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Impaired endothelin-induced vasoconstriction in coronary arteries of Zucker obese rats is associated with uncoupling of $[\text{Ca}^{2+}]_i$ signaling

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Katakam, Prasad V. G., James A. Snipes, Christina D. Tulbert, Keita Mayanagi, Allison W. Miller, and David W. Busija. Impaired endothelin-induced vasoconstriction in coronary arteries of Zucker obese rats is associated with uncoupling of $[\text{Ca}^{2+}]_i$ signaling. \textit{Am J Physiol Regul Integr Comp Physiol} 290: R145–R153, 2006; doi:10.1152/ajpregu.00405.2005.—Although insulin resistance (IR) is a major risk factor for coronary artery disease, little is known about the regulation of coronary vascular tone in IR by endothelin-1 (ET-1). We examined ET-1 and PGF$_2\alpha$-induced vasoconstriction in isolated small coronary arteries (SCAs; SCAs: $\sim$250 $\mu$M) of Zucker obese (ZO) rats and control Zucker lean (ZL) rats. ET-1 response was assessed in the absence and presence of endothelin type A (ET$_A$; BQ-123), type B (ET$_B$; BQ-788), or both receptor inhibitors. ZO arteries displayed reduced contraction to ET-1 compared with ZL arteries. In contrast, PGF$_2\alpha$, elicited similar vasoconstriction in both groups. ET$_A$ inhibition diminished the ET-1 response in both groups. ET$_B$ inhibition alone or in combination with ET$_A$ blockade, however, restored the ET-1 response in ZO arteries to the level of ZL arteries. Similarly, inhibition of endothelial nitric oxide (NO) synthase with N-nitro-L-arginine methyl ester (l-NAM) enhanced the contraction to ET-1 and abolished the difference between ZO and ZL arteries. In vascular smooth muscle cells from ZO, ET-1-induced elevation of myoplasmic intracellular free calcium concentration ($[\text{Ca}^{2+}]_i$) (measured by fluo-4 AM fluorescence), and maximal contractions were diminished compared with ZL, both in the presence and absence of l-NAM. However, increases in $[\text{Ca}^{2+}]_i$ elicited similar contractions of the vascular smooth muscle cells in both groups. Analysis of protein and total RNA from SCA of ZO and ZL revealed equal expression of ET-1 and the ET$_A$ and ET$_B$ receptors. Thus coronary arteries from ZO rats exhibit reduced ET-1-induced vasoconstriction resulting from increased ET$_B$-mediated generation of NO and diminished elevation of myoplasmic $[\text{Ca}^{2+}]_i$.

Insulin resistance; endothelial nitric oxide synthase

Compelling scientific evidence has established that individuals with impaired glucose tolerance [prediabetes or insulin resistance (IR)] and/or obesity are at a substantially increased risk of type 2 diabetes (6). Clinical evidence has also identified IR as an independent risk factor for hypertension, coronary artery disease, and stroke (27, 39a). However, the mechanisms underlying the coronary artery disease in IR have not been thoroughly investigated.

It is believed that impaired ability to vasodilate and/or an enhanced sensitivity to vasoconstrictor agonists underlie the vascular dysfunction associated with IR. Endothelin-1 (ET-1), a potent endothelium-derived vasoconstrictor peptide, plays an important role in regulation of vascular tone in the coronary circulation. The actions of ET-1 are mediated by two major receptors, endothelin type A (ET$_A$) and type B (ET$_B$), which are present on vascular smooth muscle (VSM) cells and endothelial cells (5, 33). Vascular contraction induced by ET-1 is predominantly mediated by activation of VSM cell ET$_A$ receptors but may also occur by stimulation of VSM cell ET$_B$ receptors (5, 33). ET-1 regulates the contraction of VSM cells primarily by increasing intracellular $\text{Ca}^{2+}$ concentration ($[\text{Ca}^{2+}]_i$) via opening various $\text{Ca}^{2+}$ channels (5, 33). In addition, ET-1 also modulates the contraction through activating the Ca$^{2+}$-independent RhoA/Rho-kinase pathway (14). Activation of endothelial ET$_B$ receptors results mostly in increased endothelial nitric oxide (NO) synthase (eNOS) activity and release of NO.

Abnormal ET-1 activity has been implicated in the pathogenesis of cardiovascular diseases associated with diabetes. However, studies of ET-1 activity in coronary arteries were mostly limited to models of diabetes (25, 39, 40). ET-1 regulation of coronary circulation, in the setting of IR, has not been adequately studied. Hyperinsulinemia, pathognomonic of IR, has been shown to promote the expression of ET-1 (30) and its receptors (17). Moreover, we previously reported enhanced insulin-induced vasoconstriction in young male Zucker obese (ZO) rats compared with the lean (ZL) controls, a widely accepted model of IR (22). We hypothesized that hyperinsulinemia associated with IR promotes enhanced activity of ET-1 in ZO arteries. Therefore, we examined the role of ET-1 in coronary vascular dysfunction in 12-wk-old ZO and ZL by evaluating $J$) the vascular response to ET-1 and PGF$_2\alpha$; 2) the contribution of ET$_A$ and ET$_B$ receptors to ET-1 responses; 3) the interaction of NO and ET-1 in regulation of coronary vascular tone; 4) the expression of ET-1, ET$_A$, and ET$_B$ receptors; and 5) the role of VSM cell $[\text{Ca}^{2+}]_i$ in ET-1-induced contraction and the relationship between $[\text{Ca}^{2+}]_i$ and contractility of VSM cells.

MATERIALS AND METHODS

The experimental protocol was approved by the Animal Care and Use Committee of Wake Forest University Health Sciences. All experiments complied with the National Institutes of Health \textit{Guide for...
the Care and Use of Laboratory Animals. Experiments were performed on male 12- to 13-wk-old ZL (n = 16) and ZO (n = 20) rats (Harlan, Indianapolis, IN). Animals were fed standard rat diet and drank tap water ad libitum.

Isolation and cannulation of the arteries. These methods have been described previously (13, 21, 22). Briefly, rats were anesthetized with pentobarbital sodium (50 mg/kg ip) and anticoagulated with heparin (500 U ip). The heart was removed after thoracotomy and placed in a chilled oxygenated modified Krebs-Ringer bicarbonate solution concentration (in mM): 118.3 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, and 11.1 dextrose. SCA (=250 μm diameter) from the septum and/or the epicardium were isolated from the surrounding perivascular tissue and removed. A section of the small coronary artery (SCA) was transferred to a vessel chamber, mounted between two glass micropipettes, and secured with 10-0 ophthalmic suture. The vessel bath was mounted on an inverted microscope with a video camera attached. Oxygenated and warmed (20% O₂-5% CO₂-75% N₂ at 37°C) Krebs solution was circulated continuously through the vessel bath. The lumen of the artery was then flushed with Krebs solution; one micropipette was clamped off, and the other was connected to a pressure servo controller (Living Systems, Burlington, VT) to maintain a constant intraluminal pressure of 60 mmHg. Drugs were added abuminally to the bath solution. Only one concentration-response experiment was performed per arterial segment; however, several arterial segments were taken from each rat. The video camera on the microscope was connected to a TV monitor and also to a video dimension analyzer (Living Systems) that measures intraluminal diameter. Vascular responses were recorded on a calibrated chart paper recorder for analysis.

Vascular reactivity experiments. Coronary arteries were allowed to equilibrate for 30 min in the tissue bath. Subsequently, concentration-response studies to ET-1 (10⁻¹²–10⁻⁸ M) were performed. To evaluate the role of NO in the vascular response to ET-1, arteries were pretreated for a period of 30 min with Nω-nitro-l-arginine methyl ester (L-NAME) (100 μM), an inhibitor of eNOS. To evaluate the role of the ET₄ or ET₅ receptors in the vascular response to ET-1, arteries were pretreated for a period of 30 min with specific inhibitors BQ-123 (1 μM) or BQ-788 (1 μM), respectively, or both. To establish the specificity of ET-1 activity, responses to PGE₂ (10⁻⁶–10⁻³ M), a vasodilator, were also determined. At the end of each experiment, the quality of vascular preparation was tested by administration of ACh (10⁻⁴ M), an endothelium-dependent vasodilator, followed by sodium nitroprusside (10⁻⁴ M), an exogenous NO donor. Data were reported as % constriction (the change in diameter for each concentration of ET-1 administered expressed as % baseline diameter).

Western blot analysis. Equal amounts of protein for each sample were separated by 4–20% SDS-PAGE and transferred onto a PVDF sheet (PolyScreen PVDF, PerkinElmer Life Sciences, Boston, MA). Membranes were incubated in a blocking buffer (Tris-buffered saline, 0.1% Tween 20, 5% skim milk powder) for 1 h at room temperature, and then blots were incubated with monoclonal anti-ET₄ (1:3,000; BD Transduction Laboratories) or monoclonal anti-ET₅ (1:25,000; Sigma) antibodies overnight at 4°C. Membranes were then washed three times in Tris-buffered saline with 0.1% Tween 20 and incubated for 1 h in the blocking buffer with anti-mouse IgG (1:10,000; Jackson ImmunoResearch Laboratories, West Grove, PA) conjugated to horseradish peroxidase. Final reaction products were visualized using enhanced chemiluminescence (SuperSignal West Pico; Pierce, Rockford, IL) and recorded on X-ray film. The bands were scanned and the densities of the bands were quantitated by using Foto Dyno (FOTO/Analyt PC Image and Image J). Subsequently, data were expressed by normalizing the immunoblot band density of the protein of interest to the corresponding band density of β-actin.

RT-PCR. Total RNA was obtained from isolated SCA using the SV Total RNA Isolation System (Promega, Madison, WI), and RT-PCR experiments were carried out as described previously (23). Expression of ET-1, ET₄ receptor, and ET₅ receptor mRNA was analyzed using specific primers (Table 1). Expected lengths of the RT-PCR products were 500, 416, and 386 base pairs, respectively. β-Actin primers (Promega) were also included in the RT-PCR reaction to normalize the RT-PCR results with an expected product length of 285 base pairs. Each PCR reaction was repeated at least three times. The number of PCR cycles varied according to the expression level of the target gene.

A suitable number of cycles was determined to ensure that PCR was taking place in the linear range, to guarantee a proportional relationship between input RNA and densitometric readout.

Isolation of vascular smooth muscle cells. SCAs were isolated as described in Isolation and cannulation of the arteries. The arterial preparation was exposed to a solution (in mM: 140 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES; pH 7.0) containing albumin (4 g/l) followed by mild trituration. The solution was centrifuged at 1,000 g, and the supernatant was discarded. SCAs were isolated as described by Giulumian and colleagues (12, 28). Briefly, isolated SCAs and cerebral arteries were placed in dissociation medium (in mM): 110 NaCl, 5 KCl, 2 MgCl₂, 0.16 CaCl₂, 10 HEPES, 10 NaHCO₃, 0.5 KH₂PO₄, 0.5 NaH₂PO₄, 0.49 EDTA, 1.6 CaCl₂, and 5.5 glucose. With the aid of a dissecting microscope, middle, anterior, and posterior cerebral arteries and basilar arteries were carefully isolated. All the arteries were transferred to a vessel chamber and mounted onto a glass micropipette, and the blood inside the arteries was flushed out by careful infusion with cold Krebs solution. To measure the ET-1-induced changes in fluo-4 AM fluorescence, single smooth muscle cells were prepared freshly as described by Giuluman and colleagues (23). Briefly, isolated SCAs and cerebral arteries were placed in dissociation medium (in mM): 110 NaCl, 5 KCl, 2 MgCl₂, 0.16 CaCl₂, 10 HEPES, 10 NaHCO₃, 0.5 KH₂PO₄, 0.5 NaH₂PO₄, 0.49 EDTA, 10 taurine, and 10 glucose (pH 6.9) containing albumin (4 mg/ml) with papain (1.52 mg/ml) and dithiothreitol (0.54 mg/ml). Cells were dispersed for 30 min in a shaking water bath at 37°C followed by mild trituration. The solution was centrifuged at 1,000 g for 12 min, and the pellet was resuspended in dissociation medium to obtain freshly dispersed smooth muscle cells.

[Ca²⁺]i imaging and data analysis. VSM cells were loaded for 30 min at room temperature with 0.5 μM fura-4 AM loaded with 1× volume of Pluronic F-127 (0.1%). Subsequently, cells were washed twice with dissociation medium and resuspended in normal Ringer solution (in mM: 140 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES; pH 7.0) imaging and data analysis.

Table 1. Sequences of primers

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence Type</th>
<th>Sequences</th>
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<tr>
<td>Endothelin-1, 500 bp</td>
<td>Forward</td>
<td>5'-CTCGTCTCTATGTAATGCTAGCG-3'</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5'-GCTCTGCTCTCTGGTATGCTCG-3'</td>
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<tr>
<td>ET₄ receptor, 416 bp</td>
<td>Forward</td>
<td>5'-GTGCTGCTCTTGGTATGCTCG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-CATGGCTCTCTCTGGTATGCTCG-3'</td>
</tr>
<tr>
<td>ET₅ receptor, 386 bp</td>
<td>Forward</td>
<td>5'-TGCTGCTCTCTCTGGTATGCTCG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-GATTGCTCTGGTATGCTCGGTTG-3'</td>
</tr>
<tr>
<td>β-Actin, 505 bp</td>
<td>Forward</td>
<td>5'-GTGCAAGGCTCTCTGGTATGCTCG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-GTCACAGCTCTGGTATGCTCG-3'</td>
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ET₄, endothelin type A; ET₅, ET type B. Accession no. NM_031144 was used with ET₅ to get separation of the bands.
7.4). The cells were imaged using a Zeiss LSM 510 laser scanning microscope (Carl Zeiss, Jena, Germany). VSM cells were placed in a four-chambered coverglass (Nalge Nunc, Naperville, IL) and mounted on the stage of an Axiovert inverted microscope. The excitation beam of 488 nm was produced by an argon laser, attenuated in intensity with an acoustooptical tunable filter, and delivered to the specimen via a Zeiss Apochromat × 63 water immersion objective. Emitted fluorescence was captured at wavelengths >510 nm using LSM 510 software (version 2.01; Carl Zeiss, Jena, Germany) running on a Windows XP work station. During a time series protocol, x-y axes 2-D images (typically 50–80 images per series; image depth of 8 bits) 512 × 512 pixels, were taken every 5 s. All experiments were done at room temperature (20–23°C). Responses to ET-1 (10−10 and 10−8 M) and potassium chloride (KCl; 0.5 mM) were assessed in the presence and absence of L-NAME (100 μM). Images were processed and analyzed using the software provided by Carl Zeiss. Global [Ca2+]i was estimated across the entire cell by selecting the regions of interest in the overlay function tool of Zeiss LSM Image Browser (Jena, Germany). The %change in length of VSM cells was calculated in the same manner as the fluorescence measurements. Data were expressed as means ± SE for the number of cells (n) analyzed. On any given day, VSM cells from a pair of ZL and ZO rats were prepared and the experiments were conducted simultaneously under the same conditions.

Chemicals. All chemicals used in this study were obtained from Sigma (St. Louis, MO). Fluo-4 AM and Pluronic F-127 were purchased from Molecular Probes (Eugene, OR). Fluo-4 AM stock solution was made in DMSO, and aliquots were stored at approximately −20°C and protected from light.

Data analysis. All data are reported as means ± SE. All statistical comparisons for concentration response experiments were performed using ANOVA with repeated measures followed by Tukey’s post hoc test. The criteria for significance was P < 0.05.

RESULTS

Vascular reactivity. The resting intraluminal diameter of SCAs did not differ between ZL (249 ± 8 μm, n = 45) and ZO (246 ± 7 μm, n = 48) rats. In ZL arteries, ET-1 induced a concentration-dependent vasoconstriction with a maximal constriction of 82 ± 2% (n = 9; Fig. 1). Similarly, ET-1 induced a dose-dependent vasoconstriction in ZO arteries, but the maximal constriction was significantly reduced to 58 ± 3% (n = 15, P < 0.05; Fig. 1).

Pretreatment of arteries with L-NAME resulted in a constriction in both ZL (4 ± 1%, n = 8) and ZO arteries; however, it was significantly greater in ZO arteries (12 ± 2%, n = 5, P < 0.05). In the presence of L-NAME, significantly increased contraction was observed at all concentrations of ET-1 in ZO arteries, whereas enhanced contraction to ET-1 was observed at 10−10 M in ZL arteries (Fig. 1). The maximal constriction to ET-1 was significantly enhanced only in arteries from ZO rats (74 ± 5%, n = 5; P < 0.05 vs. baseline) compared with ZL arteries [82 ± 4%, n = 8; P = not significant (NS) vs. baseline]. In contrast, the EC50 of ET-1 response was significantly decreased in both groups, indicating enhanced sensitivity to ET-1 in the presence of L-NAME (Table 2).

Pretreatment of the arteries with BQ-123 did not affect the baseline diameter in either group (data not shown). However, BQ-123 significantly inhibited the ET-1 response in both ZL (44 ± 7%, n = 7) and ZO (11 ± 5%, n = 6) arteries by reducing the maximal constriction (P < 0.05; Fig. 2) and increasing the EC50 (P < 0.05; Table 2) compared with corresponding baseline responses. In contrast, BQ-788 signifi-

<table>
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<th>Table 2. Vascular response to ET-1</th>
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<tr>
<td><strong>EC50</strong></td>
</tr>
<tr>
<td>Base</td>
</tr>
<tr>
<td>BQ-123 (ET&lt;sub&gt;a&lt;/sub&gt; blockade)</td>
</tr>
<tr>
<td>BQ-788 (ET&lt;sub&gt;a&lt;/sub&gt; blockade)</td>
</tr>
<tr>
<td>BQ-123 + BQ-788</td>
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<td>L-NAME (eNOS inhibition)</td>
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Values are nanomoles, means ± SE. ET-1, endothelin-1; BQ-123 and BQ-788, endothelin with inhibitors; L-NAME, N′-nitro-l-arginine methyl ester; eNOS, endothelial nitric oxide synthase. *P < 0.05 compared with Zucker lean rat baseline; †P < 0.05 compared with Zucker obese rat baseline; ‡P < 0.05 compared with BQ-123.

Fig. 1. Cumulative concentration-response experiments to endothelin-1 (ET-1; 10−12–10−8 M) in small coronary arteries (SCAs) from Zucker lean (ZL) and Zucker obese (ZO) rats with and without N’-nitro-l-arginine methyl ester (l-NAME; 100 μM) pretreatment. *Statistically significant difference (P < 0.05) in vasoconstriction to ET-1 compared with the baseline response in ZL and ZO arteries, respectively.

Fig. 2. Cumulative concentration-response experiments to ET-1 (10−12–10−8 M) in SCAs from ZL and ZO rats. Arteries were pretreated with ET<sub>a</sub> receptor inhibitor BQ-123 (1 μM), ET<sub>a</sub> receptor inhibitor BQ-788 (1 μM), or both. **Statistically significant difference (P < 0.05) in vasoconstriction to ET-1 compared with the baseline response in ZL and ZO arteries, respectively.
icantly reduced the baseline diameter in ZO arteries (2.2 ± 0.8%; n = 7, P < 0.05) compared with ZL (0 ± 0.6%; n = 7). Furthermore, BQ-788 significantly enhanced the constriction to ET-1 only at 10⁻⁸ M concentration in ZO arteries, whereas the ET-1 response in ZL arteries was unaffected. The maximal constriction to ET-1 in BQ-788-treated arteries was 72 ± 3% in ZO (n = 7; P < 0.05 vs. baseline) and 77 ± 3% in ZL (n = 7; Fig. 2). BQ-788 did not change the EC₅₀ of ET-1 response in both groups (Table 2). Pretreatment of arteries with BQ-123 and BQ-788 together reduced the contraction to ET-1 in ZL arteries, whereas ZO arteries were unaffected. The maximal contraction to ET-1 was 42 ± 9% in ZL (n = 6; P < 0.05 vs. baseline) and 42 ± 5% in ZO arteries (n = 7; P = NS vs. baseline). In contrast, the EC₅₀ of ET-1 response was significantly increased in the presence of BQ-123 and BQ-788 in both groups compared with baseline (Table 2).

PGF₂α induced contraction of SCA in a dose-dependent manner. Although ZO arteries (23 ± 4%, n = 8) showed a tendency toward enhanced maximal contraction to PGF₂α compared with ZL arteries (33 ± 4%, n = 8), the difference was not statistically significant (Fig. 3).

Expression of ET-1 and ET receptors. Western blot analysis with specific antibodies revealed that ZO and ZL arteries exhibit equal expression of ETA receptors. Densities of respective immunoreactive bands, normalized to β-actin and expressed in arbitrary units, were 0.64 ± 0.17 in ZO arteries (n = 8) and 0.67 ± 0.14 in ZL arteries (n = 8, P = NS; Fig. 4). In addition, RT-PCR experiments showed that expression of ET-1 mRNA was similar in the ZO and ZL arteries; densities of bands normalized to β-actin and expressed in arbitrary units, were 0.4 ± 0.02 (n = 6) in ZO and 0.35 ± 0.04 (n = 5) in ZL (P = NS). Similarly, densities of normalized bands of ETA and ETB receptor mRNAs were 0.88 ± 0.05 and 0.82 ± 0.05 (n = 6 each) in ZO and 0.75 ± 0.07 and 0.79 ± 0.07 (n = 5 each) in ZL, respectively (P = NS; Fig. 5).

[Ca²⁺]i measurement. ET-1 induced a concentration-dependent elevation of fluo-4 AM fluorescence in VSM cells of both ZO and ZL arteries. The elevation in fluorescence intensity in response to ET-1 or KCl was associated with contraction of the VSM cells. Fig. 6A shows a typical response of VSM cells at baseline and after administration of ET-1 and KCI. Maximal...

Fig. 3. Cumulative concentration-response experiments to PGF₂α (10⁻²–10⁻⁵ M) in SCAs from ZL and ZO rats.

Fig. 4. A: images of Western blots using antibodies directed against the ETA receptor and β-actin in isolated SCAs of ZO and ZL arteries. B: each lane was loaded with equal amounts of protein, and densities of the immunoreactive bands were normalized to β-actin. Normalized intensities of the immunoreactive bands were similar in ZO and ZL arteries.

Fig. 5. A: detection of ET-1, ETA receptor, and ETB receptor mRNA with RT-PCR in SCAs of ZO and ZL rats with corresponding β-actin mRNA. B: intensities of the mRNA bands normalized to those of corresponding β-actin were similar in ZO and ZL arteries.
compared with 12 length in response to ET-1 between L-NAME-treated and n/H11005 in response to ET-1 was significantly reduced in ZO (15 was 69 -untreated cells. In addition, L-NAME (10 and 100 n/H11001 treatment alone had no effect on [Ca2 P 27, Fig. 6). In addition, maximal contraction in [Ca2 increase in [Ca2]i to 10–9 M ET-1 was 41 ± 11% in ZL (n = 22) compared with 12 ± 8% in ZO (n = 21, P < 0.05; Fig. 6B). Similarly, maximal increase in [Ca2+]i to 10–8 M ET-1 was 69 ± 11% in ZL (n = 15) compared with 28 ± 9% in ZO (n = 19, P < 0.05; Fig. 6B). In addition, maximal contraction of VSM cells in response to ET-1 was significantly reduced in ZO (22 ± 4%, n = 22; P < 0.05) compared with ZL (46 ± 2%, n = 21; Fig. 6C). In the presence of l-NAME, maximal increase in [Ca2+]i to ET-1 was 75 ± 14% in ZL (n = 18) compared with 36 ± 12% in ZO (n = 27, P < 0.05). Similarly, in the presence of l-NAME, maximal contraction of VSM cells in response to ET-1 was significantly reduced in ZO (15 ± 4%, n = 19; P < 0.05) compared with ZL (39 ± 5%, n = 18; Fig. 6C). However, there was no significant difference in either peak increase in [Ca2+]i, or maximal decrease in VSM cell length in response to ET-1 between l-NAME-treated and -untreated cells. In addition, l-NAME (10 and 100 μM) pre-treatment alone had no effect on [Ca2+]i, or VSM cell length (data not shown).

Maximal contraction of each VSM cell was divided by the corresponding maximal increase of [Ca2+]i in response to ET-1, and the fraction was used as an index of the [Ca2+]i-contractility relationship. This index, however, was not significantly different between ZL (0.91 ± 0.16) and ZO (0.94 ± 0.22) rats. Thus a given increase in [Ca2+]i in ZO rats elicited similar contraction of the VSM cells compared with ZL, suggesting that the sensitivity of contractile apparatus to [Ca2+]i was not altered in ZO arteries. In other words, both groups of rats exhibited a linear relationship between VSM cell [Ca2+]i and contractility, indicating ET-1-induced contraction is proportional to the elevation of [Ca2+]i. In addition, maximal increase in [Ca2+]i, from baseline in response to KCl was similar in ZL (74 ± 12%, n = 6) compared with ZO (86 ± 9%, n = 9, P = NS; Fig. 6B).

In VSM cells of cerebral arteries from both ZO and ZL rats, ET-1 readily induced elevation of fluo-4 AM fluorescence followed by contraction at a much lower concentration (10–10 M) compared with SCA. Figure 7A shows a typical fluo-4 AM fluorescence of VSM cells at baseline and after administration of ET-1 (10–10 M). ET-1 induced a similar elevation of [Ca2+]i in VSM cells of ZO and ZL cerebral arteries (Fig. 7). Maximal increase in [Ca2+]i, from baseline in response to ET-1 was 92 ± 13% in ZL (n = 15) compared with 76 ± 10% in ZO (n = 17, P = NS; Fig. 7B) arteries. Similarly, maximal contraction in response to ET-1 was 48 ± 3% in ZL (n = 15) compared with 48 ± 3% in ZO (n = 17, P = NS; Fig. 7B) arteries. The index
of \([Ca^{2+}]_i\)-contractility relationships was also not significantly different between ZL (0.93 ± 0.33) and ZO (0.99 ± 0.35) arteries, suggesting a similar \([Ca^{2+}]_i\) sensitivity of contractile apparatus.

DISCUSSION

Major findings of this study are as follows: 1) ET-1-induced vasoconstriction in SCAs of ZO rats was reduced compared with ZL; 2) inhibition of NO production or ET\(_B\) receptor blockade restored the contraction to ET-1 in ZO arteries; 3) studies of protein from SCAs revealed similar expression of ETA, whereas mRNA studies showed identical expression of ETA and ET\(_B\) receptors and ET-1 peptide in both rat groups; 4) freshly isolated VSM cells of ZO coronary arteries exhibited reduced \([Ca^{2+}]_i\), elevation and contraction in response to ET-1 compared with ZL arteries, suggesting the uncoupling of ET-1 receptor activation and \([Ca^{2+}]_i\) signaling; and 5) VSM cell \([Ca^{2+}]_i\)-contractility relationship revealed an intact \(Ca^{2+}\) sensitivity of the contractile apparatus in ZO arteries.

The ZO rat model has been well defined in terms of biochemistry and vascular function. We reported previously that, when on a regular diet, 12-wk-old ZO rats exhibit IR alone (hyperinsulinemia) but without overt diabetes (fasting euglycemia) (9, 10). However, the ZO rats have greater weight and display dyslipidemia characterized by elevated triglycerides and elevated total cholesterol (11). Thus ZO rats exhibit characteristics of the typical metabolic syndrome accompanying prediabetes or IR found in humans.

Previously, our vascular reactivity studies (10) of basilar artery in ZO rats found impaired vasodilation to ACh with intact vasoconstriction to ET-1. In contrast, we (22) observed that coronary arteries of ZO rats displayed normal vasodilation to ACh. Interestingly, in ZO coronary arteries, insulin-induced vasodilation was diminished, whereas vasoconstriction was enhanced (22). Thus it is apparent that vascular dysfunction associated with IR was mediated by a variety of mechanisms based on the vascular bed studied. In the present study, contrary to our hypothesis, we found that ET-1-induced vasoconstriction was reduced in the SCA of ZO rats compared with ZL rats. However, contractile response to vasodilator prostanoioid PGF\(_{2\alpha}\) was similar in ZO and ZL arteries. Thus the decreased vasoconstriction to ET-1 in ZO arteries appears to be a specific defect unique to endothelin receptors.

A majority of the studies in coronary arteries of models of diabetes reported enhanced constriction to ET-1 (25, 39, 40). In Zucker rats, ET-1-induced vasoconstriction has been examined in mesenteric (1, 17, 38), basilar (9, 20), skeletal muscle (37) arteries, and thoracic aorta (17–19, 38, 43). Conflicting findings reported either an increased or normal contraction to ET-1 in ZO arteries. Similar variation was also observed in response to vasodilators with either an impaired (10, 32, 38, 44) or normal (19, 22, 38) endothelium-dependent relaxation in ZO rats. Reduced ET-1-induced vasoconstriction was also observed by others in various vascular beds in diabetes (3, 26, 29, 43), hypertension (12, 13, 28), and aging (35). In contrast, several conflicting studies reported either a normal (37) or enhanced (7, 19, 24, 39–41) ET-1-induced constriction in models of diabetes. The apparent difference in ET-1 response has not been adequately explained; however, it is believed that local activity of ET-1 modulates the ETA expression, thereby influencing ET-1 response. Alternatively, these differences may, in part, be due to the differences between arterial preparation (conduit vs. resistance arteries) or age of the animals investigated (16).
We evaluated the role of ET_A and ET_B receptors in the ET-1 response by using specific receptor inhibitors. Inhibition of ET_A significantly reduced the majority of the ET-1-induced contractions in both ZO and ZL rats, indicating that ET_A primarily mediates the contraction to ET-1. Inhibition of ET_B, however, enhanced the maximal contraction to ET-1 in ZO arteries, whereas ZL arteries were unaffected. This suggests that ET_B primarily opposes ET-1-induced contraction only in ZO arteries but not in ZL arteries. Combined inhibition of ET_A and ET_B receptors reversed the ET_A inhibitor-induced diminution of contraction to ET-1 in ZO arteries. In contrast, in ZL arteries, the maximal contraction to ET-1 after ET_A inhibition was unaffected by the addition of ET_B inhibitor. However, the EC_{50} of ET-1 dose response was significantly increased in both groups, suggesting modulation of ET-1 sensitivity of arteries by ET_B receptors. Interestingly, inhibition of ET_B receptor alone or in combination with ET_A inhibition in ZO arteries restored the contraction to ET-1 and abolished the difference in ET-1 response between ZO and ZL arteries, implicating ET_B receptors in the diminished ET-1 response. Thus it is likely that in ZO arteries, ET_B receptor activation, possibly via enhanced NO production, may have reduced the contraction to ET-1.

We previously reported an enhanced eNOS expression and increased NO production in the coronary (22) and basilar (10) arteries from ZO rats. NO generated via ET_B-receptors (5, 33) regulates ET-1 activity by inhibiting its production in vascular endothelial cells (4, 42), displacing ET-1 from receptors and reducing the [Ca^{2+}]_{i} mobilization in response to ET-1 (15). Therefore, it appeared important to evaluate the involvement of NO in diminishing the contraction to ET-1. Inhibition of eNOS resulted in an increase in ET-1-induced maximal contraction in ZO arteries and a leftward shift of ET-1 response curves in arteries from both groups. Similar to ET_B blockade, inhibition of eNOS abolished the difference in contraction to ET-1 between ZO and ZL arteries. Taken together, ET_B and eNOS inhibition studies implicate both in a concerted action to enhance the generation of NO to reduce the ET-1-induced contraction. It may be explained that elevated eNOS enzyme activity promotes enhanced NO generation in response to ET_B activation, thus resulting in diminished ET-1-induced contraction in ZO arteries.

Alternatively, a decrease in expression of ET_A receptors or increased expression of ET_B receptors can also lead to reduced response to ET-1. It has been known that increased vascular expression and activity of ET_A results in downregulation of ET_A receptors in various disease models (12, 13) where decreased ET-1 response was observed. However, our RT-PCR studies found normal ET-1 and ET_A mRNA expression in SCA of ZO compared with ZL rats. Similarly, Western blot analysis of vascular ET_A receptor protein showed similar expression in both rats. Because ET_B receptors may also mediate ET-1-induced contraction, we evaluated the expression of the ET_B receptor mRNA in ZO and ZL arteries and found them to be similar. Thus altered expression of ET_A or ET_B receptors does not appear to mediate reduced ET-1 response in ZO arteries. Interestingly, Molero et al. (28) reported decreased ET-1 binding, despite normal ET_A protein levels in mesenteric microvessels of DOCA-salt hypertensive rats with diminished ET-1 response. Thus normal ET_A protein levels do not rule out decreased ET-1 vascular binding by an unknown mechanism, resulting in decreased ET-1 response in ZO coronary arteries.

Several studies in diabetes models reported increased VSM cell [Ca^{2+}]_{i} elevation as the mechanism underlying enhanced responses to ET-1 (18, 25, 31). This finding led us to evaluate whether decreased elevation of [Ca^{2+}]_{i} in VSM cells might precede diminished ET-1 response in ZO arteries. By using fluo-4 AM, a calcium-sensitive fluorescent dye, we found that ET-1 induced elevations of [Ca^{2+}]_{i} in freshly isolated VSM cells of both ZO and ZL coronary arteries at 10^{-7} and 10^{-8} M concentrations. Elevation of [Ca^{2+}]_{i} was followed immediately by contraction of VSM cells. Similarly, membrane depolarization by KCl also induced elevation of [Ca^{2+}]_{i}, followed by contraction of VSM cells. VSM cells from ZO arteries, however, displayed decreased peak elevation of [Ca^{2+}]_{i}, in response to ET at both doses tested compared with ZL arteries. In addition, the decrease in length of the VSM cells was also significantly less in ZO compared with ZL. The decreased ET-1-induced peak increase [Ca^{2+}]_{i} and maximal contraction in VSM cells of ZO compared with ZL persisted even after L-NAME treatment. This suggests an additional abnormal VSM contraction to ET-1 independent of endothelium.

To compare and also to verify our findings of [Ca^{2+}]_{i} experiments in SCAs, we performed similar experiments in VSM cells of cerebral arteries in which we observed identical ET-1-induced contraction in both groups of Zucker rats. In contrast to the SCAs, ET-1-induced [Ca^{2+}]_{i} elevation and contraction was found to be similar in VSM cells from cerebral arteries in ZO compared with ZL arteries. Our [Ca^{2+}]_{i} and contraction studies in isolated VSM cells were consistent with our previous vascular reactivity studies in basilar arteries in which we reported normal ET-1-induced contraction in ZO arteries. Interestingly, cerebral arteries and SCAs in ZO rats exhibited a fascinating variation in the underlying mechanisms of vascular dysfunction. Thus our isolated VSM cell uncovered reduced smooth muscle contraction to ET-1 in ZO in addition to the endothelial dysfunction involving the ET_B receptors.

On the basis of the evidence from various pathological states with abnormal ET-1 response, the potential mechanisms of decreased ET-1-induced elevation of [Ca^{2+}]_{i}, may include 1) decreased ET receptors on VSM cells, 2) altered expression of various Ca^{2+} channels, 3) decreased sensitivity of the contractile apparatus to the [Ca^{2+}]_{i}, or 4) uncoupling of receptor activation and Ca^{2+} influx. Western blot analysis of ET_A expression and RT-PCR studies of ET-1, ET_A, and ET_B receptor expression clearly indicated normal receptor numbers in ZO arteries. Furthermore, we observed an identical elevation of [Ca^{2+}]_{i} in VSM cells of ZO and ZL arteries in response to depolarization with KCl. This intact [Ca^{2+}]_{i} response to KCl in ZO, though not conclusive, suggests normal nonreceptor-mediated Ca^{2+} mobilization. Further studies, however, are required to study the expression and to characterize the electrophysiological properties of calcium channels in ZO arteries.

Several VSM cell [Ca^{2+}]_{i} measurement studies in diabetic (25) and hypertensive models (34, 36) have shown that altered Ca^{2+} sensitivity of contractile apparatus leads to an abnormal response to ET-1. Typically, Ca^{2+} sensitivity was assessed by quantitating the relationship between various parameters of contraction and change in myoplasmic free [Ca^{2+}]_{i}. In our data analysis, we derived an arbitrary index of Ca^{2+} sensitivity by dividing %maximal contraction by %maximal increase in [Ca^{2+}]_{i} for individual VSM cells in response to ET-1. In both ZO and ZL cells, the index was close to unity, indicating a
linear relationship between elevation of [Ca$^{2+}$]i, and contraction of VSM cells. No significant difference in the index of relationship between [Ca$^{2+}$]i, and contraction was observed in VSM cells from ZO and ZL arteries, suggesting that Ca$^{2+}$ sensitivity was unlikely to have contributed to reduced ET-1 response. Similar to SCAs, the VSM cells from cerebral arteries also displayed identical [Ca$^{2+}$]i-contractility relationship, as indicated by the arbitrary index of calcium sensitivity. Thus we report for the first time an uncoupling of receptor activation and [Ca$^{2+}$]i, as a likely mechanism underlying the abnormal ET-1-induced contraction in SCAs of ZO rats.

Our data revealed a multitude of mechanisms underlying the diminished ET-1 response in SCAs of ZO rats that involve both endothelium and vascular smooth muscle. Increased ETa-mediated generation of NO and uncoupling of [Ca$^{2+}$]i, elevation from receptor activation are the likely mechanisms underlying the diminished ET-1 response in ZO arteries. Although it is unclear, the abnormal ET-1 response may be a compensatory mechanism to mitigate the pronounced vasoconstrictor burden and vascular remodeling known to occur in IR. The significance of our novel findings is that IR promotes vascular dysfuction via distinctly diverse mechanisms in the cerebral and coronary arteries of identical diameter in ZO rats.

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