Potential role of insulin signaling on vascular smooth muscle cell migration, proliferation, and inflammation pathways

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Cersosimo E, Xu X, Musi N. Potential role of insulin signaling on vascular smooth muscle cell migration, proliferation, and inflammation pathways. Am J Physiol Cell Physiol 302: C652–C657, 2012. First published November 16, 2011; doi:10.1152/ajpcell.00022.2011.—To investigate the role of insulin signaling pathways in migration, proliferation, and inflammation of vascular smooth muscle cells (VSMCs), we examined the expression of active components of the phosphatidylinositol 3 (PI-3) kinase (p-Akt) and mitogen-activated protein kinase (MAPK) (p-Erk) in primary cultures of VSMCs from human coronary arteries. VSMCs were treated in a dose-response manner with insulin (0, 1, 10, and 100 nM) for 20 min, and Akt and Erk phosphorylation were measured by Western blot analysis. In separate experiments, we evaluated the effect of 200 μM palmitate, in the presence and absence of 8 μM pioglitazone, on insulin-stimulated (100 nM for 20 min) Akt and Erk phosphorylation. The phosphorylation of Akt and Erk in VSMCs exhibited a dose dependency with a three- to fourfold increase, respectively, at the highest dose (100 nM). In the presence of palmitate, insulin-induced Akt phosphorylation was completely abolished, and there was a twofold increase in p-Erk. With addition of pioglitazone, the phosphorylation of Akt by insulin remained unchanged, whereas insulin-stimulated Erk phosphorylation was reduced by pioglitazone. These data in VSMCs indicate that high palmitate decreases insulin-stimulated Akt phosphorylation and stimulates MAPK, whereas preexposure peroxisome proliferator-activated receptor-γ agonist pioglitazone preserves Akt phosphorylation and simultaneously attenuates MAPK signaling. Our results suggest that metabolic and mitogenic insulin signals have different sensitivity, are independently regulated, and may play a role in arterial smooth muscle cells migration, proliferation, and inflammation in conditions of acute hyperinsulinemia.

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MATERIALS AND METHODS

Primary Cell Cultures

Healthy human coronary arterial smooth myoblasts (C-12511 HCASMC-c) were obtained from Promo Cell (Heidelberg, Germany). Cells were grown in PromoCell SMC growth medium-2 (C-22062 and C-39267) that was replaced every 48 h; the cell adherence rate was checked periodically, thereafter. The presence of mature and differentiated smooth muscle cells was confirmed under a light microscope by the typical “spindle-cell” appearance. Further confirmation of the presence of smooth muscle cells was obtained in selected culture dishes by immunohistochemistry and by immunoelectrophoresis, after the addition of a specific α-actin antibody (Sigma-Aldrich, St. Louis, MO).

Cell Viability Assays

The effects of various doses of palmitate and pioglitazone on cell viability were evaluated in our VSMC preparations using the methods proposed by Esfandiarei et al. (8) and by Pan et al. (8, 23). The results obtained in these experiments are depicted in the online Supplementary Fig. S1, A and B (see Am J Physiol Cell Physiol website). VSMC exposed to the palmitate dose of 200 mM, which was chosen in all of our experiments, was associated with greater than 85% percent cell survival. The addition of pioglitazone at the dose of 8 μM also was associated with an acceptable percentage survival of VSMC in all studies conducted (see Fig. 1B). Of note, both the palmitate dose of 200 mM and the pioglitazone dose of 8 μM reproduce clinically relevant circulating concentrations described in obese and type 2 diabetic patients and pharmacological levels attained during therapy, respectively.

Experimental Design

VSMC incubation. Primary VSMCs differentiated into myotubules were confirmed under light microscopy before each experiment. After ascertaining of viability, VSMCs were seeded in triplicate in working plates (6-well culture plates at a density of 2 × 10^5/well and incubated until they were ~80% confluent at 37°C in the incubator). For the insulin dose-response experiments, the cells were treated with 1, 10, or 100 nM of insulin (Humulin, Eli Lilly, Indianapolis, IN) for 20 min and then collected in lysis buffer (20 mM Tris, pH 7.5, 5 mM EDTA, 10 mM NaPO4, 100 mM NaF, 2 mM Na3VO4, 1% Nonidet-P40, 10 μM leupeptin, 3 mM benzamidine, 10 μg/ml apropin, and 1 mM PMSF) and subsequently frozen. To examine the effect of palmitate and pioglitazone, VSMCs were treated with/without 200 μM palmitate conjugated with BSA (or control BSA alone) and with/without 8 μM pioglitazone for 24 h, followed by 20 min stimulation with 100 nM insulin. Cells were then collected in lysis buffer and frozen. Stock palmitate solution was prepared with 8 mM sodium palmitate conjugated with 10,5% BSA (Sigma), as previously described (27). Pioglitazone was dissolved in DMSO at a final concentration of 0.1%. Control samples not treated with pioglitazone were incubated in 0.1% DMSO.

Western blot analysis. Proteins from the cell lysates were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were then blocked in Tris-buffered saline with 0.05% Tween 20 (TBST) and 5% nonfat milk for 1 h at room temperature and incubated overnight at 4°C with the specific antibodies against phospho-Akt (Ser473), Akt, phospho-Erk (Thr185/Tyr187, Invitrogen), Erk, phospho-mTor rabbit polyclonal anti-body (Ser2448), phospho-p38 (Thr180/Tyr182), p38, and β-actin (all from Cell Signaling, Danvers, MA). Bound antibodies were detected with a secondary antibody (anti-rabbit immunoglobulin-horseradish peroxidase-linked antibody using enhanced chemiluminescence reagents; Perkin Elmer). Bands were quantitated with ImageQuant (18, 21, 27).

Statistical Analyses

Our main objective was to determine and compare the expression/activity of phosphorylated Akt and phosphorylated-ERK1/2, components of insulin signaling pathway, measured in different experimental conditions using primary cultures of human vascular smooth muscle cells. All analyses were performed either in duplicate or triplicate and repeated in six to nine separate experiments.

The final number of experiments needed was calculated with a two-tailed test using the preliminary data shown in Table 1. All data are expressed as means ± SE. Comparisons between groups were performed with one-way ANOVA with repeated measures. Analyses were performed using SigmaStat software.

RESULTS

Treatment of VSMCs with increasing concentrations of insulin in the first set of experiments was used to document the presence of insulin-responsive elements and to establish an insulin dose-response curve for the selected insulin signaling molecules, Akt and MAPK pathway. In all subsequent studies high palmitate and/or insulin at the fixed concentration of 100 nM were utilized to reproduce the metabolic milieu encountered in conditions of acute hyperinsulinemia and insulin resistance. Pioglitazone, a peroxisome proliferator-activated receptor-γ (PPAR-γ) agonist and known insulin-sensitizing agent, was added to the surrounding media in selected experiments to enable the examination in vitro of the effects of improved insulin sensitivity in insulin signaling molecules. A representative Western blot with bands corresponding to phospho-Akt and phospho-Erk are shown in Fig. 1. Treatment with 1 nM of insulin caused minimal Akt phosphorylation; however, Akt phosphorylation doubled at 10 nM insulin and at 100 nM Akt phosphorylation increased fourfold over baseline. Insulin also augmented Erk phosphorylation in a dose-dependent manner. Figure 2 demonstrates that insulin treatment caused a significant increase in phospho-Akt; however, this effect was completely inhibited by palmitate. Despite some heterogeneity, the average density of the bands under conditions of insulin with insulin is greater than that of conditions of insulin without insulin, as depicted in the bar graph in Fig. 2.

Figure 3 summarizes the results obtained in the experiments designed to examine the effects of pioglitazone on Akt and Erk phosphorylation. In the presence of insulin there was a sevenfold increase in phospho-Akt (P < 0.001), and when pioglitazone was added this was also enhanced but not statistically

Table 1. Expression/activity of phosphorylated Akt and phosphorylated-ERK1/2, components of insulin signaling pathway

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control Media</th>
<th>n</th>
<th>Plus Glucose and FFA</th>
<th>n</th>
<th>Plus PIO</th>
<th>n</th>
<th>Power</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI3-kinase (p-Akt)</td>
<td>100 ± 16</td>
<td>9</td>
<td>78 ± 15</td>
<td>9</td>
<td>85 ± 12</td>
<td>8</td>
<td>0.770</td>
</tr>
<tr>
<td>MAPK (p-ERK)</td>
<td>1.9 ± 0.4</td>
<td>9</td>
<td>4.9 ± 0.4</td>
<td>8</td>
<td>1.2 ± 0.6</td>
<td>8</td>
<td>0.911</td>
</tr>
</tbody>
</table>

PI3-kinase; phosphoinositol-3 kinase; p-Akt, phosphorylated Akt; MAPK, mitogen-activated protein kinase; p-ERK, phosphorylated-ERK1/2; PIO, pioglitazone; FFA, free fatty acid. n, number of experiments.
significant \( (P = \text{not significant}) \). In this same experiment, we were able to demonstrate a significant difference between phospho-Akt in the presence of insulin with or without pioglitazone \( (P < 0.05) \). In contrast, cells treated with insulin showed a marked increase in Erk phosphorylation, but preexposure to pioglitazone (Pio) attenuated down this increase \( (P < 0.05, \text{Pio} + \text{Ins vs. Insulin}) \).

Results for Akt and Erk phosphorylation in response to palmitate treatment, both in the presence and absence of pioglitazone, are shown in Fig. 4. The complete abolishment of insulin-stimulated Akt phosphorylation by palmitate was overcome by preexposure of VSMCs to pioglitazone. In the same experiment, preexposure of cells to pioglitazone was accompanied by a simultaneous and significant attenuation of the insulin-induced threefold increase in p-Erk/ERK \( (P < 0.05) \). These findings are supported by the data presented in Supplementary Figures S2 and S3, which demonstrate similar effects of palmitate (Suppl. Fig S2) and of pioglitazone (Suppl. Fig S3) on p38 and mTOR phosphorylation in human vascular smooth muscle cells under the same above detailed experimental conditions.

**DISCUSSION**

In this series of in vitro experiments, we have confirmed that insulin-responsive elements are present in human coronary artery smooth muscle cells and that the metabolic and mitogenic insulin signaling pathways have different sensitivity to some common stimuli. The activation of both components appears to be dose dependent. These observations provide further evidence that the handling and preservation of the VSMCs exposed to experimental conditions in our laboratory

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**Fig. 1.** Effects of insulin in human vascular smooth muscle cells. Cells were treated either with no insulin \( (0 = \text{Control}) \) or increasing concentrations of insulin \( (1, 10, 100 \text{ nM}) \) each for 20 min. Akt \( (A) \) and Erk phosphorylation \( (B) \) were measured by Western blotting. The experiments were performed in triplicate and data are expressed as means ± SE.

**Fig. 2.** Effect of palmitate in human vascular smooth muscle cells. Cells were preincubated in the presence or absence of 200 \( \mu \text{M} \) palmitate (or BSA) for 24 h, followed by a 20-min treatment with 100 nM insulin. Akt and Erk phosphorylation were measured by Western blotting. The experiment was performed in triplicate \( (n = 9) \). Data are means ± SE.
behave much similar to what has been previously reported in the literature (7, 21). Exposure to high palmitate significantly attenuates the phosphorylation of Akt, an important signaling intermediary involved in glucose regulation, whereas the phosphorylation of the mitogenic regulator Erk is augmented. Preexposure to the insulin sensitizer pioglitazone preserves insulin-stimulated Akt phosphorylation, and perhaps more importantly, it significantly reduces the phosphorylation of Erk. Insulin stimulation of the (Akt) component of the metabolic signaling pathway is completely abolished in the presence of high palmitate, whereas the addition of pioglitazone is accompanied by a sevenfold increase in Akt phosphorylation. In contrast, high palmitate induces a three- to fourfold increase in Erk phosphorylation, an important component of the proliferative pathway, which is attenuated by pioglitazone. These observations in human coronary arteries indicate that the activation and inactivation processes of the metabolic and mitogenic insulin signaling pathways are independent and may actually change in opposite directions when exposed to the same stimulus. If confirmed in experiments with long-term exposure to high insulin, our findings would provide an additional potential explanation for the molecular basis underlying the concomitant metabolic insulin resistance and stimulation of proliferative pathways within the VSMCs of the arterial wall. In other words, these data raise the possibility that in the presence of hyperinsulinemia in conjunction with elevated fatty acids, glucose transport may be impaired simultaneously with overactivation of the molecular pathways leading to smooth muscle cell proliferation and inflammation. As a consequence, these alterations could favor the formation of arterial plaques.

We have shown that in the presence of high palmitate concentrations, the activity of the insulin-mediated metabolic (Akt) pathway is attenuated while the mitogenic transduction...
signal is activated in VSMCs. Treatment with insulin for 20 min at the concentration of 100 nM partially restores the activation of the metabolic and mitogenic molecules, although not to baseline levels. These in vitro findings are consistent with various reports from clinical studies showing a close relationship between circulating fatty acids and vascular dysfunction (2, 36) and other in vitro data indicating that the accumulation of intracellular fatty acid derivatives inhibits insulin-mediated glucose metabolism (2, 5, 24, 29, 30). Although the proinflammatory activity of fatty acids has been documented (17), a direct stimulation of the mitogenic (MAPK) pathway, resulting in VSMC proliferation and migration has not yet been demonstrated. These observations, if confirmed, in long-term high insulin exposure conditions might aid in the understanding of the molecular mechanisms underlying the accelerated process of atheroma formation in the arterial wall in individuals with chronic elevated fatty acids and insulin resistance, including patients with type 2 diabetes.

The demonstration that insulin promotes phosphorylation and activates the MAPK pathway, which is followed by stimulation of the cell mitogenic activity, has long been known and by documenting similar findings in VSMCs derived from human coronary arteries, we provide further support to the theory that this pathway is involved in cell proliferation, migration, and inflammation (7, 21). Gogg et al. (9) recently reported similar findings in type 2 diabetes microvascular endothelial cells and showed a “selective” insulin resistance involving IRS-1 and PI3-kinase pathways concomitant with increased MAPK activity. Our data are also in agreement with earlier reports in human skeletal muscle and with the concept that hyperinsulinemia, in insulin-resistant conditions, although ineffective in stimulating the PI3 kinase-Akt branch, it is capable of activating the mitogenic and inflammatory pathways. Of greater interest, our findings that preexposure of smooth muscle cells to the PPAR-γ agonist pioglitazone maintains the activity of the phospho-Akt further confirm the role for this insulin sensitizer agent in slowing the progression of arterial plaques by directly or indirectly reducing the activity of the MAPK pathway. Hsueh, Law, and colleagues (3, 13, 14) have already demonstrated the effects of PPAR-γ agonists in major cell types in the vasculature, including intimal macrophages and VSMCs extracted from human atheroma. It is believed that PPAR-γ agonists bind to its ligand and promote cell cycle arrest via inhibition of protein phosphorylation (13). As a consequence, the proliferation and migration of monocytes and smooth muscle cells are suppressed, which may explain some of the anti-atherogenic properties of thiazolidinediones (14). It should be mentioned that there are also numerous reports indirectly linking the effects of PPAR-γ agonists on VSMC insulin signaling pathways via endothelial nitric oxide synthase activation and changes in other intermediary signals in vascular endothelial and inflammatory cells in the presence of PPAR-γ agonists (10, 33, 34). The physiological relevance of these in vitro findings, much similar to ours, has not yet been fully established. Our data confirm the anti-atherogenic properties of PPAR-γ agonists and further raise the possibility that suppression of the MAPK insulin signaling may represent an additional mechanism by which vascular atheroma formation can be attenuated. These in vitro observations also are consistent with most clinical studies in type 2 diabetes treated with thiazolidinediones, which demonstrated slow progression of the carotid intimal media thickness (20) and reduction in intracoronary wall atheroma size (22).

It should be emphasized that there are some inherent limitations to in vitro experiments and the clinical relevance of these findings requires further investigation. These should be confirmed in primary cell cultures obtained in subjects with insulin resistance in whom arterial tissue samples can be harvested. Moreover, clinical studies including obese subjects and diabetic patients with and without coronary artery disease should be sought to substantiate the hypothesis that intracellular activation of the MAPK axis by sustained hyperinsulinemia is responsible, at least in part, for the atheroma formation and expansion.

In summary, these original experiments in VSMCs derived from human coronary arteries indicate that in the presence of high palmitate and insulin treatment there is a profound inhibition of the PI-3 kinase signaling pathway, whereas the activation of the MAPK signaling pathway is enhanced. Exposure to the PPAR-γ agonist pioglitazone preserves insulin-stimulated Akt phosphorylation and attenuates the augmented mitogenic signaling activity induced by palmitate. These results suggest that these two cellular signaling pathways have differential sensitivity to insulin and are independently regulated in VSMCs. In conclusion, our data support the notion that fatty acids in combination with hyperinsulinemia facilitate smooth muscle cell migration, proliferation, and inflammation, thereby promoting the formation/progression of arterial wall atheroma. Further studies will be required to elucidate the involvement of these pathways in the process of accelerated atherosclerosis in individuals with insulin resistance.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: E.C. and N.M. conception and design of research; E.C., X.X., and N.M. performed experiments; E.C., X.X., and N.M. analyzed data; E.C., X.X., and N.M. interpreted results of experiments; E.C. and N.M. prepared Figs.; E.C. and N.M. drafted manuscript; E.C. and N.M. edited and revised manuscript; E.C., X.X., and N.M. approved final version of manuscript.

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