Hyperinsulinemia fails to augment ET-1 action in the skeletal muscle vascular bed in vivo in humans

Amale A. Lteif, Angie D. Fulford, Robert V. Considine, Inessa Gelfand, Alain D. Baron, and Kieren J. Mather

Division of Endocrinology and Metabolism, Indiana University School of Medicine, Indianapolis, Indiana; and Amylin Pharmaceuticals, Inc., San Diego, California

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Am J Physiol Endocrinol Metab 295: E1510–E1517, 2008. First published October 28, 2008; doi:10.1152/ajpendo.90549.2008.—Endogenous endothelin action is augmented in human obesity and type 2 diabetes and contributes to endothelial dysfunction and impairs insulin-mediated vasodilation in humans. We hypothesized that insulin resistance-associated hyperinsulinemia could preferentially drive endothelin-mediated vasoconstriction. We applied hyperinsulenic-euglycemic clamps with higher insulin dosing in obese subjects than lean subjects (30 vs. 10 mU·m−2·min−1, respectively), with the goal of matching insulin’s nitric oxide (NO)-mediated vascular effects. We predicted that, under these circumstances, insulin-stimulated endothelin-1 (ET-1) action (assessed with the type A endothelin receptor antagonist BQ-123) would be augmented in proportion to hyperinsulinemia. NO bioactivity was assessed using the nitric oxide synthase inhibitor G-monomethyl-L-arginine. Insulin-mediated vasodilation and insulin-stimulated NO bioavailability were well matched across groups by this approach. As expected, steady-state insulin levels were approximately threefold higher in obese than lean subjects (109.2 ± 10.2 pmol/l vs. 51.8 ± 8.0, P = 0.03). Despite this, the augmentation of insulin-stimulated vasodilation by BQ-123 was not different between groups. ET-1 flux across the leg was not augmented by insulin alone but was increased with the addition of BQ-123 to insulin (P = 0.01 BQ-123 effect, P = not significant comparing groups). Endothelin antagonism augmented insulin-stimulated NO bioavailability and NOx flux, but not differently between groups and not proportional to hyperinsulinemia. These findings do not support the hypothesis that insulin resistance-associated hyperinsulinemia preferentially drives endothelin-mediated vasoconstriction.

endothelin-1; insulin; obesity

ENDOTHELIAL DYSFUNCTION IS A FEATURE of human obesity and type 2 diabetes mellitus, and parallel impairments in insulin’s vascular and metabolic actions are seen in proportion to insulin resistance (3, 23). This has been attributed to impaired bioavailability of nitric oxide (NO) due to reduced actions of insulin to stimulate NO production, in part supported by observations that insulin’s actions to stimulate the production of NO are mediated by insulin receptor substrate-1, phosphatidylinositol 3-kinase (PI3-kinase), and protein kinase B (17, 20, 56). Derangements in signal transduction in these pathways contribute to impairments in insulin’s metabolic actions in states of insulin resistance and appear to also underlie impairments in this vascular action of insulin (8, 27, 34, 46, 50).

We and others have shown, however, that excess endogenous action of endothelin also contributes importantly to endothelial dysfunction in human obesity and type 2 diabetes (5, 6, 29). This is presumed to reflect increased production, and observations in vitro and in vivo implicate insulin as a driver of endothelin-1 (ET-1) production (14, 18, 36). Insulin appears to modulate ET-1 production via mitogen-activated protein kinase (MAPK)/MEKK pathway systems (11, 40). Importantly, these response pathways do not seem to be affected by insulin resistance. This has been described as “selective” insulin resistance (19, 33), the notion that insulin’s various actions may be differentially affected by impairments in insulin response in states of metabolic insulin resistance. In this context, the possibility arises that the compensatory hyperinsulinemia of the insulin-resistant state would concurrently drive the overproduction of endothelin, resulting in a net impairment of vascular function on this basis.

The principal experimental difficulty in exploring these questions in vivo is the presumed concurrent action of insulin on both NO and endothelin. This is particularly important because NO can itself contribute to the regulation of endothelin production and action (1, 16, 26, 30, 47, 51). One approach to separate these two actions is to experimentally match effects of insulin on one of these two pathways across groups. In the current studies, we have applied higher insulin dosing in obese subjects than lean subjects, with the goal of matching insulin’s effects on NO but magnifying the group difference in insulinemia. If the premise is true that insulin’s stimulation of ET-1 is not subject to insulin resistance, then this circumstance would be expected to produce greater stimulation of ET-1 in response to the greater hyperinsulinemia achieved. We have assessed ET-1 action as the vasodilator response to the type A endothelin receptor antagonist BQ-123, comparing circumstances with insulin alone vs. insulin plus endothelin antagonism. We hypothesized that the mismatched hyperinsulinemia would produce matched NO bioavailability but mismatched ET-1 action, with magnified vasodilator response to BQ-123 in obese subjects in response to the greater insulin exposure.

METHODS

Subjects were recruited through newspaper advertisement and classified as either lean or obese according to body mass index cut points of ≥26 for men or ≥28 for women. Exclusion criteria included hypertension (systolic blood pressure >140/diastolic blood pressure >90) or antihypertensive therapy, elevated serum lipids (total choles-

Address for reprint requests and other correspondence: K. Mather, Division of Endocrinology & Metabolism, Dept. of Medicine, Indiana Univ. School of Medicine, CL459, 541 North Clinical Dr., Indianapolis, IN 46202 (e-mail: kmather@iupui.edu).
terol >5.2 mmol/l, low-density lipoprotein >2.3 mmol/l, or triglyceride >2.0 mmol/l), biochemical evidence of renal or hepatic dysfunction, or significant underlying medical conditions. All subjects underwent a standard 75-g oral glucose tolerance test to screen for diabetes mellitus and had body composition assessed by dual-energy X-ray absorptiometry measurement. This study was approved by the local Institutional Review Board, and all subjects gave written informed consent. All procedures were performed in accordance with institutional guidelines.

Technique. A 6-Fr sheath (Cordis, Miami, FL) was placed in the right femoral vein to allow the insertion of a custom-designed 5-Fr double-lumen thermodilution catheter (Baxter Scientific, Edwards Division, Irvine, CA) to measure leg blood flow (LBF). The right femoral artery was cannulated with a 5.5-Fr double-lumen catheter to allow simultaneous infusion of vasoactive agents and invasive blood pressure monitoring via a vital signs monitor (Spacelabs, Redmond, WA). All hemodynamic measurements were obtained with the subjects in the supine position in a quiet temperature-controlled room. Basal LBF and mean arterial pressure (MAP) measurements were obtained following a 30-min rest after the insertion of the catheters. Femoral vein thermodilution curves were used to measure rates of LBF, calculated by integration of the area under the curve, using a cardiac output computer (model 9520A; American Edwards Laboratories). Initially, 24 LBF measurements were obtained at ~30-s intervals and averaged to obtain basal readings. During subsequent drug infusions, the mean of 10 measurements was taken at each study stage. Invasively determined MAP was recorded with every other LBF determination.

Protocol. Vascular responses were measured as changes in LBF as above, with vasoactive agents administered via the ipsilateral femoral artery. Continuous bedside monitoring of heart rate and intravascular blood pressure allowed full hemodynamic assessment.

ET-1 blockade was achieved with BQ-123 (Clinalfa, Basel, Switzerland), a high-affinity competitive inhibitor of ET-1 type A receptors. We have previously demonstrated increased endogenous endothelin activity in obese and diabetic subjects compared with lean subjects using an intrafemoral arterial infusion rate of 0.6 mg/min (1 μmol/min) (29), and this rate was used in the current study protocol. Nω-monomethyl-L-arginine (L-NMMA) (Clinalfa), a competitive antagonist of L-arginine, was infused at 16 mg/min in the femoral artery to block NO generation by nitric oxide synthase (NOS). This is the standard infusion rate used in our laboratory (4, 30), chosen on the basis that it provides near-maximal effect across all populations. Four-hour hyperinsulinemic-euglycemic glucose clamps were performed using different insulin exposure for lean (10 μU·m⁻²·min⁻¹) and obese (30 μU·m⁻²·min⁻¹) subjects, by design imposing hyperinsulinemic conditions that would provide matched effects on skeletal muscle glucose metabolism and therefore on insulin-stimulated NO across the groups (28) but achieving different circulating insulin levels. Insulin was administered systemically via an antecubital vein.

The protocol is presented diagrammatically in Fig. 1. All studies were performed following an overnight fast. Female subjects with a regular menstrual cycle were studied in the menstrual phase (days 1–7) of their cycle. Subjects were studied in random sequence on two occasions, receiving insulin alone on one occasion and insulin with concurrent BQ-123 on the other occasion. On each study day, basal vascular responses were measured before the initiation of the hyperinsulinemic-euglycemic clamp. Steady state was defined as the stage when glucose infusion rate changes of ≤5% were required to maintain euglycemia, and was at least 210 min from the initiation of the clamp procedure. Further vascular measurements were made at this stage, followed by measurements during the coinfusion of L-NMMA.

Laboratory. Blood for serum glucose determinations was put in untreated polypropylene tubes and centrifuged using an Eppendorf microcentrifuge (Brinkman, Westbury, NY). The glucose concentration of the supernatant was then measured by the glucose oxidase method using a glucose analyzer (model 2300; Yellow Springs Instruments, Yellow Springs, OH). Blood for determination of plasma insulin was collected in heparinized tubes, processed immediately, and frozen at −20°C. Insulin determinations were made using a dual-site radioimmune assay, specific for human insulin and with cross-reactivity with proinsulin <0.2%. The lower detection limit is 0.56 pmol/l, and in our laboratory the inter- and intra-assay coefficients of variation (CV) are 4.1 and 2.6%, respectively. Total serum nitrate/nitrite (NOx) was determined by colorimetric assay (Cayman Chemical, Ann Arbor, MI). The limit of detection for this assay is 1 μM, with intra- and interassay CV of 2.7 and 3.4%, respectively. NOx flux was calculated as femoral venous NOx times LBF and was used as an index of net NO production. Endothelin levels were determined by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis MN). The limit of detection for this assay is 1.0 pg/ml, with intra- and interassay CV of 5.1 and 4.5%, respectively, and <1% crossreactivity to big ET-1. Endothelin flux was calculated as femoral venous ET-1 times LBF and was used as an index of ET-1 production. Adiponectin was measured with a commercially available RIA kit (Linco Research, St. Charles, MO). The limit of sensitivity of this assay is 1 ng/ml with an intra- and interassay precision of 6.2 and 6.9%, respectively. Standard methodologies for cholesterol and triglyceride determinations were performed through our local hospital's clinical laboratory.

Statistical analysis. Given the previously recognized effects of endothelin antagonism on blood pressure, we anticipated needing to account for different blood pressures after intervention between the two study days, and therefore prespecified leg vascular conductance (LVC = LBF/MAP) as the primary endpoint of interest. Data that were not normally distributed were normalized through logarithmic transformations before analysis. Comparisons between and within groups were performed by t-tests, ANOVA, and repeated-measures ANOVA for paired data as appropriate. For the latter, we performed a generalized version of the repeated-measures ANOVA using linear mixed-models procedures, using the subjects as a random factor and the presence or absence of the relevant antagonist as a contrast factor. Statistical significance was accepted at a level of P < 0.05. Population descriptive statistics are presented as means ± SD; otherwise, results are presented as means ± SE.

A priori our study was designed to include nine subjects in each group, with power to detect a group difference in the LVC increment in response to insulin with and without BQ-123 of ~12 units (i.e., a difference in the augmentation of insulin’s vasodilation in response to BQ-123 between groups of this magnitude) with P = 0.05 and 80% power, assuming within-subject correlations of 0.6 for repeated measures. In prior studies, the observed variability in LVC measures is 8.2 units (pooled across subgroups and measurement conditions). The hypothesis relates most directly to this effect of BQ-123, and therefore the primary endpoint for analysis was the BQ-123-induced change in insulin-mediated vasodilation, comparing the response between the two groups. Secondary endpoints for analysis included BQ-123-

<table>
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<th>Intervention</th>
<th>NOS Antag</th>
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<tr>
<td>Time (min)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-30</td>
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Vascular Measures

<table>
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<tr>
<th>Study 1</th>
<th>Saline</th>
<th>Insulin/Glucose Clamp -NMMa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study 2</td>
<td>Saline</td>
<td>Clamp + BQ123 -NMMa</td>
</tr>
</tbody>
</table>

Fig. 1. Study schema. Subjects were studied on 2 occasions in random sequence, receiving a systemic hyperinsulinemic-euglycemic clamp (300 mU·m⁻²·min⁻¹) alone or in combination with intrafemoral arterial endothelin type A (ETₐ) antagonism (BQ-123, 1 μmol/min). The nitric oxide synthase antagonist Nω-monomethyl-L-arginine (L-NMMA) was infused at the end of each study (16 mg/min). Arrows indicate stages where vascular and hemodynamic measurements were taken.
induced changes in insulin-stimulated ET-1 levels and ET-1 flux and changes in NO bioavailability, NOx levels, and NOx flux.

RESULTS

The subject characteristics are presented in Table 1. We studied nine lean and nine obese subjects, with full paired data available in all subjects for the primary endpoint analysis. Full data to the end of the l-NMMA stage of both paired studies were available in eight lean and six obese subjects due to technical difficulties arising during these last stages. As expected, obese subjects had higher body mass index, waist circumference, and insulin levels. Obese subjects had marginally higher glucose and triglyceride levels, not statistically different from lean subjects. Adiponectin was significantly lower in the obese subjects. No differences across groups in the lipid profiles were seen.

Matched metabolic and vascular effects of insulin. The two insulin infusion rates were chosen on the basis of their anticipated equivalence in achieving insulin-stimulated glucose disposal. This was in fact achieved (whole body glucose disposal rate of $4.9 \pm 0.7 \, \text{mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ in lean vs. $5.4 \pm 0.7$ in obese, $P = 0.59$). Similarly, the steady-state arterial-venous glucose difference (lean $17.5 \pm 2.5$ vs. obese $20.7 \pm 2.7 \, \text{mg} / \text{dl}$, $P = 0.20$) and leg glucose uptake (lean $51.2 \pm 10.2$ vs. obese $54.7 \pm 11.0 \, \text{mg} / \text{min}$, $P = 0.31$) were well matched under these conditions.

As expected, insulin-mediated vasodilation was also well matched by this maneuver. Baseline blood flow rates were nonsignificantly higher in obese subjects (lean $0.216 \pm 0.010$ vs. obese $0.279 \pm 0.012 \, \text{l/min}$, $P = 0.10$). LVC was well matched at baseline (lean $24.4 \pm 6.8$ vs. obese $23.1 \pm 5.8$ units, $P = 	ext{not significant (NS)}$). The increments in LVC achieved with insulin were modest (as expected given the low insulin doses used) but statistically significant, and equivalent across groups (change from baseline in LBF: lean $4.8 \pm 2.7$ vs. obese $5.8 \pm 3.0$ units; $P = 0.01$ for insulin, $P = 0.8$ comparing groups; Fig. 2, top). Steady-state blood pressure (MAP: lean $91.9 \pm 4.1$ vs. obese $97.9 \pm 4.4 \, \text{mmHg}$, $P = 0.23$) was not statistically different across groups, suggesting that the vascular and hemodynamic effects of insulin were well matched as designed. Overall, these low-dose insulin exposures induced $-22\%$ increases in vascular conductance, and by design these changes were not statistically different across groups. Furthermore, the decrement in LVC achieved with the NOS antagonist l-NMMA was equivalent between groups (LVC reduced by $11.7 \pm 4.8$ units lean, by $13.9 \pm 6.0$ obese; $P = 0.007$ l-NMMA effect, $P = 0.8$ comparing groups: Fig. 3, top left). Therefore, both insulin-mediated vasodilation and insulin-stimulated NO bioavailability were augmented by insulin to an equivalent degree in both groups.

By design, the matching of glucose metabolic rates and vasodilation between the groups was achieved by imposing markedly different steady-state levels of insulinemia. At baseline, obese subjects had approximately twofold elevated insulin levels (Table 1). Under steady-state conditions without concurrent BQ-123 infusion, insulin concentrations were $109.2 \pm 10.2 \, \text{pmol/l}$ in lean subjects and $518.4 \pm 84.0 \, \text{pmol/l}$ in obese ($P = 0.03$). Concurrent BQ-123 did not materially change the steady-state insulin levels achieved (lean $112.8 \pm 15.0$, obese $378.6 \pm 115.2 \, \text{pmol/l}$, $P < 0.001$; $P = 	ext{NS compared with insulin alone}$). Therefore, under both study conditions, the steady-state insulin levels were approximately threefold higher in obese than lean subjects.

Insulin-stimulated endothelin action and production. The vasodilator response to insulin was markedly augmented by coinfusion of the endothelin antagonist BQ-123 ($P = 0.006$, Fig. 2, bottom left). We observed essentially a near-doubling of LVC (lean increase from $23.7 \pm 3.9$ to $44.3 \pm 16.6$; obese increase from $26.5 \pm 3.1$ to $46.1 \pm 15.4$; significant for groups combined and individually; Fig. 2). The augmentation of insulin-mediated vasodilation by BQ-123 represents endothelin action under insulin stimulation. Contrary to the anticipated response, this response was not different between groups ($P = 0.9$) despite the differing insulin exposures. Furthermore, by repeated-measures ANOVA comparing the change in LVC in response to insulin with and without BQ-123 (Fig. 2, top right), although the overall effect of BQ-123 to augment insulin-mediated vasodilation was significant for each group individually and for all subjects combined ($P = 0.03$), this effect did not differ across groups ($P = 0.60$).

Insulin alone did not significantly augment endothelin levels or flux (Table 2); in particular, this was not seen even in obese subjects despite the markedly different insulinemia both at baseline and under steady-state conditions. Because of augmentation of LBF and increased levels of endothelin in the venous effluent of the leg, coapplication of BQ-123 resulted in a significantly increased endothelin flux compared with insulin-only conditions ($P = 0.02$, $P = 	ext{NS comparing groups}$). There was no apparent effect of BQ-123 to modulate insulin levels, suggesting that this effect was not mediated by unanticipated changes in this presumed stimulus to endothelin production.

In keeping with the lack of apparent effect of hyperinsulinemia on endothelin action, we found no evidence for direct associations between insulin levels and endothelin levels or flux, at baseline or at steady state ($P = 	ext{NS for both}$). Similarly, the change in endothelin levels or change in endothelin flux was not associated with steady-state insulin levels or change in insulin levels from

### Table 1. Subject characteristics

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<thead>
<tr>
<th></th>
<th>Lean ($n = 9$)</th>
<th>Obese ($n = 9$)</th>
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<tbody>
<tr>
<td>Age, yr</td>
<td>36.5 (6.7)</td>
<td>35.6 (8.9)</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>6/3</td>
<td>4/5</td>
</tr>
<tr>
<td>Race (AA/C)</td>
<td>5/4</td>
<td>5/4</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>21.7 (1.4)</td>
<td>32.8 (4.0)*</td>
</tr>
<tr>
<td>Fat, %</td>
<td>16.0 (6.8)</td>
<td>39.7 (10.4)*</td>
</tr>
<tr>
<td>Waist, cm</td>
<td>72.2 (10.5)</td>
<td>89.6 (7.5)*</td>
</tr>
<tr>
<td>Glucose, mmol/l</td>
<td>4.7 (0.6)</td>
<td>5.7 (0.7)</td>
</tr>
<tr>
<td>Insulin, pmol/l</td>
<td>56.2 (40.5)</td>
<td>105.5 (25.6)*</td>
</tr>
<tr>
<td>NEFA, mmol/l</td>
<td>0.49 (0.28)</td>
<td>0.76 (0.61)</td>
</tr>
<tr>
<td>Endothelin-1, pg/ml</td>
<td>1.94 (1.69)</td>
<td>1.37 (0.76)</td>
</tr>
<tr>
<td>Adiponectin, µg/ml</td>
<td>8.4 (2.8)</td>
<td>6.8 (3.7)*</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>114.3 (16.0)</td>
<td>116.3 (13.7)</td>
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<tr>
<td>DBP, mmHg</td>
<td>68.7 (11.1)</td>
<td>68.7 (10.2)</td>
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<tr>
<td>Cholesterol, mmol/l</td>
<td>3.9 (0.6)</td>
<td>4.3 (1.0)</td>
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<td>LDL, mmol/l</td>
<td>2.3 (0.4)</td>
<td>2.3 (1.3)</td>
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<tr>
<td>HDL, mmol/l</td>
<td>1.2 (0.3)</td>
<td>1.3 (0.3)</td>
</tr>
<tr>
<td>Triglycerides, mmol/l</td>
<td>1.1 (0.5)</td>
<td>1.6 (1.0)</td>
</tr>
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Values represent means (SD). M, males; F, females; AA, African American; C, Caucasian; BMI, body mass index; NEFA, nonesterified fatty acids; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL, low-density lipoprotein cholesterol; HDL, high-density lipoprotein cholesterol. *$P < 0.01$ and †$P < 0.05$ across groups.
baseline to steady state (P = NS for both). Figure 4 demonstrates the lack of relationship between steady-state ET-1 levels and the insulin-induced increment in LBF. The augmentation of insulin-mediated vasodilation afforded by BQ-123 is evident in the offset of the values between treatments, but it is clear that this is not related to ET-1 levels either with or without concurrent BQ-123 (P = NS for both slopes vs. 0).

**Insulin-stimulated NO bioavailability.** The vasoconstrictor responses to l-NMMA were augmented in both groups by coapplication of BQ-123 (P = 0.04 evaluating all subjects; Fig. 3, top right), demonstrating that endothelin limits insulin-stimulated NO bioavailability. There was a nominally larger l-NMMA-induced decrement in LVC with BQ-123 in obese (28.5 ± 4.6 units) vs. lean (19.3 ± 4.6 units) subjects, but this group difference did not achieve statistical significance (P = 0.3). By repeated-measures ANOVA, there was no evident difference between groups in this response (P = 0.3). This finding is congruent with the equivalent augmentation of insulin-mediated vasodilation across the two groups afforded by BQ-123. NO flux was augmented in parallel with these vasomotor changes (Table 2), significantly increased compared with baseline by insulin plus BQ-123 (P = 0.04; P = NS comparing groups), but not increased by low-dose insulin alone (P = NS). Reductions in NOx flux in response to l-NMMA were moderately but not significantly greater with BQ-123 than without, not different across groups (Fig. 3, bottom right).

**DISCUSSION**

Insulin-mediated vasodilation is impaired in obesity and limited in part by endogenous endothelin-mediated vasoconstriction. To test the hypothesis that the hyperinsulinemia associated with insulin resistance could preferentially drive
endothelin-mediated vasoconstriction, we applied differential low-dose hyperinsulinemic glucose clamps in lean and obese humans, targeting matched insulin-stimulated NO bioavailability. Under these conditions, the differential insulinemia was predicted to preferentially augment endothelin action in obese subjects.

This approach provided well-matched metabolic and baseline vascular effects, including matched insulin-mediated vasodilation and matched responses to the NOS inhibitor L-NMMA. This suggests that vascular insulin actions via the NO system were comparable, as intended. This was accomplished with markedly different insulin levels, approximately threefold higher in obese than lean subjects at steady state. Despite this differential insulinemia, we did not find any suggestion of augmented ET-1 production or action in obese vs. lean subjects. Coinfusion with the endothelin antagonist BQ-123 allowed augmented insulin-mediated vasodilation in both groups, but without a significantly greater response in the obese subjects to reflect the increased insulinemia.

An alternate path to augmented insulin-mediated vasodilation by endothelin antagonism would be an unmasking of insulin’s actions to stimulate NO (30). However, this was also not seen. Although the response to the NOS antagonist L-NMMA was modestly but not significantly increased (in the direction of increased NO bioavailability) among obese subjects treated with insulin + BQ-123, this was not statistically different from the effect seen in lean subjects. These data failed to demonstrate the hypothesized augmentation of insulin-stimulated endothelin action with greater hyperinsulinemia in obese subjects and raise the possibility that hyperinsulinemia may not be sufficient to explain augmented endothelin biology in vivo in human obesity.

Insulin, ET-1 production, and ET-1 action. In vitro, insulin’s action to stimulate the production of endothelin is dose-dependent (14, 18, 36) across a concentration range from physiological to supraphysiological. These actions are mediated by MAPK pathways (11, 40), which are not typically impaired under conditions of insulin resistance. Experimental conditions that specifically impose insulin resistance to NO stimulation (for example, using PI3-kinase inhibitors) fail to block insulin’s actions on endothelin production in vitro (9, 11, 40). We predicted that the differential hyperinsulinemia afforded by the current design would result in augmented ET-1 production and action in obese compared with lean subjects. Instead, we observed matched insulin action with and without endothelin antagonism between the groups despite the greater hyperinsulinemia to which obese subjects were exposed. To assess the validity of this unexpected result, we have critically evaluated the following major underlying assumptions.

Assumptions in the experimental design. The design assumes that an action of insulin to stimulate the endothelin system will be evident after a 4-h exposure to hyperinsulinemia. Unlike insulin’s action on NO production, which is mediated by modulation of the activity of existing enzyme systems, insulin action on endothelin requires manufacture, processing, release, and action of new peptides. In vitro, 2 h were sufficient for production and secretion of endothelin from cultured endothelial cells (14, 36). Furthermore, other laboratories have demonstrated changes in ET-1 levels with acute metabolic challenges like oral glucose tolerance testing, where the duration of stimulation is considerably shorter than we have applied (12, 49, 55). Studies with hyperinsulinemic clamps analogous to ours have also previously been shown to evoke increases in circulating ET-1 (14). Although in vivo dose-response data in humans are not available, the assumption that higher levels of insulin should have produced a greater effect in obesity is supported by in vitro data demonstrating a clear dose response across the range of insulin levels we produced (14, 31). Overall, it seems likely that the insulin exposure was itself sufficient in both concentration and duration to have elicited an effect on endothelin production.

Slow vasodilator responses to endothelin antagonism are well recognized, with full actions often requiring 60 min. We provided exposure to BQ-123 throughout the 4-h duration of the insulin infusion, using an infusion rate that has been previously shown by us, using the current techniques, to demonstrate midrange vasodilation with a signal range suffi-
cient to recognize increases in LVC of as little as 30% (29). A priori our study was designed to detect a group difference in the BQ-123-induced increase in the insulin-induced increment in LVC of ~12 units; the observed group differences of ~1 units were unequivocally negative (i.e., reflecting a true lack of effect rather than problems with statistical power). Also, as described above, the range of vasodilation responses observed was well below the maximum range of our technique. Overall, we cannot account for this unexpectedly negative result on the basis of design or technical issues.

**Assumptions in the hypothesis.** The notion of selective insulin resistance underlies the presumption that greater hyperinsulinemia might have induced proportionally greater ET-1 production and action. This in turn is based on the premises that 1) insulin actions to NO are affected by insulin resistance, whereas insulin actions to ET-1 are not; and 2) insulin’s actions to regulate ET-1 are direct and proportional. What is the evidence in support of these assumptions?

**Premise 1** appears to be well grounded. The literature contains convincing evidence from in vitro and animal studies in favor of selective insulin resistance (9, 10, 40, 41). This has been most unequivocally demonstrated in experimental circumstances where insulin’s actions via PI3-kinase are inhibited by wortmannin, leaving residual actions to ET-1 unaffected (9, 11, 40). Of note, only one of these studies evaluated dose-response relationships (11), and this study was done in non-obese rats. The question remains open whether this effect also applies to vessels from insulin-resistant animals.

**Premise 2** (whether the actions to stimulate ET-1 are proportional to hyperinsulinemia) has been evaluated in vitro but not confirmed in vivo. The maximal increment in ET-1 secretion appears to be in the range of fourfold, but, with insulin in physiological (nM) ranges, the increment is reported to be 20–200% (14, 18, 31, 35). Interestingly, in one study, insulin appeared to be acting to stimulate ET-1 production via the insulin-like growth factor (IGF)-I receptor. This may be germane, since endothelial cells express both insulin and IGF-I receptors, and the insulin concentrations achieved in the obese subjects are at the bottom end of the range that can signal via IGF-I receptors (24).

Dose-dependent effects of insulin on endothelin-mediated vasoconstriction have been reported in arterioles isolated from normal rats during concurrent NOS synthase antagonism (9) and in vascular rings from spontaneously hypertensive rats (43). These observations suggest that a second source of vascular dysfunction may be necessary to demonstrate insulin-mediated vasoconstriction via endothelin.

Although these in vitro data are consistent, effects of insulin infusions on ET-1 levels reported in humans are surprisingly inconsistent. Some authors report reductions in ET-1 with insulin (15, 39, 48, 51), some report neutral effects (21, 31), and others report increases in ET-1 levels (13, 38, 49, 54). Where increases are found, they have been on the order of 30% and are not obviously larger with greater insulin exposure. Interestingly, insulinoma patients do not exhibit elevated levels of ET-1 (38). In the current study, under insulin stimulation, we saw a significant increase in ET-1 flux (Table 2) but nominally less in obese subjects than lean subjects and clearly not proportional to insulinemia. Regardless of the underlying explanation, the current data argue against the notion of a dose response between insulin and endothelin production or action in humans, at least in physiological concentration ranges under the clamp conditions studied.

**An alternate hypothesis.** We observed matched effects of insulin on endothelin action under the experimental circumstance where insulin’s actions on NO were matched between groups. Regulatory and functional interactions of NO and ET-1 are well documented, including in vivo in humans (1, 7, 30). In particular, acute increases in bioavailable nitrates can acutely suppress ET-1 levels and action (9, 37). The current observations may therefore reflect the matching of insulin’s NO-dependent vascular actions, rather than separate effects via differential hyperinsulinemia. This may explain why the balanced insulin exposure produced equal insulin-stimulated BQ-123-induced vasodilation. With this perspective, under normal circumstances for subjects with insulin resistance, the failure of insulin to activate NO (or reduced NO bioavailability more generally) would fail to suppress ET-1, leading to augmented ET-1 action. The capacity of insulin to directly stimulate ET-1 may therefore be misleading, and this action may not play an important role in the in vivo determination of ET-1 action. Separate studies will be required to test this idea.

**Endothelin limits insulin-mediated vasodilation.** These studies unequivocally demonstrate augmented insulin-mediated vasodilation following application of BQ-123, which was not different between groups. An effect of endothelin to limit insulin-stimulated vasodilation has been reported in both animals models (10, 22, 32, 44, 45, 52) and in insulin-resistant humans (2, 25, 42). The careful matching of insulin’s actions on NO between insulin-sensitive and insulin-resistant subjects before the application of the endothelin antagonist is unique to our study. This approach circumvents any concerns about whether the stimulus of insulin to NO was comparable and simultaneously provides mismatched hyperinsulinemia. In this circumstance, we have demonstrated a parallel effect of endothelin antagonism to augment insulin-mediated vasodilation in both lean and obese subjects. Therefore, this phenomenon is not unique to the insulin-resistant state but a generalized feature of insulin’s vascular action.

Is endothelin directly limiting insulin-mediated vasodilation? If so, once the limiting action of endothelin was removed, the obese subjects (exposed to 3-fold higher insulin concentrations) should have demonstrated a proportionally greater vasodilator or NO stimulation response to insulin compared with the lean subjects. This might have been manifest in the following three ways: as a magnified vasodilatory response to endothelin antagonism, as a magnified vasoconstrictor response to NOS antagonism, and/or as a magnified production of nitrates by the leg. Of these, we observed only a modestly and nonsignificantly augmented constrictor response to 1-NMMA with insulin when BQ-123 was applied compared with insulin alone ($P = 0.14$; Fig. 3). There was no apparent magnification of BQ-123-induced vasodilation compared with lean subjects, and, similarly, the augmentation of NOx flux that was seen with insulin + BQ-123 vs. insulin alone did not differ between lean and obese subjects. Overall, endothelin antagonism alone was not sufficient to normalize vascular insulin sensitivity in obese subjects, suggesting that endothelin alone does not account for vascular insulin resistance in humans.

Increased endothelin action is a feature of insulin resistance in both animals and humans (5, 6, 29). The question at hand is whether this is attributable to the attendant hyperinsulinemia or...
to some other feature(s) of the insulin-resistant state. These findings do not support the hypothesis that insulin resistance-associated hyperinsulinemia preferentially drives endothelin-mediated vasoconstriction.

**Limitations.** In these studies, we have assessed actions of endothelin via type A receptors only. Endothelial responses to endothelin include NO production via type B receptors, and it is of interest whether the balance of ET receptor activation is impaired in circumstances of impaired endothelial function. In the current study, we can comment that no apparent compensatory augmentation of endothelin-stimulated NO production appeared to have confounded the findings, but potential contributions of actions via the type B receptor have not been explicitly evaluated. t-NMMA has long-lasting effects and therefore was only applied last in the current study’s protocols. Future work will evaluate effects of insulin on endothelin-mediated vasodilation in the context of NOS antagonism, which will contribute to this question.

We did not observe consistently measurable increases in ET-1 levels with the insulin exposures used, and the isolated observation of increased ET-1 flux during combined exposure to insulin and BQ-123 is difficult to reconcile with the lack of apparent effect under other conditions. Such increases are not uniformly evident in the literature (reviewed above), and it is unclear whether this is attributable to biological variability or technical issues in the measurement. Arguably, the circulating levels are of secondary interest because of the recognized abluminal secretion of endothelin from endothelial cells (53). Our focus on ET-1 action (response to the endothelin receptor antagonist) rather than levels circumvents this issue somewhat, but our results would be more definitive if we had observed more consistent increases in circulating ET-1. The interpretation of our data is not compromised, however, because of our choice of vascular function endpoints in the design of the study.

Not all subjects completed the final stages of the protocol to provide t-NMMA data. With this reduced sample, it is possible that a positive finding was missed because of compromised power for these secondary endpoints. However, the apparent group difference in t-NMMA response between insulin-only and insulin + BQ-123 conditions was ~9 units, and even with the full complement of subjects we were only powered to detect differences of ~12 units, so it is unlikely that this reduction in power materially changed the overall findings relating to this secondary endpoint. Also, the inclusion of the full complement of patients for the primary endpoint allows us to be unequivocal about the core findings of this study.

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**References**

SELECTIVE INSULIN RESISTANCE AND ET-1


