Effect of Insulin and Angiotensin II on Cell Calcium in Human Skin Fibroblasts

Giulio Ceolotto, Roberto Valente, Elisabetta Baritono, Stefania Reato, Elisabetta Iori, Alessandra Monari, Roberto Trevisan, Andrea Semplicini

Abstract—We have recently shown that insulin attenuates angiotensin II–induced intracellular Ca$^{2+}$ mobilization in human skin fibroblasts from normotensive subjects. This study was designed to investigate the effects of angiotensin II and the interactions between insulin and angiotensin II on intracellular Ca$^{2+}$ mobilization in skin fibroblasts from patients with essential hypertension. Fibroblasts were obtained from 9 normotensives and 18 hypertensives. Spectrofluorophotometric free Ca$^{2+}$ measurement was performed in monolayers of 24-hour serum-deprived cells. Resting intracellular Ca$^{2+}$ level and angiotensin II–stimulated intracellular Ca$^{2+}$ peak were higher in fibroblasts from hypertensives compared with those from normotensives. The effect of acute insulin exposure was evaluated in fibroblasts from hypertensives subdivided on the basis of insulin sensitivity. In insulin-sensitive hypertensives, insulin significantly blunted the effects of angiotensin II on intracellular Ca$^{2+}$ response, whereas in insulin-resistant patients, insulin did not modify intracellular Ca$^{2+}$ response to angiotensin II. Pertussis toxin, a G$i$-inhibitor, reduced angiotensin II–stimulated Ca$^{2+}$ peak in insulin-sensitive but not in insulin-resistant hypertensives. In conclusion, the effects of angiotensin II on intracellular Ca$^{2+}$ mobilization are more pronounced in fibroblasts from hypertensives compared with those from normotensives, and the inhibitory effect of insulin is blunted in insulin-resistant hypertensives by a G$i$ pertussis toxin–sensitive abnormality. (Hypertension. 2001;37:1486-1491.)

Key Words: angiotensin II ■ calcium ■ fibroblasts ■ G proteins ■ insulin ■ insulin resistance

Insulin resistance and hyperinsulinemia are often found in hypertensive patients. The relative role of hyperinsulinemia versus that of impaired insulin action in the pathogenesis of hypertension in these patients has not been fully elucidated. In fact, hypertension has been attributed to selective insulin resistance resulting in hyperinsulinemia, with stimulation of both sympathetic neural activity and renal sodium retention. However, it has also been shown that in vivo, insulin infusion induces vasoconstriction by an endothelium-dependent mechanism and that the presence of insulin resistance may lead to a partial loss of this insulin-mediated effect, resulting in an increase in pressure.

In vitro, acute insulin exposure attenuates intracellular calcium (Ca$^{2+}$) mobilization and contractile response to pressure agents in rat aorta, cultured vascular smooth muscle cells, and in platelets. This may result from an action on Ca$^{2+}$-ATPase, voltage- and receptor-operated Ca$^{2+}$-channels, vasoconstrictor receptors, G protein, phospholipase C, or inositoltrisphosphate (IP$_3$)-sensitive Ca$^{2+}$ release channels, but its precise mechanism remains to be defined.

We have recently found that in human skin fibroblasts from normotensive subjects, insulin blunts the angiotensin (Ang) II–induced Ca$^{2+}$ response by modulating the transmembrane signal transduction. Most of the known actions of Ang II are exerted through the AT$_1$ receptor, a G protein–coupled receptor, which stimulates phospholipase C, IP$_3$, and Ca$^{2+}$ release, but recently, it has been shown that Ang II exerts biological actions also through G$_i$-coupled AT$_2$ receptors. Insulin acts through its receptor, a protein tyrosine kinase that undergoes a rapid autophosphorylation inducing the phosphorylation of several intracellular protein substrates. Insulin receptors are also able to phosphorylate some G-protein subunits coupled to vasoconstrictor receptors inhibiting ADP ribosylation of G$_s$ subunit and therefore influencing their intracellular pathway.

The intracellular cross-talk between insulin and Ang II signaling could be pathophysiologically relevant for Ca$^{2+}$ regulation, and an abnormal interaction may be involved in the association among high blood pressure, insulin resistance, and target organ damage in patients with essential hypertension and diabetes mellitus. The aim of this study was therefore to characterize the effects of insulin on Ang II–stimulated free Ca$^{2+}$ in human skin fibroblasts from insulin-sensitive and insulin-resistant hypertensive patients. In particular, we compared Ca$^{2+}$ responses induced by Ang II in normotensives and hypertensives and the effect of acute insulin exposure and of pertussis toxin on Ang II–induced Ca$^{2+}$ in insulin-sensitive and insulin-resistant hypertensives.
We used cultured skin fibroblasts in vitro, which can be easily obtained through a skin biopsy. They express a variety of different receptors, including G protein–coupled AT1 receptors, activating well-known pathways of intracellular signal transduction. They can be cultured for several passages in standardized conditions, offering a useful model for the investigation of intrinsic (possibly genetic) defects of cell function, independent of the environmental abnormality caused by hypertension and hyperinsulinemia in vivo. Furthermore, they are actively involved in the process of renal and cardiovascular fibrosis and in the development of target organ damage in hypertensive and diabetic patients.

Methods

Subjects
We have recruited 9 healthy normotensive volunteers, without a family history of hypertension and diabetes, and 18 patients with essential hypertension from the Hypertension Outpatient Clinic of the Department of Clinical and Experimental Medicine, University Hospital of Padua (Italy). All gave informed consent to the study, which had been approved by the local ethical committee. Arterial hypertension was diagnosed according to the ISH/WHO guidelines (systolic pressure >140 mm Hg and/or diastolic pressure >90 mm Hg). All the hypertensives but three underwent a 75 g oral glucose tolerance test (OGTT) and were classified according to their insulin sensitivity. This parameter was calculated by the glucose/insulin ratio during OGTT and, as previously described, is the criterion, 9 hypertensives were insulin sensitive and 6 insulin resistant. The clinical characteristics of the normotensives and of the hypertensives are reported in the Table. In comparison to normotensives, both insulin-sensitive and insulin-resistant hypertensives were heavier, but by no means were insulin-sensitive and insulin-resistant hypertensives different.

Cell Culture
Human fibroblasts were derived from a skin biopsy taken from the anterior surface of the left forearm by excision, under topical anesthesia with ethyl chloride, and cultured in Nutrient Mixture F-10 HAM medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 4 mmol/L glutamine. Cells were seeded onto a 25-cm² flask and incubated at 37°C, and the medium was changed every 2 to 3 days. Fibroblasts obtained from each subject and grown separately were used for the experiments at the 5th passage. They were identified morphologically. In particular, they were strictly diploid and, on morphological confluence, they appeared oriented with respect to one another, forming a typical parallel array of cells with no dividing nuclei visible by microscope. The cells were in the plateau phase of growth, in which there is a steady-state condition with an almost completely ceased cell proliferation, as confirmed by the evaluation of [3H]-thymidine incorporation in cells grown in the same conditions. At confluence, [3H]-thymidine incorporation in fibroblasts was 10 to 15 times lower than that observed in cells during the log phase of growth and similar to those observed in cells cultured in serum-free medium (data not shown). Moreover, they make type I collagen and were not factor VIII positive. There were no morphological differences in fibroblasts from hypertensives, either insulin-sensitive or insulin-resistant, and normotensives and frequent observations with phase-contrast optic revealed no differences in granularity and vacuolation, which may have a bearing on the health of the culture.

Measurement of Intracellular Calcium
The cells (5×10⁶) were seeded onto coverslips (3 cm×1 cm) and allowed to grow to confluence. The medium was then changed to a quiescent medium without serum, and the cells were used after 24

### Clinical Characteristics of Study Groups

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normotensives</th>
<th>Insulin Sensitive</th>
<th>Insulin Resistant</th>
<th>P (NT vs HT)</th>
<th>P (IS vs IR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects (male)</td>
<td>9 (5)</td>
<td>9 (6)</td>
<td>6 (2)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Age, y</td>
<td>40±3</td>
<td>50±4</td>
<td>54±4</td>
<td>NS</td>
<td>NS</td>
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<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>110±4</td>
<td>157±3</td>
<td>158±8</td>
<td>&lt;0.01</td>
<td>NS</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>74±3</td>
<td>99±2</td>
<td>98±2</td>
<td>&lt;0.01</td>
<td>NS</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>22±1</td>
<td>29±1</td>
<td>30±1</td>
<td>&lt;0.01</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are mean±SEM. Insulin sensitivity was defined as reported in Methods. NT indicates normotensives; HT, hypertensives; IS, insulin sensitive; and IR, insulin resistant.
Effect of Insulin on Ang II–Stimulated Cell \( \text{Ca}^{2+} \) Response

Acute insulin exposure (100 nmol/L, 20 minutes) markedly reduced Ang II–induced \( \text{Ca}^{2+} \) peak in insulin-sensitive but not in insulin-resistant hypertensives (Figure 2). Basal \( \text{Ca}^{2+} \) levels were not modified by insulin in both groups (79±6 versus 75±7 nmol/L and 204±9 versus 212±14 nmol/L, respectively). Moreover, Ang II–stimulated \( \text{Ca}^{2+} \) increase was not correlated to BMI (r=0.029, NS).

The hypertensives were divided into 2 subgroups according to insulin sensitivity, but basal \( \text{Ca}^{2+} \) and Ang II–induced \( \text{Ca}^{2+} \) peak were not different in fibroblasts from insulin-sensitive and insulin-resistant patients (79±5 versus 75±7 nmol/L and 204±9 versus 212±14 nmol/L, respectively). To determine whether the different \( \text{Ca}^{2+} \) mobilization induced by Ang II in fibroblasts from hypertensives was due to an abnormal \( \text{Ca}^{2+} \) influx, we measured Ang II–induced \( \text{Ca}^{2+} \) response in the absence of extracellular \( \text{Ca}^{2+} \). The removal of extracellular \( \text{Ca}^{2+} \) significantly reduced basal \( \text{Ca}^{2+} \) levels (from 70±5 to 56±3 nmol/L, \( P<0.05 \)) without affecting Ang II–induced \( \text{Ca}^{2+} \) peak (195±9 versus 193±7 nmol/L, NS). Similar effects have been recently reported in normotensive subjects, thus ruling out a significant role of excessive \( \text{Ca}^{2+} \) influx in inducing a greater \( \text{Ca}^{2+} \) response in hypertensives in comparison to normotensives in these experimental conditions.

Thapsigargin, a \( \text{Ca}^{2+} \)-ATPase inhibitor acting on intracellular \( \text{Ca}^{2+} \) pools including the one that is sensitive to IP₃, has been used to investigate \( \text{Ca}^{2+} \) release from intracellular stores. \( \text{Ca}^{2+} \) release induced by thapsigargin was similar in normotensives and hypertensives (93±8 versus 106±8 nmol/L), suggesting that the different \( \text{Ca}^{2+} \) mobilization is unrelated to thapsigargin-sensitive calcium stores.

**Effect of Ang II, Thapsigargin, and Calcium-Free Media on Cell Calcium**

\( \text{Ca}^{2+} \) was measured in quiescent cells before and after exposure to 100 nmol/L Ang II. This concentration has been previously shown to induce a significant and maximal \( \text{Ca}^{2+} \) response. Mean resting cytosolic free calcium and \( \text{Ca}^{2+} \) peak were markedly increased in hypertensives compared with normotensives (78±3 versus 62±3 nmol/L, \( P<0.01 \); 224±8 versus 160±12 nmol/L, \( P<0.01 \)). Basal and Ang II–stimulated \( \text{Ca}^{2+} \) levels in obese (body mass index [BMI] >25 kg/m²) and nonobese subjects did not differ (73±6 versus 64±3 nmol/L, NS, and 178±8 versus 165±9 nmol/L, NS, respectively). Moreover, Ang II–stimulated \( \text{Ca}^{2+} \) increase was not correlated to BMI (r=0.029, NS).

Experimental Protocol

Ang II was added to the perfusion solution when baseline fluorescence was stable and the fluorescence measurements continued until \( \text{Ca}^{2+} \) recovered to basal level. To study the effects of insulin incubation, different coverslips seeded with fibroblasts from the same biopsy were used and treated in parallel without and with insulin (100 nmol/L, for 20 minutes). Moreover, coverslips prepared in the same way were used to perform parallel experiments in the presence of pertussis toxin (20 ng/mL, overnight).

Chemicals

Ang II, insulin, pertussis toxin, thapsigargin, and Fura-2 AM were from Calbiochem. All other chemicals were from Sigma Aldrich.

Statistical Analysis

Values are expressed as mean±SEM except where otherwise indicated. Statistical comparisons between groups were performed with a Student’s \( t \) test for nonpaired variables and ANOVA. A value of \( P<0.05 \) was considered statistically significant.

Results

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therefore suggest that the impaired intracellular Ca^{2+} sensitive but not in insulin-resistant hypertensives. We demonstrated that (1) the stimulation of Ca^{2+} mobilization was completely abolished by pretreatment with pertussis toxin (Figure 3).

**Effect of Pertussis Toxin on Ang II–Stimulated Ca^{2+}**

Pertussis toxin (20 ng/mL, overnight) affected basal Ca^{2+} levels neither in insulin-sensitive nor in insulin-resistant hypertensives. However, it significantly decreased Ang II–induced Ca^{2+} peak in insulin-sensitive hypertensives (from 192±4 to 131±6 nmol/L, P<0.01). This effect was markedly reduced and no longer significant in insulin-resistant patients (from 208±10 to 177±9 nmol/L, NS). Furthermore, in insulin-sensitive hypertensives, the blunting effect of insulin on Ang II–induced Ca^{2+} mobilization was completely abolished by pretreatment with pertussis toxin (Figure 3).

**Discussion**

The present study demonstrates that (1) the stimulation of human skin fibroblasts with Ang II evokes a higher Ca^{2+} mobilization that is independent from intracellular Ca^{2+} pools and suggest that these cells have an increased responsiveness to Ang II. Although we did not determine Ang II receptor density, and therefore a receptor downregulation in hypertensives cannot be excluded, data on confluent cultured cells from spontaneously hypertensive rats and Wistar-Kyoto rats did not show differences in AT_{1} receptor density. Therefore, we propose that the abnormalities of Ca^{2+} response shown in the present work in hypertensives are accounted for an abnormal signal transduction of Ang II, probably located at postreceptor level. Similar alterations in Ca^{2+} homeostasis were present in insulin-sensitive and in insulin-resistant hypertensive patients, suggesting that arterial hypertension rather than insulin resistance accounts for the abnormal calcium handling.

The second aim of our study was to compare the effects of acute insulin exposure on Ang II–induced Ca^{2+} mobilization in fibroblasts from insulin-sensitive and insulin-resistant hypertensives.

Previous studies, in vitro, have shown that insulin attenuates Ca^{2+} mobilization in different cell types. In vascular smooth muscle cells, insulin inhibition of agonist-induced Ca^{2+} transient is due to inhibition of voltage- and receptor-operated Ca^{2+} channels, inhibition of IP_{3}-induced Ca^{2+} release from intracellular stores, and stimulation of plasma membrane Ca^{2+}-ATPase–mediated Ca^{2+} efflux. Insulin may also inhibit transmembrane signal transduction of vasoconstrictor agents acting on receptor-coupled G proteins, phospholipase C, or IP_{3}-sensitive Ca^{2+} release channels.

The inhibitory effect of insulin on Ca^{2+} responses to agonists is reduced in vascular smooth muscle cells from insulin-resistant Zucker obese rats compared with their lean controls, as the result of an impairment in the stimulation of both plasmalemma and sarcoplasmic reticulum Ca^{2+}-ATPase. Furthermore, the inhibitory effect of insulin on Ca^{2+} mobilization in fibroblasts from hypertensives and abolished the difference in basal Ca^{2+} between cells from normotensives and hypertensives. This suggests that increased calcium influx may contribute to the abnormal regulation of basal Ca^{2+} levels in fibroblasts from hypertensives.

Ang II mediates its effects on Ca^{2+} by IP_{3}-induced mobilization from reticular stores, which induces a rapid and transient Ca^{2+} response, and through entry of Ca^{2+} through Ca^{2+} channels, resulting in a prolonged and sustained Ca^{2+} response. The present study shows that under Ca^{2+}-free conditions, Ang II–induced Ca^{2+} peak is still higher in fibroblasts from hypertensives compared with normotensives, suggesting that an increased Ca^{2+} influx is not the cause of this abnormal Ca^{2+} response to Ang II. Moreover, the similarity in the thapsigargin-induced Ca^{2+} increase in fibroblasts from normotensives and hypertensives indicates that thapsigargin-sensitive Ca^{2+} stores do not differ. Therefore, our results are consistent with the hypothesis that in human fibroblasts from hypertensives, there is an abnormal Ang II–induced Ca^{2+} mobilization that is independent from intracellular Ca^{2+} pools.
Ca\textsuperscript{2+} mobilization induced by Ang II and endothelin-1 was impaired in platelets from patients with essential hypertension in proportion to the degree of hyperinsulinemia.\textsuperscript{32,33}

In this study, insulin attenuated Ang II–induced Ca\textsuperscript{2+} mobilization in fibroblasts from insulin-sensitive but not from insulin-resistant hypertensives. This indicates that insulin resistance but not hypertension is responsible for the reduced effect of insulin on Ang II–induced Ca\textsuperscript{2+} transient in insulin-resistant hypertensives.

Such effect is unlikely to be caused by insulin receptor downregulation in cultured cells from insulin-resistant patients. In adipose tissue, in muscle, and in red blood cells, there is some evidence for downregulation of insulin receptors in insulin-resistant patients because of altered insulin-stimulated tyrosine kinase activity.\textsuperscript{34} However, these abnormalities could be reversed both in vivo and in vitro, thereby suggesting that they are secondary rather than intrinsic molecular alterations. Furthermore, it has been shown that in long-term cultured skin fibroblasts, as the case of our study, insulin receptors are not reduced in insulin-resistant patients.\textsuperscript{35} The majority of the studies, therefore, now agrees that insulin resistance is caused by a postreceptor defect.

We recently reported that insulin blunts agonist-induced Ca\textsuperscript{2+} responses in human skin fibroblasts, an observation that was consistent with a specific action of insulin on the agonist-sensitive Ca\textsuperscript{2+} release cascade, and proposed that insulin can influence the Ang II transmembrane signal transduction.\textsuperscript{9} This hypothesis was supported by the fact that tyrosine kinase signaling pathways may modulate Ang II–induced Ca\textsuperscript{2+} transients in rat vascular smooth muscle cells.\textsuperscript{36} We still do not know the exact site of action and nature of this inhibition, but the results of the present study suggest that insulin can act at the AT\textsubscript{1} receptors coupled to G\textsubscript{i} protein. In fact, it has been shown that Ang II negatively modulates L-type Ca\textsuperscript{2+} channels through a pertussis toxin–sensitive G\textsubscript{i} mechanism.\textsuperscript{37} We have demonstrated an increased responsiveness to Ang II in hypertensive patients. Insulin modulates Ca\textsuperscript{2+} mobilization induced by Ang II through a pertussis toxin–sensitive G\textsubscript{i} mechanism. In fibroblasts from insulin-resistant patients, insulin failed to attenuate the increased responsiveness to Ang II. This alteration was seen after several passages in vitro. Therefore, these in vitro phenotypic characteristics of fibroblasts are likely to be genetically determined and independent of the in vivo metabolic and hemodynamic abnormalities. A disturbed balance between Ang II and insulin signaling might be relevant for the development target organ damage in insulin-resistant patients with hypertension and diabetes.

**Conclusions**

We have demonstrated an increased responsiveness to Ang II in hypertensive patients. Insulin modulates Ca\textsuperscript{2+} mobilization induced by Ang II through a pertussis toxin–sensitive G\textsubscript{i} mechanism. In fibroblasts from insulin-resistant patients, insulin failed to attenuate the increased responsiveness to Ang II. This alteration was seen after several passages in vitro. Therefore, these in vitro phenotypic characteristics of fibroblasts are likely to be genetically determined and independent of the in vivo metabolic and hemodynamic abnormalities. A disturbed balance between Ang II and insulin signaling might be relevant for the development target organ damage in insulin-resistant patients with hypertension and diabetes.

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