Vasoconstrictor effects of insulin in skeletal muscle arterioles are mediated by ERK1/2 activation in endothelium

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For the vasculature, both vasodilator and vasoconstrictor effects of insulin have been described. Nitric oxide (NO) is the main mediator of the acute dilatory effects of insulin in human (4, 24, 29) and rat (8, 25) vasculatures. Insulin stimulates NO production in endothelial cells by subsequently activating the intracellular enzymes 1-phosphatidylinositol 3-kinase (PI3-kinase) and Akt, which phosphorylates and activates endothelial NO synthase (7, 17, 34). Despite this well-described insulin-induced activation of NO synthase, several studies have not shown insulin-induced vasodilatation at physiological insulin concentrations (4, 8, 30).

In addition to its vasodilator actions, insulin also has vasoconstrictor effects (8, 11, 21, 25). These vasoconstrictor effects of insulin are mainly mediated by the vasocostric peptide endothelin-1 (ET-1; Refs. 4, 8, 30) and, in contrast with insulin-induced vasodilatation, remain intact during conditions of insulin resistance (1, 10). In a previous study (8), we showed that insulin induces endothelin-mediated vasoconstriction of skeletal muscle arterioles during inhibition of PI3-kinase. However, the intracellular signaling events involved in acute insulin-induced vasoconstriction are presently unknown.

Recently the activation of PI3-kinase by insulin was shown (13) to be impaired in adipose tissue microvessels of insulin-resistant rats, whereas the activation of extracellular signal-regulated kinase 1/2 (ERK1/2) with PD-98059 (40 μmol/l). In addition, inhibition of ERK1/2 without PI3-kinase inhibition uncovered insulin-mediated vasodilatation in skeletal muscle arterioles (up to 37 ± 10% of baseline diameter; P < 0.05). Effects of insulin on ERK1/2 activation in arterioles were then investigated by Western blot analysis. Insulin induced a transient 2.4-fold increase in ERK1/2 phosphorylation (maximal at ~15 min) in skeletal muscle arterioles (P < 0.05). Removal of the arteriolar endothelium abolished insulin-induced vasoconstriction, which suggests that activation of ERK1/2 in endothelial cells is involved in acute insulin-mediated vasocostriction. To investigate this, acute effects of insulin on ERK1/2 phosphorylation were studied in human microvascular endothelial cells. In support of the findings in skeletal muscle arterioles, insulin induced a 1.9-fold increase in ERK1/2 phosphorylation (maximal at ~15 min) in microvascular endothelial cells (P < 0.05). We conclude that acute vasoconstrictor effects of insulin in skeletal muscle arterioles are mediated by activation of ERK1/2 in endothelium. This ERK1/2-mediated vasoconstrictor effect antagonizes insulin-induced, PI3-kinase-dependent vasodilatation in skeletal muscle arterioles. These findings provide a novel mechanism by which insulin may determine blood flow and glucose disposal in skeletal muscle.

extracellular signal-regulated kinase 1/2; microcirculation; signal transduction; vasocostriction; dilation; resistance

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ERK1/2 phosphorylation were studied in homogenates of cremaster arterioles.

METHODS

This investigation conforms to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1996). The local ethics committee for animal experiments also approved the procedures.

Experiments were conducted on isolated first-order arterioles of cremaster muscles of healthy Wistar rats that weighed 323 ± 7 g (mean ± SE; n = 40). Rats were anesthetized with pentobarbital sodium (70 mg/kg ip) and were killed by heart excision. The right cremaster muscles were then carefully dissected from the surrounding tissue. First-order arterioles were isolated, cleared of connective tissue, and mounted in a pressure myograph. Arteriole diameter changes in response to various stimuli under no-flow conditions were then measured by video microscopy (8). Only those vessels that developed substantial spontaneous constriction (>40% of passive diameter) to pressure (65 mmHg) and showed a clear response (>10% of diameter before addition) to the endothelium-dependent vasodilator ACh (0.1 μmol/l; Sigma; St. Louis, MO) were used. All experiments were carried out in MOPS buffer that contained (in mmol/l) 145 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.0 MgSO₄, 1.2 NaH₂PO₄, 5.5 dextrose, 0.02 EDTA, 3.0 MOPS, and 2.0 pyruvate (all of which were from Merck; Darmstadt, Germany, except for pyruvate, which was from Sigma), and substances were added directly to the vessel bath. At the end of the experiments, passive diameter measurements were determined by exposing the vessels to papaverine (0.1 mmol/l; Sigma).

Vasoreactivity experiments. The acute effects of insulin (Actrapid; Novo Nordisk) on the diameters of cremaster first-order arterioles were studied by exposing arteriolar segments to four concentrations of insulin. Insulin was added to the vessel bath in a stepwise fashion starting at the lowest concentration, and diameter changes were recorded for 30 min after each concentration step. Because part of the insulin is lost by adhesion to the bath, a parallel series of experiments was performed in which samples were taken from the bath and insulin concentrations were determined by radioimmunoassay. The measured insulin concentrations were (means ± SE) 4 ± 0.7, 34 ± 6, 272 ± 44, and 3.4 ± 0.3 μU/ml. Because insulin concentrations measured in vivo in rats range between 3 and 300 μU/ml (13, 16), the first three concentrations were considered physiological, and the final concentration was pharmacological. The effects of insulin on vessel diameters were plotted at these measured insulin concentrations.

The first group of vessel segments (n = 5) was treated with insulin alone. To study vasoconstrictor effects of insulin, PI3-kinase-dependent vasodilator effects of insulin were blocked with wortmannin (50 nmol/l; Sigma). This concentration of wortmannin inhibits insulin-induced PI3-kinase activity by ~85% in rat adipocytes (14) but does not affect myosin light-chain kinase activity, which is a known side effect of wortmannin (19). We previously showed (8) that insulin induces vasocostriction under these conditions. The second group of vessels (n = 5) was treated with insulin and wortmannin.

To investigate the role of the endothelium in vasoconstrictor effects of insulin, responses to insulin were studied during inhibition of PI3-kinase after endothelium removal in a third group of vessels (n = 3). Removal of the endothelium was achieved by perfusing the vessel with a bolus of air after cannulation of the proximal side of the vessel segment.

To study the role of ERK1/2 in vasoconstrictor responses to insulin, a fourth group of vessels (n = 5) was treated with both wortmannin and the MAPK/ERK kinase (MEK) antagonist PD-98059 (40 μmol/l; Sigma) for 1 h before addition of insulin. To study the role of ERK1/2 in acute responses to insulin without PI3-kinase inhibition, a fifth group (n = 5) was pretreated with PD-98059 alone before the addition of insulin. The concentration of wortmannin that was used inhibited insulin-induced PI3-kinase activity by ~85% in rat adipocytes (14) but does not affect myosin light-chain kinase activity (19).

Effects of the different inhibitors on basal vessel diameters were investigated in time-matched control groups in which insulin solvent (MOPS buffer with 0.1% BSA) was added to the bath at the same time points as the insulin additions were made in the other experiments. For comparison, diameter changes after the first, second, third, and fourth solvent additions were given the same x-axis coordinates as the diameter changes after the first, second, third, and fourth insulin-concentration steps.

Western blot analysis of phospho-ERK1/2 in cremaster arterioles.

The protocol for Western blot analysis of isolated arterioles was adapted from the method of Eskildsen-Helmond and Mulvany (9). Segments of cremaster arterioles from the same rat (~3 mm long; n = 4 rats) were exposed to solvent (controls) or insulin (272 μU/ml) for 15 and 30 min in MOPS buffer on a 24-well plate. After stimulation, arterioles were snap-frozen in liquid nitrogen, homogenized, and dissolved in lysis buffer. Proteins were then separated by gel electrophoresis, blotted onto a nitrocellulose membrane, and stained with a specific primary antibody against ERK1/2 (1:1,000 dilution; New England Biolabs). ERK1/2 was visualized with a chemiluminescence kit (Amersham). Staining was quantified using a charge-couple device camera (Fuji Science Imaging Systems) in combination with AIDA Image Analyzer software (Isotopenmessgeräte: Staubenhardt, Germany). Blots were then stripped and stained for phosphorylated ERK1/2 (antibody from New England Biolabs; 1:2,000 dilution). Phospho-ERK1/2 staining in insulin-stimulated arterioles and time-matched controls was determined and expressed as fold increases over staining in time-matched controls. Differences were corrected for differences in total ERK1/2 staining.

Culture of human microvascular endothelial cells. Human foreskin microvascular endothelial cells (MVECs) were isolated, cultured, and characterized as previously described (20). MVECs were cultured on fibronectin-coated dishes in medium 199 supplemented with 20 mmol/l HEPES (pH 7.3), 10% heat-inactivated human serum, 10% heat-inactivated newborn calf serum, 150 mg/ml crude endothelial cell growth factor, 2 mmol/l L-glutamine, 5 U/ml heparin, 100 IU/ml penicillin, and 100 mg/ml streptomycin at 37°C in a 5% CO₂-95% air atmosphere. Two hours before the experiments were started, growth factor was withdrawn from MVEC cultures. To study the effects of insulin on ERK1/2 signaling, MVECs (n = 3 independent experiments) were stimulated with insulin (272 μU/ml) for 15 and 30 min and then lysed, and ERK1/2 phosphorylation was analyzed as described above.

Statistics. Steady-state responses are reported as mean changes in diameter from baseline (in percent) ± SE. The baseline diameter was defined as the arteriolar diameter just before addition of the first insulin concentration. Differences between means at each concentration were assessed by one-way ANOVA with Bonferroni post hoc tests. Diameter measurements before and after pretreatment were tested with a paired t-test. Differences in phospho-ERK1/2 staining were tested against a hypothetical value of zero by a one-sample t-test. Differences were considered statistically significant when P < 0.05.

RESULTS

To study the vasoactive effects of insulin on skeletal muscle arterioles, we used the isolated first-order arterioles of rat cremaster muscles as a model.

General characteristics. Passive intraluminal diameter values of arteriolar segments averaged 182 ± 3 μm (n = 40) when pressurized to 65 mmHg. During the equilibration period, all vessel segments developed spontaneous tone, which reduced the diameter measurement by 94 ± 2 (52 ± 1%) to 88 ± 2 μm. When stimulated with the endothelium-dependent vasodilator ACh (0.1 μmol/l), all vessels dilated by >10% for
>6 min. Responses of untreated control vessels (n = 6) to ACh at the beginning and end of experiments averaged 35 ± 9 and 45 ± 11% (P = 0.50), which indicates vessel stability in this setup.

Insulin induces dose-dependent vasoconstriction during inhibition of PI3-kinase. Insulin alone did not significantly change arteriolar diameter values (Fig. 1). Insulin induced a dose-dependent vasoconstriction of isolated cremaster arterioles (n = 6) in the presence of the PI3-kinase inhibitor wortmannin (Fig. 1) in accordance with our previous study (8). Significant vasoconstriction was observed at insulin concentrations of 34 μU/ml (−17 ± 3% change from baseline; P < 0.05 vs. insulin alone; P < 0.05 vs. wortmannin), 272 μU/ml (−22 ± 3% change from baseline; P < 0.01 vs. insulin alone; P < 0.05 vs. wortmannin), and 3.4 μU/ml (−21 ± 3% change from baseline; P < 0.05 vs. insulin). Vessel segments constricted slightly during the pretreatment period (from 86 ± 3 to 81 ± 5 μm; P = 0.01; Table 1), but this effect was unrelated to insulin-mediated vasoconstriction in the presence of wortmannin. This indicates that insulin-mediated vasoconstriction is independent of PI3-kinase activation.

Insulin-mediated vasoconstriction is mediated by arteriolar endothelium. To study the role of arteriolar endothelium in insulin-mediated vasoconstriction, we studied the effects of insulin during PI3-kinase inhibition after removal of arteriolar endothelium (see METHODS). Endothelium removal abolished the vasodilator response to ACh (1 μmol/l) in all treated arterioles (not shown). Removal of the arteriolar endothelium abolished insulin-induced vasoconstriction during inhibition of PI3-kinase (Fig. 2) at insulin concentrations of 34 μU/ml (3 ± 2 vs. −17 ± 3% change from baseline; P < 0.01 vs. insulin and wortmannin) and 272 μU/ml (3 ± 3 vs. −22 ± 3% change from baseline; P < 0.01 vs. insulin and wortmannin). Endothelium removal did not significantly alter the vasoconstrictor effect of wortmannin during the pretreatment period (Table 1).

Insulin-mediated vasoconstriction is mediated by ERK1/2. Insulin-mediated vasoconstriction was abolished by inhibition of ERK1/2 (with 40 μmol/l PD-98059; Fig. 3). ERK1/2 inhibition significantly attenuated insulin-induced vasoconstriction at insulin concentrations of 34 μU/ml (3 ± 5 vs. −17 ± 3% change from baseline; P < 0.01 vs. insulin and wortmannin) and 272 μU/ml (1 ± 5 vs. −22 ± 3% change from baseline; P < 0.01 vs. insulin and wortmannin). Inhibition of ERK1/2 activity did not alter vasoconstriction during pretreatment with wortmannin (Table 1) and did not change the diameter of time-matched controls compared with time-matched controls treated with wortmannin (not shown).

Insulin acutely activates ERK1/2 in skeletal muscle arterioles and MVECs. Western blot analysis of single cremaster arterioles showed that insulin (272 μU/ml) induces a transient

Table 1. Effects of different pretreatments on arteriolar diameter measurements

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Arteriolar Diameter, μm</th>
<th>Before treatment</th>
<th>After treatment</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>89 ± 6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PD-98059</td>
<td>90 ± 4</td>
<td>83 ± 5</td>
<td>&lt;0.03</td>
<td></td>
</tr>
<tr>
<td>Wortmannin</td>
<td>86 ± 3</td>
<td>81 ± 5</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Wortmannin with PD-98059</td>
<td>94 ± 5</td>
<td>81 ± 4</td>
<td>&lt;0.002</td>
<td></td>
</tr>
<tr>
<td>Wortmannin without endothelial cells</td>
<td>105 ± 9</td>
<td>96 ± 3</td>
<td>&lt;0.36</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. Diameter changes during pretreatment were tested with paired t-test of arteriolar diameter measurements before and after pretreatment.

ERK1/2 MEDIATES VASOCONSTRICTOR EFFECTS OF INSULIN

Fig. 1. Vasoactive effects of insulin alone and during inhibition of 1-phosphatidylinositol 3-kinase (PI3K) in rat cremaster first-order arterioles. Responses are shown as percent change from the baseline diameter (see METHODS). *P < 0.05 vs. insulin; **P < 0.01 vs. insulin; #P < 0.05 vs. PI3K inhibition.

Fig. 2. Vasoactive effects of insulin during PI3K inhibition in intact arterioles and in arterioles without functional endothelium (insulin + PI3Kin – endothelium). Responses are shown as percent change from baseline diameter. For comparison, results for the group insulin + PI3K inhibition is depicted in Figs. 1–3. #P < 0.05 vs. insulin + PI3K inhibition.

Fig. 3. Vasoconstrictor effects of insulin during PI3K inhibition and during combined inhibition of PI3K and extracellular signal-regulated kinase 1/2 (ERK1/2) activation. Responses are given as percent change from baseline diameter. For comparison, the group insulin + PI3K inhibition is depicted in Figs. 1–3. #P < 0.05 vs. insulin + PI3K inhibition.
2.4-fold increase in ERK1/2 phosphorylation (Fig. 4A; \( P = 0.048 \) at 15 min). The time profile of this activation matched that of ERK1/2 phosphorylation in cremaster arterioles with maximal phosphorylation \( \sim 15 \) min after insulin addition. 

ERK1/2-mediated vasoconstrictor effects of insulin antagonize insulin-mediated vasodilatation in skeletal muscle arterioles. To further investigate the role of ERK1/2 in the vasoactive effects of insulin, we studied responses to insulin in the presence of the MEK/ERK1/2 antagonist PD-98059. Inhibition of ERK1/2 activation uncovered a concentration-dependent vasodilator effect of insulin (Fig. 5). The diameter measurements of time-matched control vessels treated with the ERK1/2 inhibitor alone did not change during the time course of the experiments (1–3% change from baseline; Fig. 5). Responses to insulin in the PD-98059-treated group differed significantly from vasoreactivity responses to insulin alone at 34 \( \mu \)U/ml (11 \( \pm \) 5 vs. \( -4 \pm 1 \)% from baseline; \( P < 0.05 \)) and at 272 \( \mu \)U/ml (20 \( \pm \) 6 vs. \( -1 \pm 5 \)% from baseline; \( P < 0.05 \)). Vessel segments treated with PD-98059 constricted from 90 \( \pm \) 4 to 83 \( \pm \) 5 \( \mu \)m during the pretreatment period (\( P = 0.03 \); Table 1) but did not relate to diameter changes in response to insulin.

The vasodilator effect of insulin was abolished by pretreatment with the PI3-kinase inhibitor wortmannin (50 nmol/l; Fig. 5). Wortmannin significantly inhibited insulin-induced vasodilatation at insulin concentrations of 272 \( \mu \)U/ml (20 \( \pm \) 6 vs. 1 \( \pm \) 5% diameter change; \( P < 0.01 \)) and 3.4 mU/ml (37 \( \pm \) 10 vs. 6 \( \pm \) 8% diameter change; \( P < 0.05 \)).

**DISCUSSION**

The novel findings of this study are the following: 1) vasoconstrictor effects of physiological concentrations of insulin on skeletal muscle first-order arterioles are mediated by activation of ERK1/2 signaling in endothelial cells; 2) this ERK1/2-dependent vasoconstrictor effect antagonizes insulin-induced, PI3-kinase-dependent vasodilatation in skeletal muscle arterioles; and 3) activation of ERK1/2 can be directly visualized in single first-order arterioles. These findings provide important new mechanistic information about the vascular actions of insulin. Insulin is not only a vasodilator agonist; it also has antagonistic vasodilator and vasoconstrictor actions in skeletal muscle arterioles with and without inhibition of PI3K. Responses are shown as percent change from baseline diameter. For comparison, the group insulin + PI3K inhibition + ERK1/2 inhibition is depicted in Figs. 3 and 5. *\( P < 0.05 \) vs. insulin; \#* \( P < 0.05 \) vs. insulin + PI3K inhibition + ERK1/2 inhibition.
microvascular endothelium that together may determine blood flow and glucose uptake in skeletal muscle tissue.

Antagonistic vasodilator and vasoconstrictor effects of insulin were recently demonstrated in several studies on rats (18, 21, 25, 30) and humans (4). In these studies, insulin was shown to induce both NO and endothelin activity in the vessel wall. In our setup, the balance of these vasodilator and vasoconstrictor effects resulted in an apparent lack of effect of insulin under basal conditions (see Fig. 1; Ref. 8). A balance of NO-mediated vasodilator and ET-1-mediated vasoconstrictor effects leading to an apparent lack of effect of insulin under basal conditions was also demonstrated in the human forearm (4).

In this study, we demonstrated that vasodilator and vasoconstrictor effects of insulin are mediated by different intracellular signaling pathways, and we identified ERK1/2 activation as a key signaling step in insulin-mediated vasoconstriction. Our finding that different intracellular signaling pathways regulate the different vascular effects of insulin provides a novel link between biochemical and functional evidence on selective vascular insulin resistance.

In biochemical studies it was shown that activation of PI3-kinase-dependent cell signaling, which has been implicated in insulin-mediated glucose disposal and NO synthesis, is impaired in muscles from insulin-resistant human subjects (6) and in the vasculature of insulin-resistant rats (13), whereas the insulin-mediated activation of ERK1/2 in both is normal. However, in the vascular study, the effect of the observed defect on vasoreactivity to insulin was not investigated.

In functional studies it was shown that NO-dependent vasodilator effects of insulin are attenuated in insulin-resistant subjects (29), whereas acute stimulation of endothelin release is intact (10). Moreover, in the forearm of obese, insulin-resistant, hypertensive human subjects, direct insulin-mediated vasoconstriction has been demonstrated (11). However, the mechanism behind this selective impairment of the vasoactive effects of insulin has not been elucidated.

Our finding that vasoconstrictor effects of insulin are mediated by ERK1/2, together with normal activation of ERK1/2 in insulin resistance, provides a putative mechanism to explain the predominance of insulin’s vasoconstrictor effects in this condition.

Pretreatment with PD-98059 induced a slight constriction of arterioles (see Table 1). The observed vasoconstriction may be caused by PD-98059-mediated inhibition of prostacyclin release, which has been shown in human umbilical vein endothelial cells (33). A study (3) on platelets has suggested that PD-98059 inhibits prostaglandin synthesis via direct inhibition of cyclooxygenases, which is a side effect of potential influence on results obtained with this substance. However, prostaglandins do not play a major role in vasoactive effects of insulin in rat skeletal muscle arterioles (5, 25), and the vasoconstrictor effects of insulin in our model are predominantly mediated by endothelin (8). In addition, the effect of PD-98059 pretreatment on vascular tone did not relate to insulin-mediated vasodilation in the presence of the inhibitor. Therefore, the effect of PD-98059 on the vasoactive effects of insulin can only be explained by inhibition of insulin-mediated activation of ERK1/2.

The existence of vasoconstrictor effects of insulin in vivo and in vitro evokes the question, What is the function of these insulin effects in normal physiology? Although there is presently no unequivocal answer to this question, regulation of vascular ERK1/2 activation may be a target of physiological modulators of insulin sensitivity. For example, adenosine 5’-monophosphate-activated protein kinase, which is an exercise-activated intracellular enzyme that increases insulin sensitivity, is a potent inhibitor of ERK1/2 activation by insulin-like growth factor-1 (15). By subsequent activation of adenosine 5’-monophosphate-activated protein kinase and inactivation of insulin-mediated ERK1/2 signaling in skeletal muscle arterioles, increased glucose demand may result in increased glucose delivery in muscle. Clearly, this point needs additional investigation.

The evidence from this study and the studies summarized above suggests that activation of ERK1/2 is involved in endothelin release. That the vasoconstrictor effects of insulin in our setup are also abolished by endothelin receptor blockade (8) supports this hypothesis. Moreover, inhibition of ERK1/2 in our setup does not attenuate endothelin-induced vasoconstriction (unpublished observation), which suggests that ERK1/2 activation by insulin lies upstream of endothelin release. ERK1/2 may be involved in the exocytosis of secretory granules that contain endothelin, which have been demonstrated to exist in vascular endothelium (23). ERK1/2 is associated with microtubuli (32), which are involved in exocytosis of endothelin-containing granules (31). Finally, pilot experiments on cultured human umbilical vein endothelial cells in our laboratory showed a decrease in secreted endothelin by ERK1/2 inhibition in the presence of insulin (Erínga et al., unpublished observation).

In summary, we have characterized a new mechanism via which insulin regulates microvascular tone. We propose that insulin-induced activation of ERK1/2 in arteriolar endothelium mediates acute vasoconstrictor effects of insulin and antagonizes insulin-induced, PI3-kinase-dependent vasodilatation in these arterioles. Our findings suggest a novel mechanism for coupling insulin-mediated muscle blood flow to insulin-mediated glucose disposal.

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