Insulin Increases Vascular Smooth Muscle Recovery From Intracellular Calcium Loads

Young-Cheul Kim, Michael B. Zemel

Insulin has previously been shown to attenuate vasoconstrictor responses to pressor agonists and accelerate vascular smooth muscle relaxation and vascular smooth muscle Ca$^{2+}$ efflux. To further determine the role of insulin in regulating vascular smooth muscle Ca$^{2+}$, quiescent A7r5 cultured vascular smooth muscle cells and human vascular smooth muscle cells were incubated with or without 10$^{-7}$ or 10$^{-8}$ M insulin for 1 hour and then loaded with fura-2 AM; intracellular Ca$^{2+}$ responses to and rates of recovery from angiotensin II (200 nM) and arginine vasopressin (AVP) (10 μM) were studied fluorometrically in stirred suspension. Insulin (10$^-7$ M) caused an increase in the peak intracellular Ca$^{2+}$ response to angiotensin II (peak/baseline×100=469±96 versus 288±74, P<.05) and a decrease in the peak Ca$^{2+}$ response to vasopressin (288±50 versus 389±33, P<.025). However, insulin also caused a marked increase in the rate of intracellular Ca$^{2+}$ recovery to baseline after stimulation with both angiotensin II (77.3±13.8 versus 30.6±6 nM/min, P<.03) and vasopressin (P<.05), such that the cumulative exposure to elevated intracellular Ca$^{2+}$ after stimulation with either agonist (i.e., area under the intracellular Ca$^{2+}$ curve) was reduced with insulin treatment. Insulin (10$^{-7}$) caused small but still significant effects on all parameters in the A7r5 cells. Insulin also caused comparable effects on Ca$^{2+}$ recovery in the human cells but was without significant effect on peak Ca$^{2+}$ responses to AVP. It is concluded that accelerated removal of cytosolic Ca$^{2+}$ after agonist stimulation is likely to contribute to insulin attenuation of vasoconstrictor responses and acceleration of vascular relaxation. (Hypertension 1993;22:74-77)

Key Words • angiotensin II • calcium • insulin • muscle, smooth, vascular • vasopressin

Although hypertension in insulin-resistant states is most often attributed to increased sympathetic neural output and renal sodium retention secondary to hyperinsulinemia, several recent lines of evidence suggest otherwise. Insulin appears to exert direct vasodilator effects on vascular smooth muscle. We have suggested that vascular smooth muscle manifestations of insulin resistance, leading to loss of insulin-mediated vasodilation, may be responsible for the hypertension in insulin-resistant states. We have found insulin to attenuate in vitro vascular reactivity responses to vasoconstrictor agonists in endothelial-denuded aortic strips and to accelerate endothelium-independent relaxation after vasoconstriction, whereas these effects are substantially lost in aortic strips from insulin-resistant (Zucker obese) rats. Further, physiological concentrations of insulin were recently reported to inhibit angiotensin II (Ang II) and serotonin-induced contractions of canine femoral artery smooth muscle cells.

Anderson et al. found that insulin infusion with maintenance of euglycemia caused significant decreases in peripheral vascular resistance in both normotensive and hypertensive individuals despite observed increases in muscle sympathetic nerve activity. Similarly, insulin infusion was recently reported to increase forearm blood flow during concomitant norepinephrine infusion in humans. Moreover, Dubey et al. reported that treatment with pioglitazone, a thiazolidinedione compound that amplifies peripheral cellular responses to insulin, resulted in attenuation of hypertension in both Dahl salt-sensitive and one-kidney, one-clip hypertensive rats. Insulin has been shown to decrease peak intracellular Ca$^{2+}$ ([Ca$^{2+}]_{i}$) responses to vasopressin in cultured rat aortic smooth muscle cells by reducing Ca$^{2+}$ influx through receptor-operated channels and to decrease voltage-mediated Ca$^{2+}$ influx. Moreover, we have found insulin to increase the expression of both sarcoplasmic reticulum and plasma membrane Ca$^{2+}$ ATPases and to cause a corresponding increase in Ca$^{2+}$ efflux. Accordingly, we conducted the present studies to further define the role of insulin in regulating vascular smooth muscle cell (VSMC) [Ca$^{2+}]_{i}$ responses to vasoconstrictor agonists. We have studied the effects of insulin on Ang II and arginine vasopressin–induced [Ca$^{2+}]_{i}$ transients, since Ang II–induced Ca$^{2+}$ transients are mediated by release from sarcoplasmic reticulum stores rather than transmembrane Ca$^{2+}$ flux and may therefore be regulated differently by insulin than is the vasopressin-induced [Ca$^{2+}]_{i}$ transient. We report herein that although insulin exerts markedly different effects on peak Ca$^{2+}$ responses to the two agonists, with response to Ang II augmented, insulin accelerates the rate of recovery from both agonists and reduces the area under the [Ca$^{2+}]_{i}$ response curve after stimulation with either agonist.
**Methods**

**Vascular Smooth Muscle Cell Preparation**

A clonal VSMC line (A7r5) from thoracic aorta of BDIX rats was obtained from American Type Culture Collection, Rockville, Md. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 10,000 U/mL penicillin, 10 mg/mL streptomycin, and 8 mg/mL tylosin tartrate and were maintained in a 5% CO₂, 100% humidity atmosphere at 37°C. In a separate series of studies, human pulmonary artery VSMCs were obtained (Clonetics, Inc., San Diego, Calif.) in passage 3 and grown as described above in a medium containing 5% fetal calf serum, 10 ng/mL epidermal growth factor, 1 mM dexamethasone, 2 ng/mL human fibroblast growth factor, gentamicin, and amphotericin-B. For sequential passage or study, confluent cells were rinsed with Ca²⁺/Mg²⁺-free Hanks' balanced salt solution (Sigma Chemical Co., St. Louis, Mo.) and treated with 0.5 mg/mL trypsin for 5 minutes at 37°C. Released cells were centrifuged at 750g for 5 minutes, and the pellet was then resuspended in DMEM. The growth medium of confluent cells was replaced with serum-free DMEM and antibiotics (as above) for 24 hours before VSMC Ca²⁺ studies. A7r5 cells were studied in passages 4 to 22, and human cells were studied in passages 5 to 6.

**Ca²⁺ Determination**

The fluorescent dye fura-2/AM (acetylmethoxy ester, Calbiochem, San Diego, Calif.) was dissolved in DMSO to yield a final fura-2 concentration of 10 μM. Ang II, AVP, and digitonin were obtained from Sigma. Ang II and AVP were dissolved in serum-free HEPES-buffered salt solution (HBSS) containing (in mM): NaCl 138, CaCl₂ 1.8, MgSO₄ 0.8, NaH₂PO₄ 0.9, NaHCO₃ 4.0, D-glucose 25, glutamine 6.0, HEPES 20, and 5% bovine serum albumin, pH 7.4.

Confluent cell monolayers were maintained in serum-free DMEM for 24 hours, and quiescent cells were then rinsed with Hanks' solution, trypsinized, and centrifuged. The pellet was resuspended in HBSS, and cell suspensions were prechilled on ice for approximately 10 minutes before fura-2/AM loading. Fura-2-loaded cells were incubated in a shaking water bath in the dark for 20 minutes at 37°C and then sedimented by centrifugation and resuspended in HBSS. Cells were further incubated for 60 minutes in a 37°C shaking water bath with or without insulin (10⁻⁷ or 10⁻⁸ M). Cells were then washed, centrifuged, and resuspended at a concentration of approximately 10⁶ cells/mL. [Ca²⁺]i levels were then determined fluorometrically in suspensions using dual excitation (340 and 380 nm) per single emission (510 nm) fluorometry (Hitachi F-2000, Naperville, Ill.).

Maximal and minimal fluorescent signals were obtained with 40 μM digitonin and pH 8.7 Tris (100 mM) EGTA (100 mM) to calibrate the intracellular Ca²⁺ signal. Intracellular Ca²⁺ was then calculated by the computer in the fluorometer using the equation of Grynkiewicz et al.¹⁵

Peak [Ca²⁺]i response to Ang II (200 nM) and AVP (10 μM) were evaluated after establishing a stable baseline (within the first 2 minutes of study) for each cell suspension. The rate of [Ca²⁺]i recovery (ie, rate of return to baseline) after stimulation with each agonist was calculated from the slope of the [Ca²⁺]i tracings in the recovery (ie, post-peak) phase. Recovery was calculated by regression analysis. All data were captured in a microcomputer using a data acquisition program, and data points were converted to a spreadsheet format (QUATTRO PRO 4.0, Borland International, Inc., Scotts Valley, Calif.). Regression analysis was performed on the first 30-second period after each peak response. The data were uniformly linear for at least 45 seconds in all cases. Attempts to fit the full recovery data set from each curve with an exponential regression resulted in qualitatively comparable data, but the exponential regressions resulted in a poorer fit (ie, larger error term).

All experiments were replicated eight times, and data were analyzed for differences between control and insulin-treated VSMCs using the unpaired t test.

**Results**

Baseline [Ca²⁺]i levels were not significantly affected by insulin incubation. Figs 1 and 2 depict typical tracings of the effect of insulin on the VSMC [Ca²⁺]i response to Ang II and AVP, respectively, in A7r5 cells, and the data are summarized in Table 1. The insulin-treated preparations exhibited a significantly greater peak [Ca²⁺]i response to Ang II but also markedly accelerated the rate of [Ca²⁺]i recovery to baseline (77.3 ± 13.8 versus 30.6 ± 6 nM/min, P < .03). Conse-
**TABLE 1. Effects of 10^-7 Insulin on Vascular Smooth Muscle Intracellular Calcium Responses to Angiotensin II and Arginine Vasopressin**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Response to Ang II (%)</td>
<td>288±74</td>
<td>469±96*</td>
</tr>
<tr>
<td>Response to AVP (%)</td>
<td>389±33</td>
<td>288±50†</td>
</tr>
<tr>
<td>Recovery from Ang II (nM/min)</td>
<td>30.6±6</td>
<td>77.3±13.8†</td>
</tr>
<tr>
<td>Recovery from AVP (nM/min)</td>
<td>18.46±4.7</td>
<td>23.44±4.7*</td>
</tr>
<tr>
<td>Area under Ang II response curve (nM·s)</td>
<td>19,865±515</td>
<td>16,890±258*</td>
</tr>
<tr>
<td>Area under AVP response curve (nM·s)</td>
<td>37,556±920</td>
<td>14,399±689†</td>
</tr>
</tbody>
</table>

Response (%), peak/baseline×100; baseline [Ca^{2+}], 141±14; Ang II, angiotensin II; AVP, arginine vasopressin; recovery, rate of return to baseline [Ca^{2+}]. n=8 per group.

*P<.05.
†P<.03.

**Discussion**

Data from the present study demonstrate that insulin accelerates the rate of [Ca^{2+}]i recovery to baseline in cultured VSMCs after stimulation with either Ang II or AVP. We have recently reported that insulin incubation also stimulates the expression of plasmalemma Ca^{2+}-ATPase in A7r5 cultured VSMCs and causes a corresponding increase in Ca^{2+}-ATPase-mediated Ca^{2+} efflux. We have also found insulin to increase the expression of sarcoplasmic reticulum (SR) Ca^{2+}-ATPase in these cells, although the time course of this stimulation was somewhat delayed in comparison with stimulation of the plasmalemma Ca^{2+}-ATPase. Accordingly, the increase in [Ca^{2+}]i recovery rate in the present study probably reflects insulin stimulation of plasmalemma Ca^{2+}-ATPase, SR Ca^{2+}-ATPase, or both.

In support of this concept, we have previously reported that insulin stimulates rat aortic 45Ca^{2+} efflux in a dose-dependent fashion. Moreover, we have also recently reported decreases in both plasmalemma Ca^{2+}-ATPase expression and the rate of [Ca^{2+}]i return to baseline in VSMCs obtained from Zucker obese rats compared with Zucker lean rats. Since the Zucker obese rats exhibit insulin resistance, we conclude that these observations result from a vascular smooth muscle manifestation of insulin resistance and a consequent failure of insulin to stimulate Ca^{2+}-ATPase expression and Ca^{2+} efflux.

Insulin attenuation of vasoconstrictor responses may also result, in part, from blunting of the peak [Ca^{2+}]i response to Ang II, insulin treatment reduced the peak [Ca^{2+}]i response to AVP (Fig 2, Table 1). However, the rate of recovery to baseline was still significantly increased in the insulin-treated cells compared with control (Table 1), resulting in a decrease in the area under the AVP-induced [Ca^{2+}]i response curve (Table 1).

Recovery rates were slower in the human pulmonary artery VSMCs than in the A7r5 cells, but insulin exerted a comparable effect on increasing recovery (Ang II recovery rate was 15.4±1.6 versus 11.2±1.2 nM/min in insulin-treated and control cells, respectively, P<.02; AVP recovery rate was 13.7±2.5 versus 6.9±0.8 nM/min, P<.02) and decreasing the area under the [Ca^{2+}]i response curve to both agonists. However, insulin was without effect on the peak response to either agonist.
In contrast to the effects of insulin on peak [Ca\(^{2+}\)]\(_i\), responses to AVP to Ang II in A7r5 cells. However, insulin incubation also caused a twofold increase in Ang II–induced [Ca\(^{2+}\)]\(_i\) load and consequently caused a decrease in net cellular exposure to elevated [Ca\(^{2+}\)]\(_i\), (ie, area under the [Ca\(^{2+}\)]\(_i\) curve). Since insulin attenuates vasconstrictor responses to Ang II,\(^2\) it appears that the effects on recovery rate and area under the [Ca\(^{2+}\)]\(_i\) curve may be more important to the regulation of vasconstrictor responses than are insulin effects on peak [Ca\(^{2+}\)]\(_i\), responses to vasconstrictor agonists. Moreover, since insulin increased the [Ca\(^{2+}\)]\(_i\), recovery rate in the cultured human VSMCs without affecting the peak response to either agonist, the relevant effect of insulin on human VSMC [Ca\(^{2+}\)]\(_i\) regulation is likely limited to stimulation of [Ca\(^{2+}\)]\(_i\) recovery. However, whether these differences between rat and human cells will be sustained in human cells cultured from other vessels remains to be determined.

The mechanism of insulin augmentation of peak [Ca\(^{2+}\)]\(_i\), responses to Ang II in A7r5 cells is not clear. However, we have found insulin to increase SR Ca\(^{2+}\)-ATPase expression; because Ang II–induced [Ca\(^{2+}\)]\(_i\), transients are dependent on SR Ca\(^{2+}\) release, we speculated that insulin stimulation of SR Ca\(^{2+}\)-ATPase may have resulted in increased Ang II–releasable stores. To explore this possibility, we performed preliminary experiments to evaluate the effects of inhibiting the SR Ca\(^{2+}\)-ATPase on [Ca\(^{2+}\)]\(_i\) responses in insulin-treated and control cells. Treatment of cells with 1 μM of the SR Ca\(^{2+}\)-ATPase inhibitor thapsigargin resulted in a substantially greater [Ca\(^{2+}\)]\(_i\) response in insulin-treated cells than in controls as well as a faster Ca\(^{2+}\) recovery rate (unpublished observations from our laboratory). This increased release of Ca\(^{2+}\) by thapsigargin supports the concept that insulin may increase peak [Ca\(^{2+}\)]\(_i\), responses to Ang II by stimulating SR Ca\(^{2+}\)-ATPase and thereby increasing Ang II–releasable stores. In addition, the observed increase in [Ca\(^{2+}\)]\(_i\), recovery rate in the presence of an SR Ca\(^{2+}\)-ATPase inhibitor is likely to reflect insulin stimulation of plasma membrane Ca\(^{2+}\)-ATPase. However, these data are only preliminary, as we have experienced difficulty in routinely eliciting thapsigargin–induced [Ca\(^{2+}\)]\(_i\), transients. Accordingly, although these data are suggestive, we are cautious in interpreting this thapsigargin data.

Several lines of recent evidence suggest that insulin exerts vasodilator effects and protects against vasoconstriction and that loss of these normal vascular smooth muscle cellular responses to insulin in insulin resistance results in predisposition to hypertension.\(^2\)–\(^8\),12,13 Insulin attenuates vasoconstrictor responses to vasoconstrictor agonists\(^5\) and accelerates vascular relaxation,\(^6\) whereas these effects are blunted in vascular smooth muscle from insulin-resistant animals.\(^4\) Moreover, insulin infusion increases forearm blood flow\(^6\) and reduces peripheral vascular resistance and blood pressure in both normotensive and hypertensive individuals.\(^7,8\) Additional recent data also support the concept that elevated circulating insulin per se does not result in blood pressure elevations and that VSMC resistance to the actions of insulin may instead be responsible for hypertension in insulin-resistant states.\(^18\)–\(^20\) For example, Gans et al\(^19\) recently reported that induction of acute hyperinsulinemia in a euglycemic clamp study of non–insulin-dependent diabetic patients resulted in blood pressure reductions despite observed reductions in the fractional excretion of sodium. Data from the present study, demonstrating an acceleration in [Ca\(^{2+}\)]\(_i\), recovery rate after stimulation with vasoconstrictor agonists, appear to provide a physiological rationale for these observations.

Acknowledgments

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References

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