Insulin-Mediated Growth in Aortic Smooth Muscle and the Vascular Renin-Angiotensin System

Kei Kamide, Mark T. Hori, Jian-Hua Zhu, Jack D. Barrett, Peter Eggena, Michael L. Tuck

Abstract—Insulin has been shown to directly affect blood vessel tone and to promote vascular hypertrophy, but the mechanism of these actions remains uncertain. Because angiotensin I (Ang I)—converting enzyme inhibitors have been shown to improve insulin action and to impede the progression of vascular hypertrophy in hypertensive animal models, it is possible that the vascular properties of insulin may be mediated through the tissue renin-angiotensin system (RAS). To evaluate this relationship, we first investigated the effect of insulin on components of the RAS using cultured rat vascular smooth muscle cells (VSMCs). Insulin treatment (1000 μU/mL) markedly increased angiotensinogen mRNA expression and angiotensinogen production. We next investigated the role of the RAS in insulin-mediated cell proliferation, using [3H]thymidine uptake. Studies were done both with insulin alone and in the presence of captopril (1 × 10⁻⁷ to 10⁻⁵ mol/L) and losartan (1 × 10⁻⁹ to 10⁻⁷ mol/L). [3H]Thymidine uptake was increased significantly by 1000 μU/mL insulin, and this stimulation was reduced by 1 × 10⁻⁸ mol/L captopril (−38.8%, P < 0.05) and by 1 × 10⁻⁸ mol/L losartan (−37.5%, P < 0.05). Further studies showed that the degree of insulin-mediated [3H]thymidine uptake in VSMCs could be duplicated by 4 × 10⁻¹⁰ mol/L Ang II. Losartan reduced the effects of both Ang II and insulin on [3H]thymidine uptake by about 40% to 45% of baseline (P < 0.05). Captopril reduced insulin-mediated [3H]thymidine uptake but did not affect Ang II–mediated [3H]thymidine uptake. In summary, insulin induced significant stimulation of angiotensinogen expression and production and stimulated growth similar to that seen with Ang II in cultured rat VSMCs. Inhibition of Ang II production or its binding to the Ang II type 1 (AT₁) receptor inhibited insulin-mediated growth in a fashion similar to that seen with inhibition of Ang II–mediated growth. Thus, insulin can modulate the vascular RAS, and the effect of insulin on vascular growth may be via direct effects on angiotensinogen expression and translation operative through both the AT₁ receptor and the conversion of Ang I to Ang II. (Hypertension. 1998;32:482-487.)

Key Words: angiotensinogen ■ angiotensin II ■ insulin ■ muscle, smooth, vascular

Insulin resistance and hyperinsulinemia have been correlated with hypertension, dyslipidemia, glucose intolerance, and obesity, as well as with atherosclerosis, cardiac hypertrophy, ischemic heart disease, and cerebrovascular disease. The mechanisms responsible for the increased cardiovascular risk associated with insulin are not completely understood. Insulin has indirect effects on the cardiovascular system through increased renal sodium reabsorption, enhanced sympathetic nervous system activity, and direct actions on blood vessels modulating vascular tone. The mechanisms of the action of insulin on vascular tone include alterations in nitric oxide and cytosolic calcium. The growth effects of insulin may be the most important factor in increased cardiovascular risk. Different forms of insulin such as insulin-like growth factor-I (IGF-I) and proinsulin participate along with insulin to stimulate vascular growth and atherosclerosis.

It also has been established that increased activity of the renin-angiotensin system (RAS) is associated with increased cardiovascular risk, including myocardial infarction. Thus, it is possible that an interaction between insulin and the RAS may account for many high-risk cardiac conditions. However, the combined effects of insulin and the RAS on vascular function have not been fully evaluated. Clinically, blockade of the RAS with angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin type 1 receptor (AT₁-R) antagonists improves insulin action. The opposite effect (ie, whether insulin can modulate components of the RAS) has only been partially evaluated. A study in diabetic animals showed that renin release is increased by insulin and IGF-I. In addition, blockade of the RAS by ACEI or AT₁-R antagonists prevents progression of atherosclerosis in various animal models. This effect of blockade of the RAS in prevention of cardiovascular complications is now being investigated in clinical trials. These observations point to an interaction between insulin and the RAS in the control of vascular tone and growth. The action of insulin on blood vessels, especially its effects on cell growth, may be mediated through activation of the RAS.

The effect of insulin on vascular growth could be mediated through the tissue RAS. This study examines the effects of...
insulin on angiotensinogen and renin expression and production in cultured rat vascular smooth muscle cells (VSMCs). The effects of both insulin and angiotensin II (Ang II) on cell proliferation are also examined.

**Methods**

**Cell Culture**

VSMCs were isolated from rat thoracic aorta (male Sprague-Dawley rats, 250 to 300 g) by enzymatic dispersion and grown in a 1:1 mixture of Dulbecco’s modified Eagle's medium and Ham's F-12 (Sigma Chemical Co) supplemented with 10% fetal calf serum (FCS, Tissue Culture Biologicals), 50 U/mL penicillin, and 50 μg/mL streptomycin (Sigma). Confluent cultures were passaged after treatment with trypsin-EDTA and reseeding at a 1:4 ratio in fresh media. Cells (4 to 12 passages) were seeded into 100-mm (P100) culture dishes and reached confluence in 5 to 7 days. Cell lines were randomly screened for smooth muscle actin expression by immunofluorescent staining with anti-smooth muscle actin (Enzo Diagnostics Inc) and fluorescence-conjugated rabbit anti-mouse IgG (Cappel Laboratories, Organon Teknika Corp).

**Study Protocol**

Confluent cells cultured in P100 dishes were deprived of serum for 24 hours before administration of insulin. Insulin (10 to 1000 μU/mL, regular porcine insulin; U-100, Eli Lilly & Co) was then added. After 48 hours of insulin exposure, the media was collected. VSMCs were deprived of serum for 24 hours before administration of insulin. Insulin (10 to 1000 μU/mL) or Ang II (10^-10 to 10^-4 mol/L; Sigma) with or without RAS inhibition, which was via 3 doses of the ACEI captopril (1×10^-5, 1×10^-6, 1×10^-7 mol/L) or the AT1-R antagonist losartan (1×10^-5, 1×10^-6, 1×10^-7 mol/L). Doses of these drugs were determined according to previous reports in cultured VSMCs. After 48 hours, the growth rate of VSMCs was evaluated by pulse-labeled tritiated thymidine ([H]Tdr) uptake as described below.

**Detection of mRNA**

Smooth muscle cell dishes were washed with Ca²⁺-Mg²⁺–free PBS lysed in 1 mL of 4 mol/L guanidine-isothiocyanate containing 25 mmol/L trisodium citrate (pH 7.0), 0.1 mol/L 2-mercaptoethanol, and 0.5% (wt/vol) streptomycin (Sigma). Confluent cultures were passaged after treatment with trypsin-EDTA and reseeding at a 1:4 ratio in fresh media. Cells (4 to 12 passages) were seeded into 100-mm (P100) culture dishes and reached confluence in 5 to 7 days. Cell lines were randomly screened for smooth muscle actin expression by immunofluorescent staining with anti-smooth muscle actin (Enzo Diagnostics Inc) and fluorescence-conjugated rabbit anti-mouse IgG (Cappel Laboratories, Organon Teknika Corp).

**Angiotensinogen and Renin in Culture Media**

Angiotensinogen concentration in the culture media was quantified by radioimmunoassay determination of Ang I generated after incubation with homologous rat renin in the presence of angiotensinase inhibitors as described. Renin concentration in the culture media was measured by determination of the hourly rate of Ang I production in the presence of excess homologous renin-free angiotensinogen. Samples were assayed for angiotensinogen and renin in triplicate. The limit of detection of generated Ang I for both angiotensinogen and renin determinations in these experiments is 50 pg/mL.

**Cell Proliferation**

The effect of captopril or losartan on insulin-mediated cell proliferation was estimated by assessing the rate of [H]Tdr uptake during the final 4 hours of treatment. [H]Tdr (1 μCi/mL, 3 mL per well, ICN Pharmaceuticals) was added to serum-free medium during the final 4 hours of incubation. After incubation, the medium was aspirated, and the cells were washed 3 times each in ice-cold PBS, 10% trichloroacetic acid, and then distilled water and allowed to air-dry. Cells were then solubilized in 1 mL 1% SDS, and the radioactivity of each sample was determined by liquid scintillation spectrometry using a Beckman LS-5801 spectrometer. The protein concentration of each sample was measured by the micro bicinchoninic acid method (Pierce) using an MRX plate reader (Dynatech Laboratories), and total protein was estimated by comparison of OD readings against known standards using the Biolinx protein analysis program (Dynatech Laboratories). Data are presented as [H]Tdr uptake per microgram of protein normalized as percent change versus controls.

**Determination of Receptor on Insulin-Mediated Growth**

To determine whether insulin-induced effects on growth were mediated by IGF-I receptor, [H]Tdr experiments were conducted in the presence of the anti–IGF-I receptor monoclonal antibody αdR3 (25 nmol/L, Calbiochem). Cells in 12-well plates were grown to confluence and then rendered quiescent by incubation with serum-free medium for 24 hours. Insulin or IGF-I (Calbiochem; 1000 μU/mL and 25 ng/mL, respectively) was added with or without αdR3 and incubated for 48 hours. [H]Tdr uptake was determined as described above.

**Statistical Analysis**

Each experiment was performed with a minimum of 3 different cell lines. All values are expressed as mean±SEM. Radioimmunoassay, mRNA, and [H]Tdr uptake data were analyzed by unpaired t test using StatView 4.02 (Abacus Concepts Inc, 1992). A value of P<0.05 was considered statistically significant.
Results

Angiotensinogen, Renin and AT₁-R Gene Expression in Insulin-Treated VSMCs

Figure 1, top, shows the autoradiograms for angiotensinogen, renin, and AT₁-R mRNA expression in cultured VSMCs; Figure 1, bottom, shows the densitometric analysis of the autoradiograms. Insulin treatment (1000 μU/mL) increased angiotensinogen mRNA expression by 25-fold (arbitrary density units, *P < 0.05), whereas it had no effect on renin or AT₁-R mRNA expression (Figure 1, top and bottom). Northern blot analysis for insulin-stimulated angiotensinogen mRNA production in VSMCs showed a similar several-fold increase in angiotensinogen with insulin, with its signal set at approximately 18S²⁸ (data not shown).

Angiotensinogen and Renin Production in Insulin-Treated VSMCs

Addition of insulin from 10 to 1000 μU/mL to the culture media produced a significant dose-dependent increase in angiotensinogen release by VSMCs (Figure 2). Renin levels were not detectable by this assay system with or without insulin exposure.

Effects of Insulin and Ang II on [³H]Thymidine Uptake

There was a significant increase in [³H]TdR uptake by VSMCs after 48 hours of incubation with insulin (100 to 10000 μU/mL) compared with no insulin added (Figure 3, top). Ang II (1×10⁻⁸ to 1×10⁻⁷ mol/L) also produced a significant dose-dependent increase in [³H]TdR uptake in VSMCs compared with control (Figure 3, bottom). Experiments to elucidate the equivalent potencies of insulin and Ang II were performed.
Ang II on VSMC proliferation revealed that 1000 μU/mL insulin had effects similar to 4 to 6×10^{-10} mol/L Ang II (Table 1) on [3H]TdR uptake in VSMCs.

**Effects of Captopril and Losartan on Insulin-Mediated [3H]Thymidine Uptake**

Figure 4, top, shows that insulin-mediated [3H]TdR uptake was blocked by −38.8% of baseline with 1×10^{-9} mol/L captopril (P<0.05) and by −37.5% of baseline with 1×10^{-9} mol/L losartan (P<0.05). Figure 4, bottom, shows that Ang II–stimulated [3H]TdR uptake was also blocked by 1×10^{-9} mol/L losartan (−43.6%, P<0.05) and by 1×10^{-7} mol/L losartan (−52.6%, P<0.05). Captopril had no effect on Ang II–mediated [3H]TdR uptake (Figure 4, bottom). In the absence of insulin, blockade of the RAS by all doses of captopril and losartan did not alter [3H]TdR uptake in cultured VSMCs.

**Role of IGF-I Receptor in Insulin-Mediated Growth**

Table 2 shows that both insulin and IGF-I increased [3H]TdR uptake significantly compared with basal uptake (+18.2% and +15.1%, respectively; P<0.05), αR3 blocked IGF-I–induced [3H]TdR uptake significantly (P<0.05) but had no inhibitory effect on insulin-treated cells.

**Discussion**

Both insulin resistance and hyperinsulinemia\(^5\) and increased activation of the RAS\(^4\) have been associated with hypertension, accelerated atherosclerosis,\(^3\) and increased risk for cardiovascular events. However, the status of the RAS in insulin-resistant states and the effect of insulin on the RAS have not been examined in detail. Most reports describing a relationship between insulin and the RAS have been in whole animal models\(^3\) describing systemic effects. Thus, Kobayashi et al\(^8\) and Iyer et al\(^9\) reported elevated levels of plasma Ang II in the insulin-resistant, hyperinsulinemic, fructose-fed rat model. These studies did not resolve whether the high insulin levels were the cause of the elevated plasma Ang II. Additionally, in diabetic animals, IGF-I has been shown to stimulate renin release.\(^17\)

The present study demonstrates in cultured VSMCs that insulin causes an increase in angiotensinogen mRNA transcription and augmentation of its production. Moreover, in a similar temporal fashion, insulin exposure increases VSMC growth, and this effect is partially abolished by ACE inhibition and AT\(_1\)-R blockade. These data imply that insulin may participate in VSMC proliferation through the trophic effects of Ang II, an established growth factor in VSMCs.\(^40\) The observation that the growth rate induced by 1000 μU/mL insulin in VSMCs is similar to that induced by 4×10^{-10} mol/L Ang II supports the validity of an interaction between insulin and the RAS in vascular growth. The information that the [3H]TdR uptake induced by insulin and Ang II can be

**Table 1. Comparison Between Insulin- and Ang II–Mediated [3H]Thymidine Uptake in VSMCs**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Change of [3H]TdR From Baseline (−), %</th>
<th>SE, %</th>
<th>P vs Insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin, 1000 μU/mL</td>
<td>49.7</td>
<td>5.0</td>
<td>(−)</td>
</tr>
<tr>
<td>Ang II, 10^{-10} mol/L</td>
<td>5.3</td>
<td>7.3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>2×10^{-10}</td>
<td>12.6</td>
<td>5.8</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>4×10^{-10}</td>
<td>40.3</td>
<td>2.4</td>
<td>0.160</td>
</tr>
<tr>
<td>6×10^{-10}</td>
<td>63.5</td>
<td>5.5</td>
<td>0.137</td>
</tr>
<tr>
<td>8×10^{-10}</td>
<td>92.7</td>
<td>8.7</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>10^{-9}</td>
<td>77.2</td>
<td>20.9</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

**Table 2. Effect of Anti–IGF-I Receptor Monoclonal Antibody (αR3) on Insulin- and IGF-I–Mediated Growth of VSMCs**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Change of [3H]TdR From Baseline (−), %</th>
</tr>
</thead>
<tbody>
<tr>
<td>αR3, 25 nmol/L</td>
<td>−4.4±3.0</td>
</tr>
<tr>
<td>Insulin, 1000 μU/mL</td>
<td>18.2±5.4*</td>
</tr>
<tr>
<td>IGF-I, 25 ng/mL</td>
<td>15.1±2.9*</td>
</tr>
<tr>
<td>Insulin+αR3</td>
<td>17.1±1.3*</td>
</tr>
<tr>
<td>IGF-I+αR3</td>
<td>6.3±1.6†</td>
</tr>
</tbody>
</table>

Values are mean±SEM. *P<0.05 vs basal (−); †P<0.05 vs IGF-I.
reduced by a similar degree by AT\(_1\)-R blockade indicates the dependency of this interaction.

Because the inhibitory effects of losartan and captopril on insulin-stimulated \([^{3}H]\text{TdR}\) uptake were similar, it is not likely that the kallikrein/kinin pathway could be responsible for these results\(^{16,61}\). Although both captopril and losartan reduced insulin-mediated \([^{3}H]\text{TdR}\) uptake, Ang II–mediated \([^{3}H]\text{TdR}\) uptake was suppressed only by losartan. These findings indicate that the interaction with Ang II in the facilitation of insulin-mediated growth requires converting enzyme activity. Thus, endogenous renin activity, although not measurable in the present study, should be present in the system\(^{23}\). The presence of ACE activity is also suggested by these data and has been previously reported in vascular tissue\(^{43}\). Likewise, participation of the AT\(_1\)-R in insulin-mediated growth is implied by sensitivity to losartan, although AT\(_1\)-R mRNA expression was not influenced by insulin treatment. Finally, the observation that in the absence of insulin, neither captopril nor losartan altered \([^{3}H]\text{TdR}\) uptake in VSMCs indicates a permissive role for insulin in growth mediated through the RAS.

Other investigators have described a role for the RAS in vascular growth. Makita et al\(^{25}\) reported that losartan could block Ang II–mediated \([^{3}H]\text{TdR}\) uptake in human aortic smooth muscle cells. In other studies, insulin has been shown to alter AT\(_1\)-R density in vascular tissue in fructose-fed rats\(^{39}\). Insulin-mediated effects on angiotensinogen mRNA expression have also been described in nonvascular tissue, specifically in Reuber H35 hepatoma cells\(^{45}\). In these studies, angiotensinogen mRNA expression was decreased by 24-hour insulin treatment, yet cell proliferation was increased. These findings indicate that the mechanism of insulin-mediated cell growth may be tissue specific\(^{44}\).

To justify the present findings, the transendothelial transport of insulin in the vasculature must be explained. Because specific high-affinity receptors for insulin are found in VSMCs\(^{45}\), transendothelial transport of insulin must take place. In fact, both receptor- and non–receptor-mediated insulin transport mechanisms have been described. Receptor-mediated transport of insulin across endothelial cells was described by King and Johnson\(^{46}\), and transendothelial insulin transport was described by Steil et al\(^{47}\) to be insaturable.

The high doses of insulin applied in these types of in vitro experiments in vascular tissue have also been questioned. Studies of insulin-stimulated growth and RAS expression included doses from 10 to 1000 \(\mu\text{U/mL}\). Although insulin-stimulated growth was measurable at 10 \(\mu\text{U/mL}\), we did not observe significant stimulation of thymidine uptake at doses <100 \(\mu\text{U/mL}\) of insulin. One possible explanation for this may be that the growth-stimulation pathway in our time course, 48 hours, may not involve the classic insulin-stimulated growth pathways described in acute studies. Stimulation of the vascular RAS in the present experiments required a relatively high concentration of insulin, the system being activated only at doses >100 \(\mu\text{U/mL}\). The findings are in agreement with other reports using in vitro systems, in which high doses of insulin are needed to elicit a response. However, these observations also may reflect the condition noted in animal and human states of insulin resistance and hyperinsulinemia in which postmeal plasma insulin levels >100 \(\mu\text{U/mL}\) are common\(^{38}\). The question of whether insulin-induced growth effects are mediated via the IGF-I receptor was examined in experiments performed in the presence of IGF-I receptor blockade. Both 1000 \(\mu\text{U/mL}\) insulin and 25 ng/mL IGF-I stimulated \([^{3}H]\text{TdR}\) uptake. Incubation with the anti–IGF-I receptor monoclonal antibody cIR3 significantly inhibited IGF-I–mediated \([^{3}H]\text{TdR}\) uptake while having only a negligible effect on insulin-stimulated uptake. These data indicate that insulin-mediated effects occur as a result of stimulation of the insulin receptor and not the IGF-I receptor. Banskota et al\(^{22}\) also found that IGF-I receptor blockade had little effect on insulin-mediated growth of cultured VSMCs at insulin concentrations <1000 \(\mu\text{U/mL}\).

Although these findings would imply that there is little insulin-mediated activation of the IGF-I receptor in VSMCs, alternative activation of the IGF-I pathway is possible. IGF-I is a potent growth factor, structurally similar to insulin, that can cross-react with the insulin receptor\(^{12,49}\). Delafontaine et al\(^{66}\) have shown that Ang II can increase IGF-I production in VSMCs. This study opens the possibility that IGF-I activation could also occur in VSMCs via insulin-mediated activation of the vascular RAS. In H35 hepatoma cells\(^{59}\), IGF-I and IGF-II do not alter angiotensinogen mRNA expression.

It is well known that insulin can affect regulation of gene transcription and production of several hormones and proto-oncogenes such as c-fos which promote cell growth.\(^{51}\) Thus, it is possible that the mechanisms of insulin-stimulated RAS activation may be through mitogen-activated kinase cascades resulting in acceleration of angiotensinogen gene transcription. Growth factor–mediated production of intracellular hormones has also been previously reported.\(^{52}\) Studies from our laboratory show that transcription of angiotensinogen and renin genes can be accelerated by Ang II via nuclear angiotensin receptor\(^{53}\).

In conclusion, insulin increases cellular growth rate and induces production of tissue angiotensinogen in cultured rat VSMCs. Moreover, the effect of insulin is acute and operative both through the AT\(_1\)-R and at the level of conversion of Ang I to Ang II. This finding suggests that hyperinsulinemia may contribute to vascular hypertrophy via activation of the vascular RAS, increasing angiotensinogen production and intracellular Ang II, and activation of the AT\(_1\)-R. These studies indicate that inhibition of the vascular RAS by ACEI or AT\(_1\)-R antagonists could diminish the progression of atherosclerosis in insulin-resistance states.

Acknowledgments

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