Rho-Kinase Inhibition Improves Vasodilator Responsiveness During

Hyperinsulinemia in the Metabolic Syndrome

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Running Head

Rho-kinase & vascular reactivity in the metabolic syndrome.

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Abstract

In patients with the metabolic syndrome (MetS), the facilitatory effect of insulin on forearm vasodilator responsiveness to different stimuli is impaired. Whether the RhoA/Rho kinase (ROCK) pathway is involved in this abnormality is unknown. We tested the hypotheses that, in MetS patients, ROCK inhibition with fasudil restores insulin-stimulated vasodilator reactivity and that oxidative stress plays a role in this mechanism. Endothelium-dependent and -independent forearm blood flow responses to acetylcholine (ACh) and sodium nitroprusside (SNP), respectively, were assessed in MetS patients (n=8) and healthy controls (n=5) before and after the addition of fasudil (200 μg/min) to an intra-arterial infusion of insulin (0.1 mU/kg/min). In MetS patients (n=5), fasudil was also infused without hyperinsulinemia. The possible involvement of oxidative stress in the effect of fasudil during hyperinsulinemia was investigated in MetS patients (n=5) by infusing vitamin C (25 mg/min). In MetS patients, compared to saline, fasudil enhanced endothelium-dependent and -independent vasodilator responses during insulin infusion (p<0.001 and p=0.008, respectively), but not in the absence of hyperinsulinemia (p=0.25 and p=0.13, respectively). By contrast, fasudil did not affect vasoreactivity to ACh and SNP during hyperinsulinemia in controls (p=0.11 and p=0.56, respectively). In MetS patients, fasudil added to insulin and vitamin C did not further enhance vasodilation to ACh and SNP (p= 0.15 and p=0.43, respectively). In the forearm circulation of patients with the MetS, ROCK inhibition by fasudil improves endothelium-dependent and -independent vasodilator responsiveness during hyperinsulinemia; increased oxidative stress seems to be involved in the pathophysiology of this phenomenon.
Keywords

Fasudil; endothelium-dependent dilation; endothelium-independent dilation; insulin; obesity.
Introduction

Insulin resistance is considered a key factor in the pathogenesis of the metabolic and cardiovascular abnormalities that define the metabolic syndrome (MetS) (13, 16). Although the mechanisms underlying the decreased insulin sensitivity present in this condition are not completely understood, an impairment of insulin-stimulated microvascular perfusion at the level of the skeletal muscle may contribute to its pathogenesis (9). Consistent with this hypothesis, we recently demonstrated that, in healthy humans, insulin enhances the vasodilator response to a variety of endothelium-dependent and independent stimuli, whereas this effect is blunted in patients with obesity-related MetS, at least in part due to increased oxidative stress (29). The pathways involved in the blunted insulin-induced enhancement of vasodilation present in the MetS, however, have been poorly characterized.

Rho-kinase (ROCK), the main effector of the small guanosine triphosphate-binding protein Rho A, is increasingly being recognized as a major player in the cardiovascular system (22). ROCK appears to mediate the vasoconstrictor effects of angiotensin II (Ang-II) (5) and endothelin 1 (ET-1) (6) and is involved in the regulation of the nitric oxide (NO) pathway (24), thus contributing to the maintenance of basal vascular tone (4) and possibly participating to the pathogenesis of human hypertension (23). The effects of ROCK on insulin signaling remain controversial. Thus, some evidence indicates that ROCK activation enhances insulin-stimulated glucose uptake in adipose tissue and skeletal muscle (12), while disruption of ROCK-1 causes insulin resistance in mice (20). Increased ROCK activation, however, impairs insulin signaling in obese rat models of insulin resistance (17) and has also been reported in insulin
resistant patients with the MetS, in whom it correlates with the number of components of
the MetS (21). Taken together, these findings suggest that ROCK exerts complex
regulatory actions, potentially representing a pivotal link in the interplay of vascular and
metabolic homeostasis.

Whether the Rho A/ROCK pathway participates in the impaired insulin-stimulated
enhancement of vasodilator responses in patients with the MetS is unknown. Therefore,
the primary aim of the current study was to assess the effects of ROCK inhibition with
fasudil on vasomotor reactivity to endothelium-dependent and independent stimuli
during hyperinsulinemia in patients with obesity-related MetS. Additionally, we aimed to
investigate the potential involvement of oxidative stress in this mechanism.

Materials and Methods

Study subjects.

Patients with obesity-related MetS, defined according to the National Cholesterol
Education Program’s Adult Treatment Panel III (13), and age-matched healthy controls
with no history or current evidence of hypertension, hyperlipidemia, diabetes,
cardiovascular disease, or any other systemic conditions were recruited for this study.
Exclusion criteria were a history or presence of coronary artery disease, peripheral
occlusive arterial disease, coagulopathy, vasculitis, or any other systemic conditions. In
patients with the MetS taking antihypertensive and/or lipid-lowering drugs, treatment
was discontinued for two weeks prior to the vascular function studies. Aspirin and
vitamin supplements were also stopped one week before participation in the study. The
local Institutional Review Boards in Rome, Italy, approved the study protocol and all
participants gave written informed consent.

Protocols

All studies were performed in the morning in a quiet room with a temperature of approximately 22°C. Each study consisted of infusions of drugs into the brachial artery and measurement of forearm blood flow (FBF) by means of strain-gauge venous occlusion plethysmography. All drugs used in this study were prepared by the local pharmaceutical service following specific procedures to ensure accurate bioavailability and sterility of the solutions. Participants were asked to refrain from smoking, drinking alcohol or beverages containing caffeine for at least 24 hours and to fast for at least 8 hours before the studies. While participants were supine, a 20-gauge Teflon catheter (Arrow Inc., Limeric, PA) was inserted into the brachial artery of the non-dominant arm (left in most cases) for drug infusion. Another 20-gauge catheter (Abbott Laboratories, Abbott Park, IL) was inserted into a deep antecubital vein of the same arm for blood sampling. The extended arm was positioned slightly above the level of the right atrium and a mercury-filled strain gauge was placed around the widest part of the forearm. The strain gauge was connected to a plethysmograph (model EC-6, D.E. Hokanson Inc., Bellevue, WA) calibrated to measure the percent change in volume and connected to a personal computer through an analog-to-digital converter. For each measurement, a cuff placed around the upper arm was inflated to 40 mm Hg with a rapid cuff inflator (model E-10, Hokanson Inc., Bellevue, WA) to occlude venous outflow from the extremity. A wrist cuff was inflated to suprasystolic pressures 1 minute before each measurement to exclude hand circulation. Flow measurements were recorded for
approximately 7 seconds every 15 seconds; 7 readings were obtained for each mean value. Blood pressure was recorded with the use of a standard mercury manometer. Throughout all studies, volumes infused were matched by administration of variable amounts of saline. In patients with the MetS, insulin plasma concentrations were determined by electrochemiluminescent immunoassay (Roche Diagnostics, Mannheim, Germany) in an effluent deep vein of the infused forearm at baseline and following intra-arterial infusion of insulin.

Protocol 1: Assessment of the effects of ROCK inhibition on vascular reactivity during hyperinsulinemia in patients with the MetS and in healthy controls.

To determine the effects of ROCK inhibition with fasudil on forearm vascular responses during hyperinsulinemia, 8 patients with the MetS and 5 healthy controls were enrolled in this protocol. After the forearm was instrumented, normal saline was given intra-arterially for 15 minutes, at which point an infusion of regular insulin (Humulin; Eli Lilly, Indianapolis, IN) at 0.1 mU/kg/min (1 mL/min infusion rate) was started in the same line. After 45 minutes, basal FBF was measured and dose-response curves to the endothelium-dependent vasodilator acetylcholine (ACh) and the exogenous NO donor sodium nitroprusside (SNP) were obtained, separated by a 20-minute rest period. The sequence of ACh and SNP infusion was randomized to avoid bias related to the order of these procedures. Acetylcholine chloride (Clinalfa, Läufelfingen, Switzerland) was infused at 7.5, 15, and 30 μg/min, whereas SNP (Malesci, Florence, Italy) was administered at 0.8, 1.6, and 3.2 μg/min. The infusion rates at the various doses were 0.25, 0.5, and 1 mL/min, respectively, for both ACh and SNP. Each dose was given for
5 minutes and FBF was measured during the last 2 minutes of infusion. At the end of the first 2 curves, a 20-minute rest period was allowed. Then, the baseline FBF was measured again, and an intra-arterial infusion of the ROCK inhibitor fasudil (Sigma-Aldrich, Milan, Italy) at 200 μg/min (1 mL/min infusion rate) was started. The dose of fasudil was selected based on the dose (300 μg/min) used in a previous study to prevent myocardial ischemia in patients with coronary microvascular spasm (25). That dose is known to result in intravascular concentrations of fasudil higher than the half-maximal inhibitory concentration (IC50) of the drug for Rho-kinase inhibition (10). Because of the lower blood flow in the forearm compared to the coronary circulation, we calculated that a dose of 200 μg/min would be sufficient to achieve similar fasudil concentrations in the forearm. After 45 minutes, FBF was reassessed and the dose-response curves to ACh and SNP were repeated according to the same protocol and in the same order as detailed above.

Protocol 2: Assessment of the effects of ROCK inhibition with fasudil on vascular reactivity in patients with the MetS.

To assess if, in patients with the MetS, ROCK inhibition with fasudil improves endothelium-dependent and endothelium-independent vascular reactivity in the absence of hyperinsulinemia, 5 additional patients with the MetS were enrolled in the following protocol. After the forearm was instrumented, normal saline (NS) was infused intra-arterially for 15 minutes and basal FBF was measured. Then, the same dose-response curves to ACh and SNP as detailed in protocol 1 were obtained. Subsequently, a 20-minute rest period was allowed and an intra-arterial infusion of fasudil at the same
dose as in protocol 1 was started. After 45 minutes, a basal FBF measurement was obtained; then the dose-response curves to ACh and SNP were repeated in the same fashion as before.

Protocol 3: Assessment of the effects of ROCK inhibition on vascular reactivity during hyperinsulinemia and vitamin C infusion in patients with the MetS.

To investigate whether the effect of ROCK inhibition on vascular reactivity during hyperinsulinemia in patients with the MetS is related to enhanced oxidative stress, further studies were performed employing the antioxidant vitamin C. For this purpose, 5 additional patients with the MetS were recruited. After the forearm was instrumented, the concomitant intra-arterial infusion of regular insulin, as described in protocol 1, and of vitamin C (Bracco, Milan, Italy) was started. Vitamin C was administered at a rate of 25 mg/min (1 mL/min infusion rate), a dose proven effective to prevent premature NO deactivation by superoxide anions (14). After 15 minutes, baseline FBF was measured and dose-response curves to ACh and SNP were obtained as detailed in protocol 1. Then, a 20-minute rest period was allowed and an intra-arterial infusion of fasudil, at the same dose as in protocol 1, was started. After 45 minutes, the same dose-response curves to ACh and SNP were repeated.

Statistical Analysis

Within group analyses were performed by paired t test, one-way ANOVA, and two-way ANOVA for repeated measures, as appropriate. Between-group comparisons were performed using unpaired t test and two-way ANOVA, as appropriate. All calculated
probability values are two-tailed, and a \( P \) value \(<0.05\) was considered statistically significant. All group data are reported as mean±SEM.

**Results**

A total of 18 patients with the MetS and 5 healthy controls participated in this investigation. Their baseline anthropometric and biochemical characteristics are reported in the table. None of the participants was engaged in a formal exercise program, and the levels of physical activity were comparable between the two groups. Mean arterial pressure and heart rate did not change significantly after infusion of any of the drugs used in the study, thus indicating that the drug effects were limited to the infused forearm and did not extend to the systemic circulation. In patients with the MetS participating in studies with hyperinsulinemia, forearm insulin plasma levels were 15±2 \( \mu \)U/ml at baseline and rose to 208±46 \( \mu \)U/ml following intra-arterial infusion of insulin.

*Effects of ROCK inhibition with fasudil on vascular reactivity during hyperinsulinemia in patients with the MetS and in healthy controls.*

In the MetS patients, the administration of escalating doses of ACh and SNP during insulin infusion resulted in a progressive increase in FBF from baseline (\( P<0.001 \) for both). When the ACh and SNP curves were repeated after fasudil was added on top of the insulin infusion, the vasodilator responses to these drugs were enhanced, so that the FBF increases were significantly higher during fasudil administration than during insulin infusion alone (Figure 1, top panels). In contrast, in the healthy controls, the addition of fasudil did not significantly affect ACh- and SNP-induced forearm
vasodilation when compared with the response observed during insulin infusion alone (Figure 1, bottom panels).

Effects of ROCK inhibition with fasudil on vascular reactivity in patients with the MetS.

In the MetS patients, during saline administration the infusion of escalating doses of ACh and SNP resulted in a progressive increase in FBF from baseline ($P<0.001$ for both). No significant changes in the vasodilator responses to ACh and SNP were observed during ROCK inhibition with fasudil compared with saline alone (Figure 2).

Effects of ROCK inhibition with fasudil on vascular reactivity during hyperinsulinemia and vitamin C infusion in patients with the MetS.

In the MetS patients, during the concomitant infusion of insulin and vitamin C, increasing doses of ACh and SNP resulted in a progressive rise in FBF from baseline ($P<0.001$ for both). In this group of patients, no significant changes in FBF responses to ACh and SNP were observed when fasudil infusion was added on top of that of insulin and vitamin C (Figure 3).

Discussion

The main novel finding of the present study is that ROCK inhibition by fasudil improves both endothelium-dependent and -independent NO-mediated vasodilator responsiveness during hyperinsulinemia in patients with the MetS. Of note, this enhancing effect of fasudil on forearm vascular reactivity is not observed in the absence of hyperinsulinemia. Furthermore, even in the presence of high circulating insulin levels,
fasudil does not modify vascular responses to both ACh and SNP in healthy controls, hence suggesting that ROCK's contribution to the pathogenesis of the abnormal vascular responses to insulin is specific to patients with MetS.

**Insulin resistance and ROCK activity**

In a recent investigation (29), we elucidated a novel mechanism by which insulin may affect vasodilator responses in the microcirculation, thus potentially increasing the delivery of metabolic substrates to the skeletal muscle and enhancing insulin sensitivity. Specifically, we showed that insulin's improvement of vascular reactivity is not limited to the hormone's effects on the endothelium but also involves actions on vascular smooth muscle cells (VSMCs) to induce generalized enhancement of the response to different vasodilator stimuli. The findings of the current study further expand our previous observations by demonstrating a specific involvement of RhoA/ROCK as downstream mediators of the impaired facilitatory effect of insulin on vasodilatory responses in patients with the MetS.

In recent years, several in-vivo studies have elucidated the role of ROCK in pivotal cellular functions at the level of the arterial wall, such as contraction, proliferation, and apoptosis, which may play a role in the development of cardiovascular disease (28). These findings have been accompanied by the evidence that the RhoA/ROCK pathway may contribute to the increased constrictor tone present in the forearm vasculature of patients with essential hypertension (23) and that ROCK activity measured in peripheral leukocytes is inversely associated with flow-mediated dilation and the number of cardiovascular risk factors (30). Also, increased activity of ROCK has been shown to participate in the pathogenesis of endothelial dysfunction in patients with coronary
artery disease (26) and heart failure (19). Interestingly, in the MetS patients participating in our study, fasudil did not improve the responsiveness to ACh in the absence of hyperinsulinemia, suggesting that ROCK activation does not play a primary role in the pathogenesis of the endothelial dysfunction commonly seen in this population (31). By contrast, the favorable action of fasudil on forearm vascular reactivity to both ACh and SNP seen only in the presence of hyperinsulinemia suggests that this hormone is responsible for the activation of the Rho A/ROCK pathway in the VSMCs of patients with the MetS. Previous studies have shown that, under physiological conditions, insulin enhances VSMCs vasodilator capacity by inactivating the small GTPase RhoA and its target ROCK, thereby leading to decreased phosphorylation of myosin light chain and subsequent vasodilation (2). Of note, this insulin-induced inhibition of ROCK in VSMCs involves the PI3 kinase (PI3K) pathway (2), whose activity is known to be impaired in insulin resistant states (18). Thus, loss of the latter mechanism, with elevation in ROCK and compromised vasodilator capacity due to decreased myosin bound phosphatase activity, has previously been reported in VSMCs isolated from insulin resistant animal models with either type 2 diabetes (27) or hypertension (3). It may be postulated, therefore, that down-regulation of PI3K signaling in the vasculature of our insulin resistant patients is associated with enhancement of vasoconstrictor, proatherogenic pathways, thereby leading to increased activity of ROCK. This view is indirectly supported by previous results showing that, in hyperinsulinemic Zucker rats, Rho-kinase inhibition with fasudil improves IRS-1-dependent insulin signaling in skeletal muscle cells and concurrently restores the vasodilator responsiveness to both ACh and SNP (17). Besides its vascular actions, ROCK appears to play a pivotal role also in the
regulation of glucose homeostasis. In a mouse knock out model, ROCK1 deficiency impaired insulin sensitivity by decreasing insulin-stimulated PI3K activity and glucose transport in the skeletal muscle, suggesting that activation of ROCK1 is essential for the physiologic actions of insulin on glucose transport in the skeletal muscle in vivo (20). Additionally, in keeping with these findings, studies in cultured adipocytes and isolated soleus muscle have shown that ROCK activation promotes PI3K activity and enhances insulin-stimulated glucose transport (12). Also, insulin activation of the ROCK1 isoform and glucose disposal are impaired in the skeletal muscle of patients with type 2 diabetes (8). In aggregate, the above vascular and metabolic actions support the hypothesis that the RhoA/ROCK pathway represents an important link between glucose metabolism and cardiovascular disease. However, the apparently discrepant effects on insulin signaling suggest that ROCK may exert tissue-specific actions. Accordingly, in the vascular system, especially in VSMCs, ROCK2 is the predominant isoform (28) and its activation seems to result in untoward metabolic and vascular actions.

Role of oxidative stress

In recent years, a wealth of data has indicated that high levels of reactive oxygen species (ROS) in the arterial wall are involved in the pathophysiology of vascular dysfunction associated with insulin resistance (11). We tested the hypothesis that enhanced oxidative stress might be involved in the activation of ROCK in patients with the MetS by use of an antioxidant. In our patients, when fasudil was given on top of vitamin C, no further enhancement of the responsiveness to ACh and SNP during hyperinsulinemia was obtained. This finding suggests that oxidative stress is indeed involved in the effect of fasudil to improve vasodilator responses during
hyperinsulinemia in the MetS. One possible explanation for our results is that enhanced oxidative stress triggers the activation of ROCK (15) in these patients. But again, if that would be the case, augmented ROCK activity should have been observed even in the absence of hyperinsulinemia. A more likely explanation, therefore, seems to rely on increased ROS production within the vessel wall leading to disruption of the physiological insulin signaling and activation of ROCK. The merit of this hypothesis stems from previous evidence that ROS negatively regulate the PI3K mediated insulin pathway in VSMCs by inhibiting phosphorylation of IRS-1 and the downstream signaling steps (11).

Limitations and perspectives

Given the complexity and invasive nature of our vascular studies, this investigation included small numbers of patients and controls. Therefore, the power of the study may have not been sufficient to detect statistically significant differences in our groups. As we explored the vascular responses of the intact forearm circulation in vivo more direct insights into the precise molecular mechanisms leading to insulin-enhanced ROCK activation and the ensuing impaired vasomotor reactivity in patients with obesity-related MetS may not be possible. Also, a more definitive conclusion that enhanced oxidative stress might be involved in the activation of ROCK in patients with the MetS would require evidence that oxidative stress is indeed suppressed during fasudil infusion. However, such studies examining whether vitamin C further enhances insulin's facilitatory effect in MetS patients in the presence of fasudil infusion would be beyond the main scope of the investigation and could not be easily undertaken. Finally, the interpretation of the responses to vitamin C may be affected by the previous evidence...
that intra-arterial vitamin C infusion improves endothelial function (1) whereas oral vitamin C intake does not (7). This difference can be explained by the much lower plasma levels of vitamin C achieved orally compared with intra-arterial infusion. Irrespective of the underlying mechanism, however, our findings clearly suggest an involvement of the RhoA/ROCK pathway in the pathophysiology of abnormal vascular responsiveness to insulin in these patients. Studies have clearly demonstrated that insulin-mediated vasodilation is important not only for glucose disposal, but also for its own delivery to skeletal muscle (9). Our results, therefore, may also have potential clinical implications, suggesting that ROCK may represent an important promising therapeutic target for cardiovascular disease prevention in the MetS.
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All authors have no conflicts of interest to disclose.

F.S. recruited patients and collected data, M.T. collected data and reviewed the manuscript; V.R. collected data; N.D.D. contributed to the discussion and reviewed the manuscript; P.G. recruited the patients and contributed to the discussion; N.M. prepared the solutions and reviewed the manuscript; U.C. wrote the manuscript; C.C. wrote the protocols, edited and reviewed the manuscript.
References


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Figure Captions

Figure 1: Plots showing forearm blood flow responses to intra-arterial infusion of escalating doses of acetylcholine (left panels) and sodium nitroprusside (right panels) during the concomitant infusion of insulin (open circles) or insulin and fasudil (black circles) in the MetS patients (top panels) and in healthy controls (bottom panels). The \( P \) values refer to the comparisons of vascular responses to acetylcholine and sodium nitroprusside under different conditions and in different study groups by two-way analysis of variance for repeated measures. All values are reported as means \( \pm \) SEM.

Figure 2: Plots showing forearm blood flow responses to intra-arterial infusion of escalating doses of acetylcholine (left panel) and sodium nitroprusside (right panel) during the concomitant infusion of saline (open circles) or fasudil (black circles) in the MetS patients. The \( P \) values refer to the comparisons of vascular responses to acetylcholine and sodium nitroprusside under different conditions by two-way analysis of variance for repeated measures. All values are reported as means \( \pm \) SEM.

Figure 3: Plots showing forearm blood flow responses to intra-arterial infusion of escalating doses of acetylcholine (left panel) and sodium nitroprusside (right panel) during the concomitant infusion of insulin and vitamin C (open circles) or insulin and vitamin C and fasudil (black circles) in the MetS patients. The \( P \) values refer to the comparisons of vascular responses to acetylcholine and sodium nitroprusside under different conditions by two-way analysis of variance for repeated measures. All values are reported as means \( \pm \) SEM.
Table. Clinical Characteristics of the Study Population

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<th>Variable</th>
<th>Control Subjects (n=5)</th>
<th>Study 1 (n=8)</th>
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Data are expressed as mean ± SEM. BMI: body mass index; MAP: mean arterial pressure; HDL: high-density lipoprotein. *P<0.05 vs controls. There was no significant difference in any of the variables among the 3 MetS groups.
Metabolic Syndrome

Acetylcholine ($\mu$g/min)

Baseline 7.5 15 30

Forearm Blood Flow (ml/min/dl)

Saline Fasudil

Baseline 0.8 1.6 3.2

P<0.001 P=0.008

Sodium Nitroprusside ($\mu$g/min)

Control Subjects

Acetylcholine ($\mu$g/min)

Baseline 7.5 15 30

Forearm Blood Flow (ml/min/dl)

Saline Fasudil

Baseline 0.8 1.6 3.2

P=0.11 P=0.56

Sodium Nitroprusside ($\mu$g/min)
**Figure 2**

- **Acetylcholine (µg/min)**
  - Baseline: 7.5, 15, 30
  - Forearm Blood Flow (ml/min/dl)
    - Baseline: 0, 4, 8, 12, 16
    - 

- **Sodium Nitroprusside (µg/min)**
  - Baseline: 0.8, 1.6, 3.2
  - Forearm Blood Flow (ml/min/dl)
    - Baseline: 0, 3, 6, 9, 12, 15

- **Saline**
- **Fasudil**

**P = 0.25**

**P = 0.13**
Figure 3

Forearm Blood Flow (ml/min/dl)

- Acetylcholine (µg/min)
  - Vitamin C alone
  - Vitamin C + Fasudil

- Sodium Nitroprusside (µg/min)
  - Vitamin C alone
  - Vitamin C + Fasudil

P=0.15
P=0.43