Rho-kinase inhibition improves vasodilator responsiveness during hyperinsulinemia in the metabolic syndrome

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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 2 wk prior to the vascular function studies. Aspirin and a variety of endothelium-dependent and -independent stimuli, whereas this effect is blunted in patients with obesity-related MetS at least in part because of increased oxidative stress (29). However, the pathways involved in the blunted insulin-induced enhancement of vasodilation present in the MetS have been poorly characterized.

Rho-kinase (ROCK), the main effector of the small guanosine triphosphate-binding protein RhoA, is increasingly being recognized as a major player in the cardiovascular system (22). ROCK appears to mediate the vasoconstrictor effects of angiotensin II (5) and endothelin 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 in 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), whereas disruption of ROCK1 causes insulin resistance in mice (20). However, increased ROCK activation 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 RhoA/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 present 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.

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 demonstrated recently that, in healthy humans, insulin enhances the vasodilator response to
vitamin supplements were also stopped 1 wk 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 ~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 drinking beverages containing caffeine for ≥24 h and to fast for ≥8 h before the studies. While participants were supine, a 20-gauge Teflon catheter (Arrow, Limeric, PA) was inserted into the brachial artery of the nondominant arm (left arm 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, Bellevue, WA) that was 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 mmHg, with a rapid cuff inflator (model E-10; Hokanson, Bellevue, WA) to occlude venous outflow from the extremity. A wrist cuff was inflated to suprasystolic pressures 1 min before each measurement to exclude hand circulation. Flow measurements were recorded for ~7 s every 15 s; seven 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, eight patients with the MetS and five healthy controls were enrolled in this protocol. After the forearm was instrumented, normal saline was infused intra-arterially for 15 min, and basal FBF was measured. Then, a 20-min rest period was allowed, and an intra-arterial infusion of fasudil at the same dose as in protocol 1 was started. After 45 min, a basal FBF measurement was obtained; then the dose-response curves to ACh and SNP were repeated in the same fashion as before.

Protocol 2: assessment of the effects of ROCK inhibition with fasudil on vascular reactivity in patients with the MetS. To assess whether, in patients with the MetS, ROCK inhibition with fasudil improves endothelium-dependent and endothelium-independent vascular reactivity in the absence of hyperinsulinemia, five additional patients with the MetS were enrolled in the following protocol. After the forearm was instrumented, normal saline was infused intra-arterially for 15 min, 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-min rest period was allowed, and an intra-arterial infusion of fasudil at the same dose as in protocol 1 was started. After 45 min, a basal FBF measurement was obtained; then the dose-response curves to ACh and SNP were repeated in the same order as detailed above.

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 employing the antioxidant vitamin C were performed. For this purpose, five 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 min, baseline FBF was measured, and dose-response curves to ACh and SNP were obtained as detailed in protocol 1. Then, a 20-min rest period was allowed, and an intra-arterial infusion of fasudil, at the same dose as in protocol 1, was started. After 45 min, 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 measurements, 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 means ± SE.

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 1. 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 μU/ml at baseline and rose to 208 ± 46 μU/ml following intra-arterial infusion of insulin.

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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 FFB increases were significantly higher during fasudil administration than during insulin infusion alone (Fig. 1, top). 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 (Fig. 1, bottom).

Table 1. Clinical characteristics of the study population

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control Subjects (n = 5)</th>
<th>Metabolic Syndrome Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Study 1 (n = 8)</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>3/2</td>
<td>5/3</td>
</tr>
<tr>
<td>Age, yr</td>
<td>41 ± 3</td>
<td>44 ± 3</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>25 ± 2</td>
<td>38 ± 3*</td>
</tr>
<tr>
<td>Smoking (yes/no)</td>
<td>1/4</td>
<td>2/6</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>94 ± 1</td>
<td>104 ± 2*</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>167 ± 13</td>
<td>221 ± 16*</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dl</td>
<td>56 ± 4</td>
<td>42 ± 4*</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>68 ± 11</td>
<td>194 ± 17*</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>87 ± 4</td>
<td>108 ± 4*</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. BMI, body mass index; MAP, mean arterial pressure. *P < 0.05 vs. controls. There was no significant difference in any of the variables among the 3 metabolic syndrome groups.

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 (Fig. 1, top). 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 (Fig. 1, bottom).

Fig. 1. Plots showing forearm blood flow responses to intra-arterial infusion of escalating doses of acetylcholine (left) and sodium nitroprusside (right) during the concomitant infusion of insulin (○) or insulin and fasudil (●) in the metabolic syndrome patients (top) and in healthy controls (bottom). The P values refer to the comparisons of vascular responses to acetylcholine and sodium nitroprusside under different conditions and in different study groups by 2-way analysis of variance for repeated measurements. All values are reported as means ± SE.
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 (Fig. 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 (Fig. 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 either ACh or 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 facili-
utationary 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 RhoA/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 phosphatidylinositol 3-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 been reported previously in VSMCs isolated from insulin-resistant animal models with either type 2 diabetes (27) or hypertension (3). Therefore, it may be postulated that downregulation 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, ROCK inhibition with fasudil improves insulin receptor substrate-1 (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 also appears to play a pivotal role in the regulation of glucose homeostasis. In a mouse knockout 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 physiological 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 were the case, augmented ROCK activity should have been observed even in the absence of hyperinsulinemia. Therefore, a more likely explanation 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. Because 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). Therefore, our results 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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**DISCLOSURES**

None of the authors have any conflicts of interest to disclose, financial or otherwise.

**AUTHOR CONTRIBUTIONS**

F.S., M.T., V.R., and N.M. performed the experiments; F.S., M.T., V.R., N.D.D., P.G., U.C., and C.C. approved the final version of the manuscript; M.T., N.D.D., P.G., N.M., U.C., and C.C. edited and revised the manuscript; N.D.D. and C.C. interpreted the results of the experiments; P.G. and C.C. did the conception and design of the research; U.C. drafted the manuscript; C.C. edited and revised the manuscript; P.G. and C.C. did the analysis of the data.

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