Laboratory Research

Insulin induces PKC-dependent proliferation of mesenteric vascular smooth muscle cells from hypertensive patients

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Background and objectives Proliferation of human vascular smooth muscle cells (VSMCs) induced by hyperinsulinemia is a very common clinical pathology. Extensive research has focused on PKC (Protein kinase C)-MAPK (mitogen-activated protein kinase) intracellular signal transduction and the phenotypic modulation accompanied by reorganization of intracellular F-actins in VSMCs. Methods DNA synthesis, signaling of ERK1/2 MAPKs, and changes in α-smooth muscle (α-SM) actin and F-actin were studied in hypertensive and normotensive human arterial VSMCs exposed to insulin and PMA with and without the PKC inhibitor, GF109203X. Results Differences among cell types in MAPK signaling, α-SM actin, and F-actin isoforms in VSMCs harvested from the arteries of patients with essential hypertension (EH) and normotension (NT) were identified in response to insulin treatment. Proliferation and activation of MAPK were more pronounced in EH VSMCs than in NEH VSMCs. Insulin exposure decreased expression of α-SM actin and was accompanied by rearrangement of intracellular F-actins in VSMCs, especially in the EH group. These effects were reversed by treatment with the PKC inhibitor. Conclusions Human mesenteric VSMCs of EH and NT patients differed in proliferation, MAPK signaling, and degree of changes in α-SM actin and F-actin isoforms immediately following insulin exposure in vitro. (J Geriatr Cardiol 2006; 3(2):100–106.)

Key Words vascular smooth muscle cell; protein kinase C; mitogen-activated protein kinase; insulin; proliferation

Introduction

Hyperinsulinemia, including clinical insulinemia and insulin resistance syndrome, is commonly associated with pathologic changes in vascular proliferation. Normal proliferation and phenotypic modulation of vascular smooth muscle cells (VSMCs) have been shown to play an important role in the physiopathology of hypertension, atherosclerosis, and diabetes. Clinical and in vitro studies have shown that VSMCs in diabetics are phenotypically different from those in non-diabetics. In addition, phenotypic differences in protein synthesis, myosin expression, and contractility have been observed in VSMCs, depending on the site of origin. Several mediators, including insulin, may contribute to the development of VSMC proliferation. As a growth factor, insulin might act directly to influence proliferation, or it may act indirectly as an activator of growth regulatory factors. The growth factor ligand triggers a protein kinase cascade, eventually inducing modulation of nuclear gene expression. After binding of insulin to the insulin receptor, several signaling pathways are activated. The transmembrane tyrosine kinase receptors for insulin, insulin-like growth factor I (IGF-I) and platelet derived growth factor (PDGF), utilize both common and distinct pathways to mediate activation of mitogen-activated protein kinases (MAPKs) in muscle cells. Growth factor signal transduction cascades usually include the extracellular signal-regulated kinase (ERK1/2) MAP kinases, which are serine/threonine protein kinases involved in cell growth and differentiation. However, whether an insulin-responsive proliferation promoter exists in VSMCs, and whether hyperinsulinemia directly promotes VSMC proliferation and phenotypic changes directly, has not been established.

In the current study, the effects of insulin on human VSMCs from the arteries of patients with essential hypertension (EH) and normotension (NT) were evaluated. We investigated changes in α-smooth muscle (α-SM) actin, F-actin and the intracellular MAPK signal transduction pathway that might be related to this hyper-proliferation.

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Methods

Culture of mesenteric human VSMCs

Human mesenteric VSMCs were isolated from the excised gastric arteries of peptic ulcer patients who had undergone abdominal surgery at Daping and Xinqiao Hospital (the 2nd and 3rd Affiliated Hospital of the Third Military Medical University in Chongqing, P.R. China). Six EH patients and 6 NT subjects were included in our study. Samples were immediately placed in 75 mm plastic dishes with phosphate buffered saline (PBS). The outer and inner layers of tissue were carefully removed. The medium was cut into approximately 1 mm squares, transferred to asceptic plastic dishes, covered with DMEM (GibcoBRL, Inc, NY) supplemented with 10% fetal calf serum (GibcoBRL, Inc, NY), and incubated at 37 °C in a damp atmosphere of 95% air and 5% CO₂. The cells grew to confluence after 2-3 weeks. Living cells were identified by trypan blue exclusion and stained cells were eliminated. The VSMC identity of the cultured cells was further confirmed by positive immunolabeling with antibodies against α-SM actin (Santa Cruz Biotechnology, Inc, CA, USA). Cells were harvested with 0.25% trypsin/0.02% EDTA and subcultured, then propagated in DMEM with 10% FCS for 24 h, to synchronize cell cycles.

Cells were then washed twice with PBS, incubated for 24 h with serum-free DMEM and then in 2 ml of fresh serum-free DMEM containing 10 μM insulin with or without 1 μM PKC inhibitor (GF109203X, Gibco BRL, Inc, NY) for 48 h. Positive control cells of EH and NT were incubated in parallel, with 100 nM PMA (Calbiochem, San Diego, CA). Control cells of EH and NT were incubated in parallel, without insulin or PMA.

Measurements of DNA synthesis

To measure the DNA synthesis of human mesenteric VSMCs cultured as above, [3H] thymidine (0.5 μCi/ml; Atomic Energy Institute, Chinese Academy of Sciences) was added into the culture medium, and VSMCs were incubated for an additional 8 h. Excess [3H] thymidine was removed by washing with 3 ml PBS. The cells were resuspended in 10% trichloroacetic acid (TCA) with vigorous vortexing. The cellular lysates were vacuum-filtered through a glass-fiber filter, which was then washed with cold 5% TCA and dried. Incorporated [3H] thymidine was measured in a liquid scintillation counter. Values are expressed as mean ± SD.

Western blots and in-gel kinase assays of ERK1/2

Insulin-treated and untreated human mesenteric VSMCs were washed twice with PBS and treated with 0.5 ml of lysis buffer (10 mM Heps, pH 7.4, 5 mM EDTA, 5 mM EGTA, 50 mM sodium pyrophosphate, 50 mM NaF, 50 mM NaCl, 100 μM Na₃VO₄, 0.01% Triton X-100, 0.5 mM phenylmethyl sulfonide, and 10 μg/ml leupeptin). Cellular lysates were frozen in liquid nitrogen and incubated on ice for 10 minutes. Samples were then centrifuged for 30 min at 13,000 rpm at 4 °C. The protein samples were stored at -80 °C until tested.

With Western blot analysis, proteins (20 μg) were subjected to 10% SDS-PAGE in recirculating conditions. The proteins were then transferred to PVDF (Millipore, Bedford, MA, USA) at 100 V for 1 h. The membrane was blocked with 1% BSA for 1 h at room temperature, incubated for 1 h at room temperature with primary anti-phosphotyrosine ERK1/2 antibody (Santa Cruz Biotechnology, Inc, CA, USA), and then incubated for 1 h with a rabbit peroxidase-conjugated secondary antibody. Immunoreactive bands were visualized by chemiluminescence (Dakopatts, Glostrup, Denmark).

An in-gel kinase assay was performed on cellular lysates to measure MAP kinase phosphotransferase activity. Proteins were harvested from EH and NT VSMC lysates. The proteins (10 μg) were separated by 10% SDS-PAGE in a gel containing 0.4 mg/ml myelin basic protein (MBP). The following incubations steps were performed on the gel: 2 times, 30 min each, in buffer A (50 mM Heps, pH 7.4 and 5 mM β-mercaptoethanol) containing 20% isopropanol; 1 time in buffer A alone for 1 h; 2 times, 30 min each, in buffer A containing 6 M guanidine-HCl. 2 times, 16 h each, in buffer A containing 0.04% Tween 20 at 4 °C; 1 time in buffer A containing 100 μM Na₃CO₃, 10 mM MgCl₂ at 30 °C for 30 min; and 1 time in buffer A containing 100 μM Na₂CO₃, 10 mM MgCl₂, 50 μM ATP, and 50 μCi [γ-32P]ATP for 1 h at 30 °C. The reaction was terminated by washing the gel 5-8 times in a fixative solution containing 10 mM sodium pyrophosphate and 5% TCA for 15 min each time. The gel was then dried and subjected to autoradiography. Signal intensities were quantified by densitometry in the linear range of film exposure, using a Standard Documentation System 120 V (Bio-Rad Laboratories, Inc., CA, USA), and autoradiographic signal intensity was quantified by densitometry in the linear range of film exposure with the National Institute of Health Image Program 1.49. All experiments were processed three times.

α-SM actin measurement with Western blots

A portion of the samples (20 μg) remaining in the lysis buffer were run on a 10% SDS-PAGE gel in recirculating conditions. The proteins were then transferred to PVDF at 100 V for 1 h. The membrane was blocked with 1% BSA for 1 h at room temperature, incubated for 1 h at room temperature with a primary anti α-SM actin antibody (Santa Cruz Biotechnology, Inc, CA, USA), and then incubated for 1 h with a rabbit peroxidase-conjugated secondary antibody. Immunoreactive bands were visualized by chemiluminescence.
F-actin measurement with immunofluorescence
Cultured VSMCs were detached using 0.25% trypsin/0.02% EDTA and replated on coverslips at a density of 1x10^4 cells/well in 6-well culture plates. The coverslips were washed twice with PBS, fixed in 3.7% paraformaldehyde in PBS for 10 min at room temperature, and permeabilized with 0.1% Triton X-100 in PBS for 5 min. After being blocked with 1% BSA in PBS, the coverslips were washed three times with PBS. The fluorescent phallotaxins (Molecular Probes Eugene, OR, USA) were added onto coverslips and incubated for 20 min at room temperature. The coverslips were washed three times with PBS, and the cells were imaged using a confocal laser scanning image system (LSM 410, Zeiss, Germany). Microscopic images were digitally recorded.

Statistical analysis
The values are expressed as means ± SDs. Differences among groups were analyzed by one-way analysis of variance followed by Fisher’s protected least significance difference test, using the Statview software package (Abacus Concept, Inc., Berkeley, CA, USA). Significance was accepted at the P<0.05 level.

Results
In VSMCs that were exposed to increasing concentrations of insulin or PMA, DNA synthesis was assessed by [3H] thymidine incorporation. Insulin treatment stimulated [3H] thymidine incorporation in a dose-dependent manner in both groups (Fig. 1A, P<0.05). [3H]Thymidine incorporation was significantly higher in the EH group than in the NT group after treatment with the 10 μM concentration of insulin (Fig. 1A, P<0.01). Because the effect of insulin stimulation was maximal at a concentration of 10 μM, the 10 μM concentration was used for further experiments. [3H]Thymidine incorporation increased approximately 2-3 fold in insulin-treated EH and NT VSMCs compared with non-insulin treated control VSMCs (P<0.01; Fig. 1B). Insulin-stimulated [3H]thymidine incorporation was significantly greater in EH (13.1±0.19 cpm/well) than in NT VSMCs (10.6±0.21 cpm/well, P<0.05).

To elucidate whether insulin induces mitosis through a PKC signaling pathway, we cultured VSMCs from EH and NT patients with treatment of PMA, an activator of PKC. As illustrated in Figure 1B, 100 nM of PMA did not increase DNA synthesis to nearly the extent as did insulin. Administration of the PKC inhibitor GF109203X (1 μM) significantly inhibited these insulin/PMA-induced increases. This finding indicated that the intracellular effect of insulin was mediated via the PKC pathway (Fig. 1B).

As measured by phosphorylation of the specific MAPK substrate, MBP, MAPK activity with insulin treatment increased to about 3 times greater than that without insulin treatment (P<0.01, Fig. 2A). MAPK activity was significantly greater in EH VSMCs treated with insulin (492.8 pM/mg/min) than in NT VSMCs (382.4 pM/mg/min; P<0.05). VSMCs in the EH and NT groups showed no significant changes with the addition of 1 μM GF109203X for 30 min and 12 h (P>0.05). Thus, VSMCs were pretreated with 1 μM GF109203X for 12 h in further experiments. Addition of GF109203X significantly decreased the activation of MAPK in both insulin-treated groups (GF109203X + insulin = 118.2 vs insulin alone in EH VSMCs = 492.8 pM/mg/min; in NT VSMCs, GF109203X + PMA = 115.3 vs insulin= 382.4 pM/mg/min; P<0.01). Correspondingly, insulin treatment enhanced tyrosine phosphorylation of phosphotyrosine ERK1/2 bands which were identified as 42 and 44 KD MAPKs. Immunoblotting results showed that phosphorytrosine ERK1/2 in VSMCs treated with insulin or PMA was increased in both EH and NT groups compared to the negative control VSMCs, as shown in Figure 2B.

To investigate the role of PKC in insulin-mediated MAPK activation, EH and NT VSMCs were pretreated with 1 μM of GF109203X for 12 h, and then treated with insulin or PMA by the procedure mentioned above. Then MAPK activation by

![Fig. 1. Effect of insulin on vascular smooth muscle cell proliferation](image-url)
phosphorylated ERK1/2 bands was examined. As shown in Figures 2C and D, the insulin- and PMA-induced activation of MAPKs was PKC-dependent in both EH and NT groups. Insulin and PMA treatment in EH and NT VSMCs lead to a transient decrease in ERK1/2 phosphorylation, as shown in Figures 2C and D. VSMCs in the same groups showed no obvious changes with 1 μM GF109203X pretreatment for 30 min and 12 h. Proliferated human mesenteric VSMCs often display a phenotype of reduced contractility. Western blot analysis revealed that α-SM actin expression was significantly decreased in response to the treatment with 10 μM of insulin (Fig.3) in both EH and NT VSMCs. But the reduction of α-SM actin was less in EH VSMCs than in NT VSMCs (19±5 arbitrary units vs 98±7; P<0.05). This effect was not seen in GF109203X-treated VSMCs or in untreated controls.

Immunofluorescence revealed a reorganization of intracellular F-actins in insulin-treated EH and NT VSMCs (Fig. 4). Human mesenteric VSMC proliferation induced by insulin or PMA was accompanied by an obvious reorganization and disruption of F-actin structure which is a typical characteristic of proliferation. But the reorganization and disruption of the F-actin structure was more striking in the EH VSMCs than in NT VSMCs (Fig. 4A and B). This effect did not appear following treatment with 1 μM of GF109203X or in untreated VSMCs. These control VSMCs showed a well-organized cytoskeleton with abundant deeply stained fibers that were organized in bundles (Figs. 4C, D, E and F).

Discussion

The major findings of the present study indicate that insulin-induced proliferation of human mesenteric VSMCs is accompanied by increased MAPK phosphorylation and phenotypic modulation in essential hypertension when compared with cells taken from normal controls.

In clinical cardiology practice, we have found a very
common phenomenon that hyperinsulinemia usually induces proliferation-related pathological changes in arteries of patients with essential hypertension, coronary heart disease, and diabetes. Abnormal proliferation and modulation and migration of VSMCs from media into intima play a major role in the pathogenesis of hypertension and atherosclerosis. Although the effects of insulin and IGF-1 on VSMC proliferation is widely recognized across mammalian species, the mechanism by which this occurs is yet unknown. Insulin triggers two major signaling pathways in VSMCs; one is the MAPK pathway and another is the insulin receptor substrate-1 (IRS-1)-phosphatidylinositol 3 kinase (PI3K)-Akt pathway. It was reported that activation of the insulin-activated PI3K-Akt pathway resulted in translocation of the glucose transporter-4 from cytosol to the plasma membrane. Because it is well known that the main organs that consume blood glucose are skeletal muscle, adipose tissue, and brain, not the VSMCs, we chose to investigate the MAPK pathway. Hypertension may modulate insulin signaling, resulting in changes in the MAPK pathway and adverse affects on the intracellular skeleton. Modulation of insulin signaling and resultant change in MAPK pathway may affect the intracellular skeleton dysfunction that relates to essential hypertensive patients.

The findings of the current study indicated that insulin-induced proliferation, MAPK activity, and phenotypic modulation of human mesenteric VSMC are significantly different in VSMCs of EH compared with those of NT patients. The [3H]thymidine incorporation in response to insulin treatment, indicating DNA synthesis, was about 3-fold higher in VSMCs of EH than that in the VSMCs of NT patients. This suggests that an interaction between insulin and hypertension was re-
sponsible for the observed mitogenic proliferative promotion.

Both insulin and PMA treatment of VSMCs increased MAPK activity to 2-3 times that of the control group as measured by phosphorylation of the specific substrate, MBP. This effect was significantly more pronounced in the VSMCs of EH patients. This finding further indicates that EH VSMCs respond more strongly to insulin than do VSMCs of NEH patients. In the in-gel assay, there was an obvious decrease of MAPK phosphorylation in the control group. Basal MAPK phosphorylation was higher in EH than in NT VSMCs, which may reflect genetic differences among the groups. However, this difference was not observed in the MAPK activity assay. The discrepancy may be due to the distinct sensitivity of these assays.

Lysophosphatidylcholine can inhibit the AKT activation induced by insulin in cultured rat aortic vascular smooth muscle cells. Further evidence indicates that this inhibitory mechanism involves PKC-α. Here, we tested our hypothesis that the insulin-induced proliferation and MAPK phosphorylation of VSMCs are accompanied by changes in isoforms of VSMCs. We found that pretreatment of VSMCs with the PKC inhibitor GF109203X abrogated the insulin-induced proliferation and MAPK phosphorylation of VSMCs, indicating that VSMC proliferation in response to insulin is PKC-dependent.

VSMC proliferation is commonly found to be accompanied by changes in isoforms of VSMCs. Here, we observed that α-smooth muscle actin expression decreased in response to insulin treatment more obviously in EH than that in NT VSMCs. This finding is in accord with the notion that proliferation in human mesenteric VSMCs is accompanied by shifting to a kind of phenotype displaying less contractility. Consistent with this idea, we found that VSMCs cultured with insulin express lower levels of 4 α-smooth muscle actin. But pretreatment with GF109203X abolished this effect. Thus, treatment with insulin increased human mesenteric VSMC proliferation in association with a phenotypic change, and this was more pronounced in EH than in NT VSMCs.

We also found that insulin- or PMA-induced proliferation of human mesenteric VSMCs was often accompanied by a decrease in F-actin or an obvious disruption in F-actin fibers. This was more pronounced in EH than in NT VSMCs, and was reversed by pretreatment with the GF109203X. This finding is also consistent with the hypothesis that insulin-induced proliferation affects VSMC contractility.

The concentration of insulin used in our experiments most certainly promoted phosphorylation of IGF-IR and several adaptor/docking proteins, including insulin receptor substrate (IRS)-1 or-2, Shc, Grab2, and 10. In turn, these molecules interact with downstream signal transducers and effectors, resulting in activation of MAPK and phosphatidylinositol 3-kinase signaling pathways. However, the IGF-I-induced MAPK activation is a very transient event, characterized by a rapid rise and decline that occurs within minutes, and is only observed with high doses of IGF-I. Therefore, intervention with IGFs was not investigated in the present study.

Insulin appeared to increase human mesenteric VSMC proliferation; the observed increase in proliferation was accompanied by MAPK phosphorylation and VSMC phenotypic modulation that was sharper in VSMCs taken from EH patients. This indicates that insulin treatment (1) stimulates human mesenteric artery VSMC proliferation and phenotype changes in EH, (2) causes a rapid, PKC-dependent activation of MAPK, and (3) has a greater effect on the VSMCs of EH than of NT patients.

References

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