Physiological Concentrations of Insulin Induce Endothelin-Dependent Vasoconstriction of Skeletal Muscle Resistance Arteries in the Presence of Tumor Necrosis Factor-α Dependence on c-Jun N-Terminal Kinase

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Objective—Tumor necrosis factor-α (TNF-α) has been linked to obesity-related insulin resistance and impaired endothelium-dependent vasodilatation, but the mechanisms have not been elucidated. To investigate whether TNF-α directly impairs insulin-mediated vasoreactivity in skeletal muscle resistance arteries and the role of c-Jun N-terminal kinase (JNK) in this interference.

Methods and Results—Insulin-mediated vasoreactivity of isolated resistance arteries of the rat cremaster muscle to insulin (4 to 3400 μU/mL) was studied in the absence and presence of TNF-α (10 ng/mL). Although insulin or TNF-α alone did not affect arterial diameter, insulin induced dose-dependent vasoconstriction of cremaster resistance arteries in the presence of TNF-α, (−12±1% at 272 μU/mL). Blocking endothelin receptors in the absence of TNF-α uncovered insulin-mediated vasodilatation (18±6% at 272 μU/mL) but not in the presence of TNF-α (2±2% at 272 μU/mL), showing that TNF-α inhibits vasodilator effects of insulin. Using digital imaging microscopy, we discovered that TNF-α activates JNK in arterial endothelium, visible as an increase in phosphorylated JNK. Moreover, inhibition of JNK with the cell-permeable peptide inhibitor L-JNKI abolished insulin-mediated vasoconstriction in the presence of TNF-α, showing that JNK is required for interaction between TNF-α and insulin.

Conclusions—TNF-α inhibits vasodilator but not vasoconstrictor effects of insulin in skeletal muscle resistance arteries, resulting in insulin-mediated vasoconstriction in the presence of TNF-α. This effect of TNF-α is critically dependent on TNF-α-mediated activation of JNK. (Arterioscler Thromb Vasc Biol. 2006;26:000-000.)

Key Words: cytokines ■ endothelin ■ endothelium ■ insulin resistance ■ microcirculation ■ vascular biology

Impairment of insulin-mediated glucose disposal (ie, insulin resistance) is associated with obesity1 and hypertension.2 Increased production of the proinflammatory cytokine tumor necrosis factor-α (TNF-α) is associated with obesity-related insulin resistance1-4 as well as obesity-related hypertension.5 The mechanisms behind these relationships have not been elucidated.

We and others hypothesized that impaired endothelium-dependent vasodilatation plays an important role in insulin resistance and hypertension.6 Impaired endothelium-dependent vasodilatation increases vascular resistance, which, in muscle, may contribute to decreased insulin-mediated glucose disposal.7 A specific type of endothelium-dependent vasodilatation that may be relevant in this regard is insulin-mediated vasodilatation.

Vasodilator and vasoconstrictor effects of insulin have been described, and the normal response to insulin is thought to be vasodilator or neutral.8,9 We and others have shown previously that in resistance arteries of skeletal muscle, NO-dependent vasodilator effects of insulin are antagonized by endothelin-mediated vasoconstrictor effects. Selective impairment of the vasodilator effects of insulin in these arteries results in insulin-mediated vasoconstriction.8,10 Physiological factors that cause such a selective impairment of insulin-mediated vasoreactivity have not been identified.

TNF-α has been proposed to impair vasodilator effects of insulin in the skeletal muscle microcirculation.8,21 Furthermore, in macrovascular endothelial cells, TNF-α has been shown to impair insulin-mediated activation of endothelial NO synthase (eNOS).11 This is achieved through inhibition of insulin-mediated activation of phosphatidylinositol 3-kinase (PI3K) and Akt, which are crucial steps in insulin-mediated eNOS activation.12,13 In skeletal muscle resistance arteries, we have shown previously that the vasodilator effects of
insulin are mediated by PI3K and eNOS. In contrast, vasoorconstrictor effects of insulin in these resistance arteries are independent of PI3K. It is unknown whether TNF-α directly impairs insulin-mediated vasoreactivity and cell signaling in resistance arteries.

The intracellular enzyme c-Jun N-terminal kinase (JNK) has been shown to regulate whole-body insulin sensitivity as well as insulin-mediated cell signaling. Moreover, activation of JNK by TNF-α has been demonstrated in fibroblasts.

We hypothesized that TNF-α impairs vasodilator effects but not vasoconstrictor effects of insulin in skeletal muscle resistance arteries through activation of JNK and impairment of insulin-mediated activation of Akt. This selective inhibition of the vasodilator effects of insulin would result in insulin-mediated vasoconstriction in the presence of TNF-α. To test these hypotheses, we used resistance arteries from the rat cremaster muscle (diameter ~90 μm) as a model.

**Methods**

The investigation conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85 to 23, revised 1996). The local ethics committee for animal experiments approved the procedures.

Experiments were conducted on isolated resistance arteries (~90 μm) of cremaster muscles of healthy Wistar rats weighing 250 to 350 g (n=40). Cremaster resistance arteries were isolated and studied in the pressure myograph as described. Only vessels that developed substantial spontaneous constriction (>40% of the passive diameter) to pressure (65 mm Hg) and then showed a clear vasodilator response (>10% of the diameter) to the endothelium-dependent vasodilator acetylcholine (ACh; 0.1 μM; Sigma) were used.

**Vasoreactivity Experiments**

Vasoreactivity experiments were performed as described. Acute effects of insulin (Actrapid; Novo Nordisk) on the diameter of cremaster resistance arteries were studied by exposing arterial segments to 4 concentrations of insulin (4±0.7, 34±6, 272±44, and 3400±300 μU/mL, as determined by radioimmunoassay). Insulin was added to the vessel bath in a stepwise fashion, starting at the lowest concentration, and diameter changes during 30 minutes after each concentration step were recorded. Because insulin concentrations measured in vivo in the rat range between 3 and 300 μU/mL, the first 3 concentrations are considered physiological and the final concentration pharmacological. Effects of insulin on vessel diameter are plotted at these measured insulin concentrations. The first group of vessel segments (n=6) was treated with insulin alone. To study the effect of TNF-α on vasoactive effects of insulin, a second group of vessels (n=6) was pretreated for 15 minutes with TNF-α (10 ng/mL; Sigma), and a dose-response curve to insulin was generated as described above. To study the effect of TNF-α on endothelin-1 (ET-1)–dependent effects of insulin in skeletal muscle resistance arteries, a third group of vessels (n=5) was pretreated with TNF-α and the nonselective ET-1 receptor antagonist PD142983 (3 μmol/L; Kordia) before addition of insulin.

To study the role of JNK in the interaction between TNF-α and insulin, a fourth group of vessels (n=4) was treated with the JNK interacting protein 1 fused to a TAT peptide (L-JNKI; 5 μmol/L; Alexis Benelux), before exposure to TNF-α and insulin. L-JNKI is taken up in cremaster arterioles within 30 minutes (see online supplement, available at http://atvb.ahajournals.org). A fifth group (n=4) was treated with L-JNKI and insulin without TNF-α to investigate whether JNK inhibition interferes with vasoreactivity to insulin alone. To confirm the results obtained with L-JNKI, the effect of the JNK inhibitor SP600125 (0.5 μmol/L; Sigma) on insulin-mediated vasoreactivity in the presence of TNF-α was studied in an sixth group of vessels (n=4). The used concentration of SP600125 suppresses JNK activity but is below the concentrations that inhibit Akt phosphorylation, a reported side effect of SP600125 at micromolar concentrations.

To study the effects of TNF-α alone or in combination with endothelin receptor antagonism or JNK inhibition on arterial diameter in time, these effects were studied in time-matched control groups (n=3 to 5 per group) treated with solvent (3-(N-morpholino)propanesulfonic acid buffer +0.1% BSA) instead of insulin.

**Measurement of Akt Phosphorylation in Cremaster Resistance Arteries**

Western blotting of isolated resistance arteries was performed as we described. In cremaster resistance arteries, insulin (272 μU/mL) induces a time-dependent phosphorylation of Akt that reaches a plateau at 15 to 30 minutes of stimulation (see online supplement).

Therefore, arterial segments were stimulated with insulin for 15 minutes for determination of pAkt. Cremaster resistance arteries were divided into 4 segments (~1.5 mm long; n=4) that were exposed for 20 minutes to solvent (controls) or insulin (272 μU/mL) in the absence and presence of TNF-α (10 ng/mL). Specific primary antibodies against Akt phosphorylated at serine 473 (1:1000; New England Biolabs) and extracellular signal-regulated kinase 1 (ERK1; 1:1000; New England Biolabs) were used to examine insulin-mediated activation of Akt.

To investigate the role of JNK in the effect of TNF-α on insulin-mediated activation of Akt, arterial segments from another cremaster muscle were exposed to solvent (control) or insulin alone or after pretreatment with TNF-α and L-JNKI.

pAkt staining in insulin-stimulated resistance arteries and time-matched controls was determined and expressed as fold increase over staining in time-matched controls. Differences were corrected for differences in ERK1, which was used as a loading control.

**Visualization of JNK Activation in Endothelium and Smooth Muscle of Skeletal Muscle Resistance Arteries**

Cremaster resistance arteries (n=4 rats) were divided into 2 segments that were treated with TNF-α or solvent for 15 minutes, fixed, cut open longitudinally for en face staining and fluorescence imaging of phosphorylated JNK (microscope Zeiss 200 mol/L). Phospho-JNK was then examined in endothelium and smooth muscle using a primary antibody against phospho-JNK (1:100; New England Biolabs) and a fluorescein isothiocyanate–labeled secondary antibody. 4,6-diamidino-2-phenylindole was used as a nuclear counterstain.

**Measurement of TNF-α–Mediated Phosphorylation of JNK in Human Microvascular Endothelial Cells**

To confirm that TNF-α activates JNK in microvascular endothelial cells (MVECs), cultured MVECs from human foreskin were stimulated with TNF-α, and JNK phosphorylation was examined. MVECs were isolated, cultured, and characterized as we have described previously. Two hours before the stimulation was started, growth factor was withdrawn from MVEC cultures. Cells were stimulated with TNF-α (10 ng/mL) for 5, 15, and 30 minutes and lysed. After protein separation on gel (10 μg/lane) and Western blotting, blots were stained with antibodies against phospho-JNK (1:1000; New England Biolabs) and a fluorescein isothiocyanate–labeled secondary antibody. Anti-tubulin staining (1:1000; Cytoskeleton) was used as a loading control.

**Statistics**

Steady-state responses are reported as mean changes in diameter from baseline (in percent) ±SEM. The baseline diameter was defined as the arterial diameter just before addition of the first insulin concentration. Differences between diameter changes at each con-
centrations were assessed by a 1-way ANOVA with Bonferroni post hoc tests. Diameters before and after pretreatment were tested with a paired t test. Insulin- and TNF-α–mediated increases in pAkt staining were tested against a hypothetical value of 0 by a 1-sample t test. Differences in pAkt staining between vessels treated with insulin, TNF-α, and insulin plus TNF-α were tested with a paired 1-way ANOVA. Differences were considered statistically significant when \( P < 0.05 \).

**Results**

**General Characteristics**

Passive intraluminal diameters of arterial segments averaged 182±3 μm (n=31) when pressurized to 65 mm Hg. During the equilibration period, all vessel segments developed spontaneous tone, reducing the diameter by 94±2 μm (52±1%) to 88±2 μm. When stimulated with the endothelium-dependent vasodilator ACh (0.1 μmol/L), vessels diluted by 37±4% of baseline diameter.

**TFN-α Inhibits Vasodilator But Not ET-1–Dependent Vasoconstrictor Effects of Insulin in Skeletal Muscle Resistance Arteries**

Insulin alone did not significantly change arterial diameter (1±2% to 5±5% of baseline diameter; Figure 1). In contrast, insulin induced a dose-dependent vasoconstriction of isolated cremaster resistance arteries in the presence of TNF-α (Figure 1). Significant vasoconstriction was observed at insulin concentrations of 34 μU/mL (−6±2% of baseline diameter; \( P < 0.05 \)), 272 μU/mL (−12±1% of baseline diameter; \( P < 0.05 \)), and 3400 μU/mL (−15±1% of baseline diameter; \( P < 0.001 \)). TNF-α did not change arterial diameter during the pretreatment period (1±4% of baseline diameter; \( P = 0.81 \)) or in time-matched controls treated with TNF-α (−1±2 to 4±4% of baseline diameter; data not shown).

Pretreatment of cremaster resistance arteries with the nonselective ET-1 receptor antagonist PD142893 (3 μmol/L) abolished the vasoconstrictor effect of insulin in the presence of TNF-α (Figure 2). Insulin-induced vasoconstriction was attenuated at 272 μU/mL (\( P < 0.05 \) versus insulin plus TNF-α) and 3400 μU/mL (\( P < 0.01 \) versus insulin plus TNF-α).

To investigate whether TNF-α interferes with insulin-mediated vasoreactivity by inhibiting vasodilator effects of insulin, we compared insulin-mediated vasoreactivity during ET-1 receptor blockade in the absence and presence of TNF-α (Figure 2). During ET-1 receptor blockade, insulin induced vasodilatation in the absence of TNF-α (maximum 28±7% of baseline diameter) but not in the presence of TNF-α (Figure 2), demonstrating that TNF-α inhibits the vasodilator effects of insulin.

TNF-α did not impair the vasodilator response to 0.1 μmol/L ACh (37±7% of baseline without TNF-α versus 52±6% of baseline with TNF-α; \( P = 0.12 \)), showing that TNF-α specifically impairs vasodilator effects of insulin without inhibiting endothelium-dependent vasodilatation in general.

**TFN-α Inhibits Insulin-Mediated Activation of Akt in Skeletal Muscle Resistance Arteries**

To study whether TNF-α impairs vasodilator effects of insulin by inhibition of insulin-mediated activation of PI3K/Akt, we studied the effects of TNF-α and insulin on the phosphorylation of Akt at Ser 473 in arterial segments. At 15 minutes, insulin (272 μU/mL) induced a 3.4-fold increase in phosphorylation of Akt in skeletal muscle resistance arteries (\( P = 0.02 \); Figure 3) that was abolished by pretreatment with TNF-α (3.4-fold versus 1.3-fold, \( P < 0.05 \); Figure 3). Pretreatment with TNF-α alone induced a 2.7-fold increase in Akt phosphorylation in cremaster resistance arteries (Figure 3), which was more variable than insulin-mediated Akt phosphorylation (relative error 0.56 for TNF-α versus 0.30 for insulin) and did not reach statistical significance (\( P = 0.11 \)).

**Role of TNF-α–Mediated Activation of JNK in TNF-α**

First, we examined the effect of TNF-α on JNK phosphorylation in cremaster resistance arteries by immunofluorescent staining of pJNK (see Methods). Under control conditions, little activated JNK could be detected (images from 1 representative experiment are shown in Figure 4A). In contrast, treatment of arteries with TNF-α induced a marked
increase of activated JNK, which was present in endothelial cells and smooth muscle cells (Figure 4A).

Second, to confirm that TNF-α increases pJNK in microvascular endothelium, MVECs were stimulated for various time periods with TNF-α and JNK phosphorylation was analyzed. In MVECs, TNF-α increased JNK phosphorylation at 15 minutes (see online supplement), confirming that TNF-α acutely activates JNK in microvascular endothelium.

Third, we examined the effect of JNK inhibition on vasoreactivity to TNF-α and insulin. Inhibition of JNK with the peptide inhibitor L-JNKI reversed the vasoconstrictor effect of insulin in the presence of TNF-α (Figure 4B). Insulin-mediated vasoconstriction was inhibited at 34 μU/mL (P<0.05 versus insulin plus TNF-α), 272 μU/mL (P<0.001 versus insulin plus TNF-α) and 3400 μU/mL (P<0.001 versus insulin plus TNF-α). In the absence of TNF-α, inhibition of JNK did not change vasoreactivity to insulin (Figure 4C). Vasoreactivity to TNF-α during the pretreatment period was not altered by JNK inhibition (~2±3% of baseline diameter; P=0.61). In the presence of TNF-α and L-JNKI, insulin even induced a dose-dependent vasodilatation of resistance arteries (15±5% of baseline diameter at 272 μU/mL; Figure 4B). The JNK inhibitor SP600125 also reversed insulin-mediated vasoconstriction in the presence of TNF-α (P<0.05 versus insulin plus TNF-α at 272 and 3400 μU/mL), but insulin did not induce vasodilatation in the presence of this compound (4±2% of baseline diameter at 272 μU/mL; Figure 4B).

Finally, we investigated whether the inhibition of insulin-mediated activation of Akt by TNF-α was reversed by JNK inhibition with L-JNKI. Indeed, in the presence of L-JNKI, TNF-α pretreatment did not impair insulin-mediated phosphorylation of Akt at Ser 473 (Figure 4D).

**Discussion**

We have shown for the first time that: (1) insulin induces vasoconstriction of skeletal muscle resistance arteries in the presence of TNF-α; (2) this shift in vasoreactivity is caused by TNF-α-mediated inhibition of vasodilator effects of insulin, whereas ET-1–dependent vasoconstrictor effects of insulin remain intact; (3) this inhibition of insulin-mediated vasodilatation by TNF-α is associated with inhibition of insulin-mediated activation of Akt in resistance arteries; and (4) TNF-α interferes with insulin-mediated vasoreactivity through activation of JNK in microvascular endothelium. These data provide a new mechanistic link between TNF-α, JNK, insulin, and microvascular dysfunction.

Antagonistic vasodilator and vasoconstrictor effects of insulin have been demonstrated recently in a number of studies in rats \(^8\)–\(^10,17\) and in man. \(^26\) In these studies, insulin was shown to induce NO and endothelin activity in the vessel wall. In our setup, insulin-mediated vasodilatation, which is entirely NO dependent, \(^9\) is offset by an ET-1–dependent vasoconstrictor effect of insulin under basal conditions (Figure 1). \(^8\)–\(^17\) This balance of NO-mediated vasodilator and ET-1–mediated vasoconstrictor effects leading to an apparent lack of effect of insulin under basal conditions has also been demonstrated in the human forearm. \(^26\)

The fact that insulin does not induce vasodilatation during ET-1 receptor blockade in the presence of TNF-α (Figure 2) demonstrates that TNF-α inhibits the vasodilator effects of insulin. In the absence of TNF-α, ET-1 receptor blockade uncovers insulin-mediated vasodilatation in our setup (Figure 2). \(^8\) The impairment of insulin-mediated vasodilatation by TNF-α that we have shown here is in agreement with previous studies in the rat hindlimb \(^24\) and in the human forearm. \(^27\)

In contrast, TNF-α does not inhibit the ET-1–mediated vasoconstrictor effects of insulin in skeletal muscle resistance arteries, resulting in insulin-induced, ET-1–mediated vasoconstriction in the presence of TNF-α (Figure 2). These data confirm that TNF-α primarily inhibits the vasodilator actions of insulin in skeletal muscle resistance arteries. In agreement with our in vitro data, the combination of insulin and TNF-α decreases blood flow in the human forearm. \(^27\) In addition, in human obesity-associated insulin resistance, a condition associated with increased TNF-α expression, insulin has been shown to decrease forearm blood flow. \(^28\) This can be explained by impaired insulin-mediated activation of NO synthesis \(^18,29\) but normal insulin-mediated production of ET-1, \(^30\) which have been reported in insulin-resistant subjects. These data and ours show that insulin-mediated vasoreactivity is selectively impaired in obesity-related insulin resistance and that TNF-α may contribute to this selective impairment.

As a mechanism for the inhibition of insulin-mediated vasodilatation, TNF-α has been proposed to inhibit insulin-mediated activation of PI3K/Akt signaling in vascular endothelium. \(^11\) Our data (Figure 3) show that this indeed takes place in skeletal muscle resistance arteries. In previous studies, we have shown that vasodilator effects of insulin in skeletal muscle resistance arteries are mediated by PI3K/Akt/eNOS, and inhibition of this signaling pathway results in insulin-mediated, ET-1–dependent vasoconstriction. \(^8,18\) These findings and the data presented here suggest that inhibition of insulin-mediated activation of PI3K/Akt is...
sufficient to explain the interference of TNF-α with insulin-mediated vasoreactivity. A number of studies have shown elevated serum levels of TNF-α in type 2 diabetes but only by 2- to 3-fold compared with lean, healthy controls. Local levels of TNF-α may be higher because TNF-α has been proposed to work in a paracrine manner. In the present study, we used a concentration in the range seen in sepsis, a condition associated with insulin resistance. In contrast to in vivo studies, TNF-α failed to inhibit ACh-mediated vasodilatation in our study. These contrasting results may be explained by a difference in the relationships between Akt activity and ACh-mediated vasodilatation in vivo and in vitro. Unlike insulin-mediated NO production, ACh-mediated NO production in isolated arteries is independent from activation of PI3K and Akt.

Our results (Figure 4) demonstrate that TNF-α–mediated activation of JNK is a critical step in this interference. TNF-α activates JNK in microvascular endothelium (Figure 4A; Figure II, available online at http://atvb.ahajournals.org) and inhibition of JNK abolishes interference of TNF-α with insulin-mediated vasoreactivity (Figure 4B) and insulin-mediated activation of Akt (Figure 4D). In cultured fibroblasts, JNK has been shown to inhibit insulin-mediated activation of PI3K by phosphorylating insulin receptor substrate 1 (IRS-1) at Ser 307. Because ET-1–mediated vasoconstrictor effects of insulin in skeletal muscle resistance arteries are independent of PI3K, this JNK-induced phosphorylation of IRS-1 may explain JNK-dependent, insulin-induced vasoconstriction in the presence of TNF-α. Indeed, preliminary experiments in our setup showed that TNF-α induces Ser 307 phosphorylation of IRS-1 in cremaster resistance arteries (data not shown). Based on these findings, we propose that JNK mediates the interaction of TNF-α with insulin-mediated activation of PI3K/Akt signaling in skeletal muscle resistance arteries (Figure 5).

Although both JNK inhibitors reversed insulin-mediated vasoconstriction in the presence of TNF-α, their effect on

Figure 4. Role of JNK in interaction of TNF-α with insulin-mediated vasoreactivity. A, TNF-α (10 ng/mL) increases phosphorylation of JNK in endothelium and smooth muscle of skeletal muscle resistance arteries. Magnification ×63. One representative example of 3 experiments is shown. B, Inhibition of JNK with L-JNKI or SP-600125 abolishes interaction of TNF-α with insulin-mediated vasoreactivity in skeletal muscle arterioles. Responses are given as percent change from the baseline diameter. L-JNKI indicates inhibitor of JNK. C, Inhibition of JNK with L-JNKI does not alter insulin-mediated vasoreactivity in the absence of TNF-α. D, TNF-α does not inhibit insulin-mediated Ser 473 phosphorylation of Akt during inhibition of JNK. E, TNF-α does not inhibit insulin-mediated Ser 473 phosphorylation of Akt during inhibition of JNK.
insulin-mediated vasoreactivity differed to a certain extent. Whereas insulin induced dose-dependent vasodilation in the presence of TNF-α and L-JNKI, it did not in the presence of TNF-α and SP600125 (Figure 4B). This attenuation of the vasodilator effects of insulin may be explained by a side effect of SP600125. Whereas L-JNKI has been reported to be specific for JNK, SP600125 has also been shown to partially inhibit phosphoinositide-dependent protein kinase 1,22,35 which is needed for insulin-mediated activation of Akt and insulin-mediated vasodilation.11,13

Functional Implications

Increased production of TNF-α has been linked to obesity-related insulin resistance and hypertension, and impairment of insulin-mediated vasodilatation may contribute to both. The relationship between the vascular and metabolic actions of insulin is incompletely understood at this moment, but the available evidence from human and rat studies suggests that insulin-mediated vaselodilatation accounts for 25% to 40% of insulin-mediated muscle glucose uptake.7,36 According to Poiseuille’s law, the insulin-mediated vasocstriction of 6% to 12% (Figure 2) that we observed at physiological levels of insulin (34 to 272 μU/mL) would result in a 23% to 41% decline in local muscle blood flow in vivo.

In summary, we have shown that TNF-α inhibits vasodilator but not vasoconstrictor effects of insulin in skeletal muscle resistance arteries, resulting in insulin-mediated vasoconstriction. This interference is mediated by activation of JNK and impairment of insulin-mediated activation of Akt. This differential inhibition of vasodilator effects of insulin may lead to impairment of muscle blood flow and increased peripheral resistance in obesity-related insulin resistance and hypertension. Inhibition of JNK may provide a new target of therapy for microvascular dysfunction in these conditions.

References


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Supplemental Data

Physiological concentrations of insulin induce endothelin-dependent vasoconstriction of skeletal muscle resistance arteries in the presence of TNFα: Dependence on c-jun N-terminal kinase

**Supplementary figure I.** Time-dependent increase in phosphorylation of Akt at Ser 473 in response to insulin (272 microU/ml) in cremaster arterioles. Segments of equal length of one arteriole (~3 mm per segment) were isolated as described in Methods, followed by incubation with insulin (272 microU/ml) for 5-30 minutes. Arteriolar proteins were separated by SDS-PAGE and immunoblotted with anti-pAkt (Ser473) antibody (top panel). The membrane was then stripped and re-blotted with anti-Akt antibody (bottom panel). Protein of an unstimulated control segment was loaded in the left lane (0).

**Supplementary figure II.** TNFα (10 ng/ml) increases phosphorylation of JNK in human microvascular endothelial cells (MVEC). One representative example of three experiments is shown. MVEC isolated from the human foreskin (see Nieuw Amerongen GP et al., *Arterioscler Thromb Vasc Biol* 2003;23:211-217) were stimulated with TNFα (10 ng/ml) for various time periods, lysed and
phosphorylation of JNK was analyzed by Western blot (see Methods). Tubulin was used as a loading control.

Supplementary figure III. Uptake of L-JNKI in endothelium and smooth muscle of cremaster resistance arteries. Resistance arteries (n = 3) were exposed to FITC-labeled L-JNKI, fixed in 2% formaldehyde and fluorescence was examined with a fluorescence microscope. L-JNKI/FITC staining was observed in smooth muscle cells (yellow arrows) as well as endothelial cells (white arrows). One representative example is shown.