the Akt/eNOS pathway.

In conclusion, increasing evidence suggests that the vasculature is an insulin-responsive tissue and that one of the
major vascular actions of insulin is its vasodilatory effect, which is mediated by enhanced production of NO. AII-induced insulin resistance in endothelial cells may play an important role in the pathophysiology of cardiovascular disease associated with hypertension and insulin resistance. The characterization of the molecular mechanism involved in AII-induced insulin resistance in the endothelium provides an important mechanistic link implicating JNK and ERK1/2 in the inhibitory effect of AII on insulin vascular action and may help to design efficacious pharmacological molecules to treat endothelial dysfunction associated with insulin resistance states.

Figure 5. Effects of AII on insulin-stimulated activation of Akt and eNOS in HUVECs. A, Ser<sup>473</sup> Akt phosphorylation. B, Thr<sup>308</sup> Akt phosphorylation. C, Ser<sup>1177</sup> eNOS phosphorylation. To normalize the blots for protein levels, after being immunoblotted with anti-phosphospecific antibodies, the blots were stripped and reprobed with anti-Akt or anti-eNOS antibodies. Each bar represents the mean±SD of 3 independent experiments, and autoradiographs of a representative experiment are shown. *P<0.01 vs control; †P<0.01 vs insulin; ††P<0.01 vs All+insulin by ANOVA.

Figure 6. Reversibility of the negative effect of AII on insulin-stimulated Akt and eNOS activation and NO production by JNK and MEK1 inhibitors in HUVECs. A, Effects of JNK and MEK1 inhibitors on AII-induced inhibition of Ser<sup>473</sup> Akt phosphorylation. B, Effects of JNK and MEK1 inhibitors on AII-induced inhibition of Ser<sup>1177</sup> eNOS phosphorylation. C, Effects of JNK and MEK1 inhibitors on AII-induced inhibition of NO production. D, Dose-response analysis for AII-induced IRS-1 phosphorylation at Ser<sup>312</sup> and Ser<sup>616</sup>. To normalize the blots for protein levels, after being immunoblotted with anti-phosphospecific antibodies, the blots were stripped and reprobed with anti-Akt, anti-eNOS, or anti-IRS-1 antibodies. Each bar represents the mean±SD of 3 independent experiments, and autoradiographs of a representative experiment are shown. *P<0.01 vs control; †P<0.01 vs insulin; ††P<0.02 vs All+insulin; †††P<0.01 vs AII+insulin by ANOVA.
Acknowledgments

This study was supported in part by grants from the European Community EuroDiabetesGene (No. QLG1-CT-1999-00674 to G.S.), Progetto di Ricerca Finalizzato, Ministero della Sanità (to G.S.), and PRIN-COFIN 2001 and 2002, Ministero dell’Istruzione, dell’Università e della Ricerca (to G.S. and F.P.).

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Circ Res. 2004;94:1211-1218; originally published online March 25, 2004;
doi: 10.1161/01.RES.0000126501.34994.96
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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