Enhanced Endothelin Activity Prevents Vasodilation to Insulin in Insulin Resistance

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Abstract—Although insulin-mediated vasodilation is impaired in insulin resistance, the mechanisms of this are unknown. We investigated factors mediating vasoactive responses to insulin in control and insulin-resistant rats. Responses to insulin in small mesenteric arteries from control and insulin-resistant rats were investigated after blocking endothelin-A receptors, cyclooxygenase, nitric oxide synthase, and potassium channels. In addition, insulin’s effect on prostacyclin production in small mesenteric blood vessels was assessed by enzyme immunoassay. Insulin induced a concentration-dependent vasodilation in control arteries that was absent in arteries from insulin-resistant rats. However, in the presence of BQ610, an endothelin-A receptor antagonist, the response to insulin was normalized in insulin-resistant arteries. In control arteries, insulin-induced vasodilation was completely inhibited by indomethacin, meclofenamate, glibenclamide, or potassium chloride. In contrast, neither n-nitro-L-arginine nor the combination of charybdotoxin and apamin altered vasodilation to insulin. In insulin-resistant arteries the presence of BQ610, vasodilation was also inhibited by indomethacin, glibenclamide, and potassium chloride. Insulin increased prostacyclin production in small mesenteric blood vessels from both groups of rats to a similar degree. Insulin-induced vasodilation in small rat mesenteric arteries is mediated through prostacyclin- and ATP-dependent potassium channels. However, insulin-resistant arteries do not vasodilate to insulin unless endothelin-A receptors are blocked. Thus, impaired relaxation to insulin in insulin-resistant rats is due to enhanced vasoconstriction by endothelin, which offsets a normal vasodilatory response to insulin.

Key Words: insulin resistance • endothelin • prostacyclin • potassium channels • insulin

Insulin resistance is associated with vascular dysfunction, hypertension, and increased susceptibility to ischemic events.1–3 In insulin-resistant (IR) animals, insulin levels are increased by several-fold and it seems that hyperinsulinemia alone may alter vascular responsiveness to physiological stimuli, including insulin itself.4–8 For example, in isolated mesenteric arteries from Zucker obese rats, a well-described model of insulin resistance, normal dilator effects of acute insulin treatment are absent. The underlying basis for this impaired responsiveness is not understood. It is possible that IR endothelial cells become resistant to insulin’s vasodilatory effects. Indeed, a selective resistance to insulin signaling has been shown in the vasculature of Zucker obese rats.8 Conversely, insulin resistance and hyperinsulinemia up-regulate vascular smooth muscle endothelin (ET) receptors and have been shown in some, but not all studies, to increase ET-1 serum concentrations.9–12 Thus, altered vascular responsiveness may be due to reduced vascular sensitivity to insulin or to an increased role of counteracting constrictor stimuli.

Thus, the purposes of the current study were (1) to evaluate insulin-induced vasodilation in small mesenteric arteries from control and IR rats, (2) to determine if antagonism of the ET-1A receptor alters the magnitude of insulin-mediated vasodilation, and (3) to determine the mechanism by which insulin induces vasodilation in this arterial bed.

Methods

The Animal Care Committee at Wake Forest University School of Medicine approved all procedures. Male Sprague-Dawley rats were obtained at 6 weeks of age and randomized into one of two groups: (1) IR (n=40) and (2) control (n=46). Animals in the IR group were fed a fructose-rich diet for 4 weeks (Teklad Labs), whereas control animals received standard rat chow. The fructose-fed model is a widely accepted model of insulin resistance.13–17 Rats were anesthetized with pentobarbital (50 mg/kg IP) and anticoagulated with heparin (500 U IP), and a section of the small intestine was removed and placed in a chilled oxygenated Krebs-Ringers bicarbonate solution (containing, in mmol/L: NaCl 118.3, KCl 4.7, CaCl2 2.5, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25, and dextrose 11.1). Fourth-order branches of the superior mesenteric artery were isolated and removed for functional studies. The intraluminal diameter of pressurized (60 mm Hg) arteries was measured as previously described.5,16,17

Vascular Reactivity Experiments

After a 30-minute equilibration period, arteries were preconstricted to ~40% of their resting diameter with phenylephrine (PE). Cumulative concentration-response experiments were performed with insulin (0.1 to 100 ng/mL). The concentrations used were based on

Received January 30, 2002; first decision February 19, 2002; revision accepted May 9, 2002.
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Hypertension is available at http://www.hypertensionaha.org

DOI: 10.1161/01.HYP.0000022806.87281.62

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data showing a concentration-dependent vasodilation in canine coronary microvessels with this regimen.\textsuperscript{18} It should be noted that this concentration ranges from \textasciitilde 17 pmol/L to 17 000 pmol/L and that the maximum concentration used exceeds that found in the IR rats by \textasciitilde 50-fold. On the basis of initial findings in arteries from IR rats and previous findings regarding the role ET-1 in the presence of hyperinsulinemia,\textsuperscript{9–11} we repeated these initial studies in the presence of the ETA receptor antagonist BQ610 (100 nmol/mL).

Additional studies were performed to determine the mechanism of insulin-mediated vasodilation in control and IR arteries. All mechanistic studies with IR arteries were performed in the presence of BQ610. Microvessels were incubated for 20 minutes in the presence of one of several enzyme inhibitors before PE constriction. To assess the role of nitric oxide, N-nitro-l-arginine (LNNA; 100 \( \mu \)mol/L) was used, but the role of cyclooxygenase (COX) was assessed using indomethacin (10 \( \mu \)mol/L) or meclofenamate (10 \( \mu \)mol/L). To assess whether \( K^+ \) channels contribute to insulin-induced vasodilation, KCl (50 \( \mu \)mol/L) rather than PE was used to constrict arteries. Furthermore, to assess the role of calcium-dependent potassium channels (\( K_{Ca} \)) or ATP-dependent potassium channels (\( K_{ATP} \)) in insulin-induced vasodilation, we pretreated arteries with either charybdotoxin (CTX; 0.1 \( \mu \)mol/L) plus apamin (0.5 \( \mu \)mol/L) or glibenclamide (10 \( \mu \)mol/L), respectively, before PE constriction. The combination of CTX and apamin was used because it has been shown to inhibit large, intermediate, and small conductance \( K_{Ca} \) channels.\textsuperscript{19,20} In a separate set of arteries, the endothelium was denuded by air perfusion. Endothelial disruption was verified as previously described.\textsuperscript{21}

**Determination of 6-Keto-Prostaglandin \( \text{F}_1\alpha \) Concentration**

In separate experiments, a section of mesentery was removed from both control (n=6) and IR rats (n=6). Third- and fourth-order blood vessels were isolated and placed in 10 mL of Krebs solution, which was equilibrated with a 21% O\textsubscript{2}/5% CO\textsubscript{2}/balance N\textsubscript{2} gas mixture and maintained at 37°C. After 30 minutes, a 1 mL sample of bathing solution was withdrawn and frozen for baseline 6-keto-prostaglandin \( \text{C}_{1} \). After 30 minutes, a 1 mL sample of bathing solution was withdrawn and frozen for baseline 6-keto-prostaglandin \( \text{F}_{1\alpha} \) (6-Keto-PGF\textsubscript{1\alpha}) concentrations. 6-Keto-PGF\textsubscript{1\alpha} is the stable hydrolys product of prostacyclin. Insulin (100 ng/mL) was added to the bath for 20 minutes, and another milliliter of bathing solution was withdrawn and frozen for assessment of 6-Keto-PGF\textsubscript{1\alpha} concentrations after insulin. In a second set of experiments, BQ610 (100 nmol/mL) was added to the bathing solution 20 minutes before insulin. The 6-Keto-PGF\textsubscript{1\alpha} concentrations were determined using a competitive binding immunoassay kit (Assay Designs). The resulting values of 6-Keto-PGF\textsubscript{1\alpha} concentrations in the supernatant were normalized for tissue weight (pg/mg tissue).

**Biochemical Measurements**

Plasma insulin was assayed using an ELISA kit (Crystal Chem, Inc) with rat antibody. Glucose concentrations were measured using a Glucose Trinder Kit (Sigma).

**Chemicals**

Insulin was obtained from Eli Lilly, and indomethacin was a generous gift from Merck (Rahway, NJ). All other chemicals were obtained from Sigma. Agents were dissolved as previously described.\textsuperscript{16,17}

**Data Analysis**

Statistical comparisons for concentration-response experiments were performed using ANOVA with repeated measures followed by a Fisher’s pair-wise, least-significant-difference test for multiple comparisons. Differences in 6-Keto-PGF\textsubscript{1\alpha} within groups were assessed with a Student’s paired \( t \) test, whereas differences between groups were assessed with an unpaired \( t \) test. Likewise, differences in biochemical measurements were assessed with an unpaired \( t \) test. Data are reported as mean±SEM. The criteria for significance was \( P<0.05 \).

**Figure 1.** Cumulative concentration-response experiments to insulin in mesenteric arteries from control and IR rats in the presence or absence the ETA antagonist BQ610. \( P<0.05 \) compared with the response in control arteries. &\( P<0.05 \) compared with the response in IR arteries.

**Results**

**Vascular Reactivity Experiments**

The resting intraluminal diameter of small mesenteric arteries did not differ between groups (215±4 \( \mu \)m for control and 211±5 \( \mu \)m for IR arteries). Moreover, percent arterial constriction after PE or KCl was similar between groups (41±2% for control and 39±2% for the IR group). Neither endothelial denudation nor any of the pharmacological inhibitors significantly altered the resting diameter in either group compared with the arteries without intervention. In arteries that were denuded of endothelium or pretreated with pharmacological inhibitors, preconstriction was induced to a degree similar to control arteries by altering the concentration of PE. For all of these experiments, approximately half of the PE concentration was required compared with those arteries without intervention. For example, arteries pretreated with BQ610 required 1.04±0.08 \( \mu \)mol/L PE to induce preconstriction, whereas arteries with no pretreatment required 2.2±0.04 \( \mu \)mol/L. The concentrations of PE required did not differ between control and IR arteries for any experiments. In arteries from control rats, insulin induced a concentration-dependent vasodilation with a maximal relaxation of 48±9% (Figure 1). In contrast, insulin-induced relaxation was absent in arteries from IR rats (Figure 1). BQ610 pretreatment of arteries from control rats did not alter insulin-induced relaxation (Figure 1); however, blockade of the ETA receptor restored insulin-induced relaxation in IR arteries (maximal relaxation, 63±9% in the presence of BQ610 versus 3±7% in IR arteries without BQ610; \( P<0.01 \); Figure 1). It should be noted that in time control experiments, BQ610 did not alter diameter in either control or IR arteries (data not shown).

**Mechanisms of Vasodilation**

Endothelium denudation of control arteries completely abolished relaxation to insulin, whereas LNNA had no effect on vasodilation (Figure 2). Pretreatment of control arteries with
indomethacin not only abolished relaxation to insulin, but induced significant vasoconstriction (Figure 2). To confirm our results with indomethacin, we repeated the experiments in the presence of meclofenamate. Indeed, insulin also induced vasoconstriction in the presence of this COX inhibitor (maximum effect, \( \frac{15}{100} \% \); \( P < 0.01 \) versus control; data not shown). Furthermore, vasodilation to insulin was abolished both after depolarization with KCl (maximal effect, \(-12 \pm 5\%\); \( P < 0.05 \) versus control; data not shown) and in the presence of glibenclamide (Figure 2). In contrast, pretreatment with the combination of CTX plus apamin had no effect on insulin-induced vasodilation (Figure 2).

In IR arteries with ET\(_{A}\) blockade, LNNA pretreatment did not affect insulin-induced vasodilation (maximal relaxation, 51 ± 10%; \( P = \text{NS} \) versus IR arteries with BQ610; data not shown). However, consistent with the control arteries, indomethacin pretreatment abolished vasodilation to insulin in BQ610-treated IR arteries (Figure 3). Likewise, depolarization with KCl and glibenclamide pretreatment also completely abolished insulin-induced relaxation (Figure 3).

**Determination of 6-Keto-PGF\(_{1\alpha}\) Concentration**

Figure 4 summarizes 6-Keto-PGF\(_{1\alpha}\) production in small mesenteric blood vessels from control and IR rats. Under basal conditions, vessels from both groups produced 6-Keto-PGF\(_{1\alpha}\) to a similar degree. After incubation with insulin (100 ng/mL), the 6-Keto-PGF\(_{1\alpha}\) concentration increased to a similar extent in both groups. The addition of BQ610 (20 minutes before insulin) to the bathing solution did not alter insulin-stimulated 6-Keto-PGF\(_{1\alpha}\) production (data not shown).

**Biochemical Measurements**

Mean body weight (316 ± 8 g for control and 320 ± 6 g for IR rats) and fasting glucose (142 ± 10 mg/dL for control and 153 ± 9 mg/dL for IR rats) were similar among control and IR rats. In contrast, fasting plasma insulin (92 ± 27 pmol/L for control and 229 ± 37 pmol/L for IR rats; \( P < 0.05 \)) was significantly elevated in IR rats compared with control.

**Discussion**

There are 2 new major findings from this study. First, insulin dilates small mesenteric arteries from normal rats. The mediator of this endothelium-dependent vasodilation seems to be prostacyclin. Second, insulin fails to dilate small mesenteric arteries from IR rats. The primary reason for this abnormal responsiveness is the enhanced role of ET in the control of this vascular bed during insulin resistance. Thus, our study demonstrates that an imbalance of opposing endothelium-based vasoactive influences participate in deranged vascular function in insulin resistance.

On the basis of data showing that COX inhibitors, KCl, or a K\(_{ATP}\) channel antagonist completely abolish insulin-induced vasodilation, the present study provides evidence that insulin induces an endothelium-dependent vasodilation in small mesenteric arteries through the production of prostacyclin and subsequent activation of K\(_{ATP}\) channels. Moreover, we have shown that insulin increases prostacyclin production to a similar extent in both control and IR arteries. These biochemical results are supportive of our pharmacological results.

This mechanism of vasodilation is present in both control and IR arteries; however, it seems that enhanced ET activity counters this process in IR arteries.

Several other laboratories have shown that insulin induces an endothelium-dependent vasodilation in normal arteries;\(^{18,22,23}\) however, this study is the first to demonstrate that...
the mechanism is primarily COX dependent. The majority of previous studies have demonstrated that insulin induces vasodilation through its effects on nitric oxide production.\textsuperscript{24–26} One reason for the difference is that most of the studies demonstrating this mechanism of vasodilation were performed in large conduit arteries such as the aorta, superior mesenteric, and epicardial coronary arteries. Before the current study, only 2 groups had published studies using resistance arteries.\textsuperscript{18,23} Ottman and colleagues\textsuperscript{18} demonstrated that insulin induced an endothelin-dependent vasodilation in small (\(\approx 112 \mu m\)) dog coronary arteries that could be inhibited by KCl or tetrabutyrammonium chloride but not by LNNA, indomethacin, tetroethylammonium chloride, glibenclamide, 4-aminopyridine, or CTX plus apamin. Conversely, Chen and Messina\textsuperscript{23} showed that insulin-induced vasodilation in isolated rat skeletal muscle arteries (\(\approx 80 \mu m\)) could be completely inhibited by the nitric oxide synthase inhibitor LNNA. The differences in these findings from our study and from each other could be either species- or vascular bed-dependent because similar concentrations of inhibitors and of insulin were used in all 3 experiments.

In contrast to experiments with control arteries, arteries from IR rats did not dilate to insulin. Similar findings have been published in another model of insulin resistance, in which insulin had no vasoactive effect in otherwise untreated mesenteric arteries from Zucker obese rats but induced vasodilation in the lean control.\textsuperscript{6} In addition, it has been shown that insulin’s ability to attenuate vasoconstriction by norepinephrine and angiotensin II is attenuated in arteries from both the fructose-fed and the Zucker obese rat, again illustrating impairment of insulin’s vasodilatory effects in models of insulin resistance.\textsuperscript{6,27,28} It should be noted that this defect in insulin-mediated dilation, although likely related directly to insulin resistance, could also be due to other metabolic derangements that are present in this syndrome including, but not limited to, elevated triglycerides and free fatty acids.

On the basis of the current study, the mechanism for this derangement in IR arteries is due to enhanced activity of the $\left(E_{T\alpha}\right)$ receptor: vasodilation to insulin was restored to normal levels after pretreatment with BQ610. It is unclear whether this is due to an increase in ET-1 tissue concentration or whether this may prove to be an important target for drug therapy with the ET receptor antagonists.

**Perspectives**

In the present study, we have shown that insulin-mediated endothelium-dependent vasodilation of small mesenteric arteries from IR rats is absent. This defect is due to enhanced ET activity in the presence of very high concentrations of insulin, which in turn overrides insulin’s vasodilatory effect. These data have important implications in the setting of insulin resistance and hyperinsulinemia such that these 2 metabolic abnormalities together may create an environment where the balanced is tipped in favor of vasoconstriction and vascular proliferation due to an overzealous ET system. If this concept holds true, this metabolically deranged environment may prove to be an important target for drug therapy with the ET receptor antagonists.

**Acknowledgments**

This work was supported by the American Heart Association (0140212N to A.W.M., 0270114N to D.W.B.) and the National Institutes of Health (HL66074 to A.W.M. and HL30260, HL46558, and HL50587 to D.W.B.).

**References**


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*Hypertension*. 2002;40:78-82; originally published online June 3, 2002;
doi: 10.1161/01.HYP.0000022806.87281.62

*Hypertension* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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