Enhanced endothelin-1 response and receptor expression in small mesenteric arteries of insulin-resistant rats

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One important aspect of determining whether ET-1 is involved in the development of insulin-resistance-induced hypertension and vascular dysfunction is to know whether the vascular response to ET-1 is altered in arteries from insulin-resistant (IR) animals. Three studies to assess the vascular response to ET-1 in arteries from fructose-induced IR rats have been performed; however, these studies provide conflicting results (7, 11, 17). Thus the question of whether the vascular response to ET-1 is augmented in the setting of insulin resistance and hyperinsulinemia has not been adequately answered. In addition, the response to ET-1 has not been assessed in isolated small mesenteric arteries, a more appropriate venue to determine the response to ET-1 because these arteries have a greater involvement in dictating peripheral vascular resistance.

This study determined 1) the ET-1 response in small mesenteric arteries from IR and control rats in the presence and absence of endothelium, 2) the ET-1 response in small mesenteric arteries from IR and control rats in the presence of ET_A- or ET_B-receptor antagonists, and 3) the ET-1 receptor binding characteristics in small mesenteric arteries from IR and control rats.

METHODS

The Animal Care Committees at the Medical College of Georgia and the Augusta Veterans Affairs Medical Center approved the current protocol. Male Sprague-Dawley rats were obtained at age 6 wk and randomized into one of two groups: IR (n = 32) or control (n = 32). IR rats were fed a fructose-rich diet (containing as percentage of total calories: 66% fructose, 22% casein, and 12% lard, plus essential vitamins and minerals) (Teklad Labs, Madison, WI) and control animals received standard rat chow. Fructose-fed rats develop insulin resistance and hyperinsulinemia within 7 days of diet therapy, endothelial dysfunction within 14 days, and borderline hypertension within 20–28 days (8, 9). Each group of animals was continued on its respective diet for a period of 4 wk so that endothelial dysfunction was consistently established (8, 9).

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Measurement of blood pressure. After 4 wk of diet treatment, rats were sedated with pentobarbital sodium (30 mg/kg ip). With the use of aseptic technique, an arterial cannula [polyethylene (PE)-10 coupled to PE-50 tubing] was placed into the femoral artery for measurement of aortic pressure. The external portion of the cannula was tunneled under the skin and sutured to the back of the neck. Animals were allowed 24 h to recover from this procedure. After the recovery period, the cannula was aligned to a fluid-filled transducer (CPXL-23, Statham, Costa Mesa, CA), and the signal was conditioned, amplified, and digitized for measurement of blood pressure in awake and unrestrained animals. Arterial blood pressure measurements were taken every 20 s for a period of 30 min. These data were averaged for each animal to determine the mean resting arterial blood pressure (MAP).

Isolation of small mesenteric arteries. After MAP measurement, fasting rats were given pentobarbital sodium (50 mg/kg ip) and heparin (500 U ip). A blood sample was taken for biochemical measurements, and a section of small intestine was removed and placed in chilled oxygenated buffer for biochemical measurements, and a section of small intestine was removed and placed in chilled oxygenated buffer (concentration in mM: 118.3 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 25 NaHCO3, and 11.1 dextrose). Third-order branches of the superior mesenteric artery (~230 μM intraluminal diameter) were isolated and removed for vascular reactivity and ET-1 receptor binding experiments. Arteries for binding experiments were frozen in liquid nitrogen and stored at −80°C.

Determination of vascular reactivity in vitro. Sections of small arteries (length 1–2 mm) were transferred to a vessel chamber and mounted and secured between two glass micropipettes (50-μm-diameter tips) with 10-0 ophthalmic suture. The vessel chamber was transferred to an inverted light microscope stage coupled to a video dimension analyzer (Living Systems Instrumentation, Burlington, VT). The video dimension analyzer was connected to both a video monitor (for visualization of the vessel) and a strip chart recorder (Kipp and Zonen) for constant recording of the intraluminal diameter of the vessel. Oxygenated Krebs solution maintained at 37°C was continuously circulated through the vessel bath. In addition, the lumen of the vessel was filled with Krebs solution through the micropipettes and maintained at a constant pressure (no flow) of 40 mmHg. Only one concentration-response experiment was performed per artery (8, 9).

ET-1 response. Mesenteric arteries were allowed to equilibrate for 30 min. Concentration-response experiments to ET-1 (1 × 10⁻¹¹–3 × 10⁻⁸ M) were performed both with and without endothelium. Endothelial denudation was performed as previously described (9). Briefly, an air bubble was placed in the lumen, after which the vessels were rolled on the glass pipette for 1 min. Endothelial disruption was verified by the absence of a dilator response to ACh, and vascular smooth muscle viability was tested by vasodilator response to nitroprusside after constriction with phenylephrine (9). In a separate set of arteries (with endothelium), the role of each receptor subtype (ETA and ETB) to the ET-1 response was assessed by pretreatment with an antagonist for each receptor before ET-1. The ETA-receptor antagonist A-192621 (1 μM) and the ETB-receptor antagonist A-127792 (0.01, 0.05, 0.1, or 1 nM) were used for these experiments.

Receptor binding assay. Binding characteristics of ¹²⁵I-labeled ET-1 (¹²⁵I]ET-1) were determined using membrane preparations obtained from small mesenteric arteries of control and IR rats. ¹²⁵I]ET-1 binding represents the total number of ETA and ETB receptors. Membrane preparations were obtained for the binding assay as previously described (14). Briefly, vascular tissue from isolated small mesenteric arteries was first weighed and pulverized at −80°C. The pulverized tissue was then added to homogenization buffer [250 mM sucrose, 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 15 μM phenylethylsulfonyl fluoride] in a glass/Teflon homogenizer at a ratio of ~1 g tissue per 5–10 ml buffer. The tissue was then homogenized for 20 strokes. The homogenate was centrifuged at 1,000 g for 30 min at 4°C. The resultant supernatant was centrifuged at 30,000 g for 45 min at 4°C. Subsequently, the supernatant was removed, and the pellet was resuspended in one-fourth the initial amount of homogenizer buffer. The protein concentration was then assessed via the Bradford method (Bio-Rad, Hercules, CA). Membrane preparations were pooled from several animals because the quantity of membrane protein obtained from a single rat was not sufficient to determine a binding curve.

¹²⁵I]ET-1 binding experiments were performed as previously described (14). In brief, a known quantity of each membrane preparation was added to each well of a microtiter plate (OptiPlate, Packard Instruments, Meridian, CT). Wheat germ agglutinin-polyvinyl toluene beads were suspended in binding buffer and added to each well. After precoupling, 25 μl of binding buffer were added to those wells required for total binding, and ET-1 was added to the other wells (final concentration of 1 μM) for nonspecific binding. ¹²⁵I]ET-1 was diluted in binding buffer and then added to each well for each ligand concentration on the binding curve. Subsequently, the plate was shaken gently for 18 h at room temperature and counted on a scintillation counter. Before establishing a binding curve, the amount of protein required was estimated by assembling a protein curve (0.5–2 μg) using vascular membrane preparations. Total and nonspecific binding were assessed for 1 nM ¹²⁵I]ET-1 at each protein concentration. Maximum binding was achieved at 2 μg per well for both groups; therefore, this concentration was used in subsequent experiments. All points were performed in duplicate and all dilutions of peptides were performed in siliconized tubes.

Biochemical measurements. Plasma insulin was assayed using a dextran-coated charcoal immunoassay with rat antibody (11). Glucose concentrations were measured with a glucose Trinder kit (Sigma Chemical, St. Louis, MO).

Materials. Wheat germ agglutinin scintillation proximity assay beads were obtained from Amersham Life Sciences (Arlington Heights, IL). ¹²⁵I]ET-1 was purchased from New England Nuclear (Boston, MA), and ET-1 was obtained from American Peptide (Sunnyvale, CA). Abbott Laboratories (Abbott Park, IL) supplied A-127792 and A-192621. American Peptide and England Nuclear (Boston, MA).

Data analysis. Data obtained from mesenteric arteries are expressed as intraluminal diameters in micrometers. Responses to ET-1 are expressed as percentage of constriction of the baseline diameter. Statistical differences between the control and IR groups for maximal relaxation (E max) and the concentration at 50% of maximal relaxation (E C50) values, and animal characteristics were calculated using an unpaired Student’s t-test. Statistical comparisons for concentration response experiments were performed by repeated-measures ANOVA with covariance followed by Fisher’s pairwise least significant difference test for multiple comparisons. Binding data were analyzed by nonlinear regression of the binding isotherm (Prism, Graphpad Software, San Diego, CA). Scatchard analysis is also shown for historical comparison purposes only. All data are reported as means ± SE, with P < 0.05 being considered significant.

RESULTS

Mean body wt (303 ± 8 g for control and 310 ± 6 g for IR) and fasting glucose (149 ± 11 mg/dl for control and
142 ± 8 mg/dl for IR) were similar between control and IR rats. In contrast, fasting plasma insulin (97 ± 27 pM for control and 234 ± 37 pM for IR, P < 0.05) and MAP (116 ± 2 mmHg for control and 132 ± 4 mmHg for IR, P < 0.05) were significantly elevated in IR rats compared with control rats.

The resting intraluminal diameters of the small mesenteric arteries (both endothelium intact and denuded) did not differ between groups (231 ± 5 μm for control vs. 238 ± 4 μm for IR rats). ET-1 elicited a concentration-dependent vasoconstriction of arteries with endothelium from both groups. The $E_{max}$ to ET-1 was similar between the groups of arteries, whereas the EC$_{50}$ for the IR group was significantly lower versus control arteries (Fig. 1, Table 1).

After endothelium denudation, the $E_{max}$ to ET-1 was enhanced in both IR and control arteries (Fig. 1) compared with arteries with endothelium. In contrast, the EC$_{50}$ was not significantly affected by the removal of endothelium in either group (Table 1, Fig. 1) compared with arteries with endothelium.

Pretreatment of arteries with the ET$_B$ antagonist A-192621 markedly increased both $E_{max}$ and EC$_{50}$ to ET-1 in IR and control arteries compared with untreated endothelium-intact arteries (Table 1, Fig. 2). However, the difference in EC$_{50}$ measurements before and after pretreatment with A-192621 were 3.8 ± 0.2 nM and 0.4 ± 0.2 nM for control and IR rats, respectively. Thus the absolute change in the concentration-response curve after ET$_B$ blockade was greater for control than IR ($P < 0.001$) arteries, suggesting a lesser role of ET$_B$ receptors in IR arteries.

In contrast, pretreatment of arteries with the ET$_A$ antagonist A-127722 inhibited the ET-1-induced vasoconstriction in arteries from both groups in a concentration-dependent manner (Fig. 3). It should be noted that ET-1 induced a greater vasoconstriction at each concentration of A-127722 (0.05 and 0.1 nM) in IR arteries compared with control arteries (Table 1, Fig. 3). The EC$_{50}$ values of A-127722 were 0.02 ± 0.01 and 0.08 ± 0.01 nM for control and IR arteries, respectively ($P < 0.001$). Additionally, the concentration required to completely abolish the response to ET-1 was greater in IR than control arteries (Fig. 3). These data suggest that ET$_A$-mediated vasoconstriction is greater in IR versus control arteries.

Receptor binding experiments showed that maximal binding (B$_{max}$) of $[^{125}I]$ET-1 was significantly increased in IR arteries (232 ± 10 fmol/mg protein) compared with control (136 ± 7 fmol/mg protein) ($P < 0.05$). In contrast, the dissociation constant (K$_d$) was similar for control (0.049 ± 0.014 nM) and IR (0.034 ± 0.008 nM) arteries (Fig. 4). These data suggest a greater number of endothelin receptors in arteries from IR compared with control rats.

<table>
<thead>
<tr>
<th>ET-1 Dose Response</th>
<th>Control Arteries</th>
<th>Insulin-Resistant Arteries</th>
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<tr>
<td></td>
<td>$E_{max}$, %constriction</td>
<td>EC$_{50}$, nM</td>
</tr>
<tr>
<td>Endothelium intact</td>
<td>58 ± 2</td>
<td>4.5 ± 1.2</td>
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<tr>
<td>Endothelium denuded</td>
<td>71 ± 2*</td>
<td>3.2 ± 0.6</td>
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<tr>
<td>ET$_B$ block (A-192621)</td>
<td>86 ± 1*</td>
<td>0.6 ± 0.2*</td>
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<tr>
<td>ET$_A$ block (A-127722)</td>
<td>0.01 ± nM</td>
<td>39 ± 4*</td>
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<tr>
<td>0.05 nM</td>
<td>22 ± 7*</td>
<td>3.3 ± 0.6</td>
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<td>0.1 nM</td>
<td>0 ± 2*</td>
<td>14 ± 4†</td>
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<td>1 nM</td>
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Values are means ± SE. ET-1, endothelin-1; $E_{max}$, maximal relaxation. $P < 0.05$; *P < 0.05 vs. respective ET-1 response in endothelium-intact arteries; †P < 0.05 vs. respective control.

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**Fig. 1.** Cumulative dose-response curve to endothelin-1 (ET-1) in small mesenteric arteries of insulin-resistant (IR) and control rats in the presence (Endo +) and absence (Endo −) of endothelium. Significant differences: *P < 0.05 in the ET-1 response between control (Endo +) and IR (Endo +) arteries, †P < 0.05 between control (Endo −) and IR (Endo −) arteries, and ‡P < 0.05 between (Endo +) and (Endo −) arteries within groups.

**Fig. 2.** Cumulative dose-response curve to ET-1 in small mesenteric arteries of control and IR rats in the presence and absence of ET$_B$ antagonist A-192621. *Concentrations where ET-1 responses in presence of A-192621 were significantly different from respective ET-1 responses in IR (Endo +) and control (Endo +) arteries without A-192621 pretreatment (P < 0.05).
DISCUSSION

The current study demonstrates an enhanced response to ET-1 in resistance arteries from IR rats. This enhanced response may be explained by two mechanisms. First, ET-1-induced vasoconstriction occurs via the activation of ETA receptors. According to the receptor binding studies, it is likely that these receptors are overexpressed in vascular tissue from IR rats. Second, impaired ETB-activated production or release of endothelium-derived relaxing factors results in an imbalance between endothelium-derived vasodilating and contracting factors, leading to enhanced vasoconstriction by ET-1.

Two distinct endothelin receptor subtypes, ETA and ETB, mediate the vascular response to ET-1 (12, 14). ETA receptors are located on the vascular smooth muscle and their stimulation results in vasoconstriction (10, 13). ETB receptors, expressed predominately on endothelial cells, have also been reported on vascular smooth muscle (10, 13). ETB receptors located on the endothelium are responsible for inducing the release of endothelium-derived relaxing factors, whereas ETB receptors on vascular smooth muscle induce vasoconstriction (10, 13).

The current data illustrate that in small mesenteric arteries from control and IR rats vasoconstriction is mediated by the ETA receptor because the presence of the ETA-receptor antagonist A-127722 was able to abolish vasoconstriction in arteries from both groups. In addition, experiments to assess the response to ET-1 in the presence of various concentrations of A-127722 suggest that ETA-receptor expression is enhanced in IR arteries because the maximal vasoconstriction to ET-1 was significantly greater in IR arteries at each concentration of the receptor antagonist (0.05 and 0.1 nM). Moreover, the concentration of A-127722 necessary to abolish the ET-1 response in IR arteries was markedly higher than that to eliminate the response in control arteries. To confirm these findings, we estimated total $^{125}$I-ET-1 binding in vascular membrane preparations from small mesenteric arteries of control and IR rats. The receptor binding experiments demonstrated a significant increase in maximal binding of $^{125}$I-ET-1 in IR arteries compared with control, suggesting an increase in expression of total endothelin receptors. In contrast, no difference was found in the $K_d$ for $^{125}$I-ET-1 binding
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curves between control and IR arteries. Taken together, these data suggest that the increased response to ET-1 in IR arteries is due to upregulation of ET_A receptors in IR arteries.

Enhanced expression of ET_A receptors in the presence of insulin resistance or hyperinsulinemia has been previously shown. In rat aortic vascular smooth muscle cells, incubation with supraphysiological concentration of insulin stimulated a selective upregulation of ET_A receptors as measured by both saturation binding and mRNA expression. Moreover, in rat tail arteries from fructose-fed IR rats, mRNA for ET_A receptors was increased approximately threefold compared with control rats. The current data confirm that endothelin (likely ET_A) receptors are overexpressed in vascular tissue from IR rats and are the first to demonstrate this in small mesenteric (near resistance) arteries.

The current data also suggest that in this arterial bed stimulation of the ET_B receptor enhances the production and release of endothelium-derived relaxing factors, because vasoconstriction was abolished after pretreatment with A-192621, with the EC_{50} for the control group decreasing dramatically and the EC_{50} for the IR group changing minimally. These data illustrate the impaired ability of the IR arteries to produce endothelium-derived relaxing factors in response to ET_B stimulation. This finding is not surprising because we and others have previously demonstrated impaired endothelium-dependent relaxation in mesenteric arteries from IR rats.

Similar to ET_B antagonist studies, endothelial denudation also induced a significant increase in maximal vasoconstriction in both groups compared with endothelium-intact arteries. However, the EC_{50} value was not altered with endothelium denudation compared with endothelium-intact arteries. It may seem contradictory that the EC_{50} was reduced with the ET_B antagonist but not by endothelium denudation. However, endothelial denudation removes the ability to produce both endothelium-derived relaxing factors and contracting factors; blocking the ET_B receptors on the endothelium only affects production of relaxing factors. Thus the resultant ET-1 response after endothelium denudation is represented entirely by the stimulation of vascular smooth muscle endothelin receptors, although the ET-1 response after ET_B-receptor blockade may be contributed to by endothelium-derived contracting factors. Importantly, the fact that the difference in EC_{50} remained between IR and control groups after endothelium denudation demonstrates that the enhanced response to ET-1 in IR arteries cannot be completely explained by impaired production of endothelium-derived relaxing factors.

Several other laboratories have assessed the vascular response to ET-1 in fructose-fed IR rats in a variety of vascular preparations; however, the results of these studies are conflicting. Enhanced maximal contraction was observed in aortic rings from IR rats, although a diminished (17) or normal (11) response was observed in superior mesenteric artery rings and the mesenteric vascular bed. It should be noted that the normal response reported by Navarro-Cid and colleagues (11) was elicited at 10 pM ET-1, which was ineffective in our experiments. None of these studies demonstrated a difference in the EC_{50} to ET-1. The apparent variation in these observations, compared with one another and to our own, may be explained by differences in artery size, vascular bed, or methodology. The current data differs from all of the above studies because we assessed the response to ET-1 in isolated small mesenteric arteries and measured intraluminal diameter under constant pressure.

Several studies in IR humans have reported increased ET-1 serum concentrations that directly correlated with the levels of hyperinsulinemia. Enhanced maximal contraction of vascular smooth muscle endothelin receptors, although the ET-1 response after endothelium denudation is represented entirely by the stimulation of vascular smooth muscle endothelin receptors, although the ET-1 response after endothelium denudation is represented entirely by the stimulation of vascular smooth muscle endothelin receptors, although the ET-1 response after endothelium denudation is represented entirely by the stimulation of vascular smooth muscle endothelin receptors, although the ET-1 response after endothelium denudation is represented entirely by the stimulation of vascular smooth muscle endothelin receptors, although the ET-1 response after endothelium denudation is represented entirely by the stimulation of vascular smooth muscle endothelin receptors, although the ET-1 response after endothelium denudation is represented entirely by the stimulation of vascular smooth muscle endothelin receptors, although the ET-1 response after endothelium denudation is represented entirely by the stimulation of vascular smooth muscle endothelin receptors, although the ET-1 response after endothelium denudation is represented entirely by the stimulation of vascular smooth muscle endothelin receptors.

In summary, the response to ET-1 is enhanced in small mesenteric arteries from IR rats, as shown by a significantly decreased EC_{50}. This enhanced response appears to be due to both increased expression of ET_A receptors on vascular smooth muscle and to underlying endothelial dysfunction.

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REFERENCES


