Obesity, insulin resistance, and skeletal muscle nitric oxide synthase

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Submitted 11 August 2011; accepted in final form 27 June 2012

Obesity, insulin resistance, and skeletal muscle nitric oxide synthase. J Appl Physiol 113: 758–765, 2012. First published July 12, 2012; doi:10.1152/japplphysiol.01018.2011.—The molecular mechanisms responsible for impaired insulin action have yet to be fully identified. Rodent models demonstrate a strong relationship between insulin resistance and an elevation in skeletal muscle inducible nitric oxide synthase (iNOS) expression; the purpose of this investigation was to explore this potential relationship in humans. Sedentary men and women were recruited to participate (means ± SE: nonobese, body mass index = 25.5 ± 0.3 kg/m2, n = 13; obese, body mass index = 36.6 ± 0.4 kg/m2, n = 14). Insulin sensitivity was measured using an intravenous glucose tolerance test with the subsequent modeling of an insulin sensitivity index (S1). Skeletal muscle was obtained from the vastus lateralis, and iNOS, endothelial nitric oxide synthase (eNOS), and neuronal nitric oxide synthase (nNOS) content were determined by Western blot. S1 was significantly lower in the obese compared with the nonobese group (−43%; P < 0.05), yet skeletal muscle iNOS protein expression was not different between nonobese and obese groups. Skeletal muscle eNOS protein was significantly higher in the nonobese than the obese group, and skeletal muscle nNOS protein tended to be higher (P = 0.054) in the obese compared with the nonobese group. Alternative analysis based on S1 (high and low tertile) indicated that the most insulin-resistant group did not have significantly more skeletal muscle iNOS protein than the most insulin-sensitive group. In conclusion, human insulin resistance does not appear to be associated with an elevation in skeletal muscle iNOS protein in middle-aged individuals under fasting conditions.

insulin resistance; nitric oxide; minimal model; obesity; iNOS; eNOS; nNOS

Peripheral insulin resistance is central to the etiology of Type 2 diabetes and cardiovascular disease (10, 30, 36), and understanding insulin resistance is thus invaluable for prevention and treatment. Considerable evidence suggests that intact insulin signal transduction in skeletal muscle is required for optimal whole-body insulin-mediated glucose transport (15, 54, 59), and defects in insulin signaling have been observed in the skeletal muscle of insulin-resistant humans (4, 9, 25, 28, 32). Specifically, insulin-stimulated insulin receptor substrate-1 (IRS-1) tyrosine phosphorylation, phosphatidylinositol-3 kinase (PI3K) activity, and Akt-2 activity have been reported to be impaired in the muscle of obese individuals or patients with Type 2 diabetes (9, 25). To date, the intracellular mechanisms responsible for these impairments remain unclear.

Inducible nitric oxide (NO) synthase (iNOS) recently has been linked to insulin resistance (27). Genetically obese diabetic (ob/ob) mice (21, 43, 52), Zucker diabetic fatty rats (43), high fat-fed mice (43), and high fat-fed rats (12) all have significantly greater skeletal muscle iNOS expression compared with their respective controls without an associated increase in endothelial nitric oxide (eNOS) synthase (eNOS) or neuronal nitric oxide synthase (nNOS). Perreault and Marette found that high fat-fed mice with a specific disruption in the iNOS gene (iNOS/−/−), which nullified iNOS activity, were protected from the decrements in both skeletal muscle insulin signaling and insulin-mediated glucose transport observed in high-fat-fed wild-type mice (43). Similarly, genetically obese diabetic (ob/ob) mice with an additional disruption of the iNOS gene (iNOS/−/− ob/ob), which eliminated iNOS activity, had significantly greater insulin-stimulated skeletal muscle IRS-1 tyrosine phosphorylation, PI3K activity, and glucose transport than iNOS+/+ ob/ob mice (52). The authors proposed that the production of NO by iNOS was responsible for inducing insulin resistance in the wild-type animals (43, 52). A possible mechanism for this impairment in insulin signaling by iNOS was recently described as a cause-and-effect relationship between an elevation in S-nitrosation (the addition of NO to the thiol group of a cysteine residue) of Akt and a reduction in Akt activity significantly reduced insulin-mediated glucose transport (12, 58). These data thus provide an explicit means by which excessive NO generated from iNOS could impair insulin signaling and insulin-mediated glucose transport in skeletal muscle (12, 58).

In humans, the relationship between iNOS and insulin action has yet to be sufficiently investigated. Relatively few publications report iNOS expression in human skeletal muscle (2, 38–40, 47, 55), and the only investigation to relate skeletal muscle iNOS expression to metabolism found that patients with uncontrolled Type 2 diabetes had approximately four times greater skeletal muscle iNOS protein than control subjects (55). The purpose of the present study was to determine whether skeletal muscle iNOS was higher in obese than in nonobese and higher in insulin-resistant compared with insulin-sensitive subjects. It was hypothesized that the insulin-resistant, obese subjects would have greater skeletal muscle iNOS protein content than nonobese individuals.

METHODS

Subjects. Men and women between the ages of 40 and 65 yr were recruited as part of a larger investigation of cardiovascular disease. All subjects were sedentary (not currently engaged in any regular exercise program), and all women were postmenopausal. Exclusion criteria included the use of medications that could alter carbohydrate metabolism and evidence of diabetes, hypertension, heart disease, or orthopedic conditions prohibiting exercise. From this cohort of subjects, 27 subjects were selected a posteriori based on tissue sample availability.
and with the explicit objective to obtain two distinct groups based on body mass index (BMI). This selection process resulted in a nonobese group (n = 13; BMI of <27.2 kg/m²) and an obese group (n = 14; BMI of >33.5 kg/m²). Both the determination of insulin sensitivity and all skeletal muscle tissue analyses were performed after this selection process. These data were alternatively analyzed in an effort to describe the relationship between insulin sensitivity and skeletal muscle NOS protein content irrespective of obesity. For this, the population was arbitrarily divided into tertile groups based on insulin sensitivity index (SI), and variables were statistically compared between the high tertile group (n = 9) and the low tertile group (n = 9). The research protocol was approved by the institutional review boards at East Carolina University and Duke University, and all subjects provided written, informed consent.

**Anthropometrics.** A digital electronic scale was utilized to measure body mass to the nearest 0.1 kg. Height was assessed with a stadiometer. BMI was calculated as weight in kilograms divided by height in meters squared (kg/m²).

**Exercise testing.** All subjects performed a standardized maximal exercise test on a treadmill with 12-lead ECG monitoring and expired gas analysis to determine the level of aerobic fitness. Ventilation, exercise test on a treadmill with 12-lead ECG monitoring and expired CO₂ consumption (V˙CO₂) were continuously monitored throughout the test via open-circuit spirometry (model 2900 U, SensorMedics, Yorba Linda, CA; or TrueMax 2400, Parvomedics, Sandy, UT). The protocol utilized increased the workload by approximately one metabolic equivalent per each 2-min stage. The peak V˙O₂ (V˙O₂peak), an indicator of aerobic fitness, was determined from the values generated during the final 40 s of the exercise test. Both the nonobese and obese groups achieved a mean peak aerobic capacity of 22.1 ± 2.0 ml/kg/min. The high tertile BMI group had a total protein content of 60 ± 3 mg/kg, and the low tertile BMI group had a total protein content of 73 ± 5 mg/kg.

**Intravenous glucose tolerance test.** A 3-h intravenous glucose tolerance test (IVGTT) was performed in the morning hours after a 12-h fast to determine insulin sensitivity based on the Bergman minimal model calculations (6). A 50% dextrose bolus (0.3 g/kg body mass) was administered after fasting samples were collected, and insulin (0.025 U/kg body mass) was then injected at minute 20. Blood samples were collected at minutes 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 19, 22, 25, 30, 40, 50, 60, 70, 80, 90, 100, 120, 140, 160, and 180. Blood samples were collected in tubes with EDTA. Plasma samples were stored at −80°C until later analyses. Plasma glucose analyses were performed using the YSI 2300 Stat Plus System (Yellow Springs, OH), whereas a chemiluminescent enzyme immunoassay (ACCESS Immunoassay System, Beckman Coulter, Brea, CA) was utilized to measure plasma insulin. SI was calculated from plasma glucose and insulin values with the software program MINMOD millennium (version 5.10, 2002) (7).

**Skeletal muscle biopsy.** Skeletal muscle was acquired from vastus lateralis of the quadriceps femoris muscle group by the percutaneous needle muscle biopsy technique (19). All biopsies were performed in the morning after a 12-h overnight fast and preceded the IVGTT. Muscle samples were frozen in liquid nitrogen and stored at −80°C until later analyses.

**Tissue homogenization and evaluation of protein content.** Frozen muscle was homogenized on ice with a ground glass homogenizer in a buffer solution (1% Trion X-100, 50 mM HEPES (pH = 7.4), 4 mM EGTA, 10 mM EDTA, 15 mM tetrasodium pyrophosphate, 100 mM β-glycerolphosphate, 25 mM sodium fluoride, 5 mM Na₃VO₄, as well as a commercial protease inhibitor cocktail (P8340, Sigma, St. Louis, MO)). Homogenized samples remained on ice for ~1 h and were then placed on a rotating wheel at 4°C for an additional hour to enhance the solubilization of proteins. Samples were centrifuged (16,000 g) at 4°C for 10 min, after which the supernatant was collected. Homogenate samples were diluted 1:5 in homogenization buffer before total protein content was collected in triplicate by bicinchoninic acid (BCA) assay kits (Pierce Biotechnology, Rockford, IL) with bovine serum albumin as the standard.

**Western blot analyses.** Muscle homogenates were mixed (1:1) with ×2 lamelli buffer (4% SDS, 20% glycerol, 20% β-mercaptoethanol, 0.004% bromphenol blue, and 0.125 M Tris-HCl, pH of ~6.8) and brought to a standard protein concentration with deionized, distilled water. Samples were then boiled for 5 min, and when eNOS, nNOS and iNOS were measured, proteins were separated by 5% SDS-PAGE at 175 V for ~1 h. For 3-nitrotyrosine analysis, proteins were separated by 4–20% SDS-PAGE at 175 V for ~75 min. Each gel was balanced with the same number of samples from nonobese and obese subjects, and each gel was also evenly balanced based on S I. For all analyses, every sample loaded onto polyacrylamide gels had a total protein content of 60 μg. Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA) at 100 V for ~2 h at 4°C in transfer buffer (25 mM Tris base, 192 mM glycine, 20% methanol). Transfer was verified by Ponceau S staining. Membranes were blocked for 90 min at room temperature in either 5% nonfat dry milk (nNOS) or 5% BSA (iNOS, eNOS, 3-nitrotyrosine) mixed in a solution of Tris-buffered saline with Tween-20 (TBS-T); 20 mM Tris base, 150 mM NaCl, 0.1% Tween-20; pH 7.5. For immunodetection, membranes were incubated overnight at 4°C with primary antibody diluted in bovine serum albumin (BSA) buffer before total protein content was assessed in triplicate by bicinchoninic acid (BCA) assay kits (Pierce Biotechnology, Rockford, IL) with bovine serum albumin as the standard.

**Solutions, staining, and protein analysis.** All antibodies against eNOS and nNOS were from Upstate Biotechnology (Lake Placid, NY) and were obtained from a commercial Western blot stripping kit (Millipore). Antibody dilutions were: 1:500 for eNOS (Upstate Cell Signaling Solutions, Lake Placid, NY); 1:750 for iNOS (BD Biosciences, San Jose, CA); 1:1,000 for nNOS (Santa Cruz Biotechnology), and a final antibody dilution for the iNOS-positive control, mouse macrophages stimulated with lipopolysaccharide and interferon-γ, was observed in the iNOS-positive control, mouse macrophages stimulated with lipopolysaccharide and interferon-γ. The relative quantification was determined by the assessment of the integrated optical density of each band performed using LabWorks 4.6 software (UVP, LLC, Upland, CA). When possible, commercial Western blot stripper buffer was utilized so that PVDF membranes could be reprobed for different protein targets. Total protein was quantified with Ponceau S according to Aldridge and colleagues (3), and for all target blots (i.e., iNOS, nNOS, eNOS, and 3-nitrotyrosine) there were no significant differences in total protein between the obese and nonobese groups as well as the low tertile SI and high tertile SI groups. Optical density units for each band were normalized to the average signal of the bands on each respective membrane.

Western blot analyses yielded two distinct skeletal muscle iNOS bands (see Fig. 2A), and these bands were aligned to a similar doublet observed in the iNOS-positive control, mouse macrophages stimulated with lipopolysaccharide and interferon-γ. This doublet is consistent with previous reports that analyzed human skeletal muscle (38, 44), rat placenta (45), macrophages (56), primary bronchial epithelial cells (31), cultured neonatal rat cardiomyocytes (51), as well as HEK293 and RT4 cells (31). Vodovoz et al. reported two distinct iNOS variants (cystolic ~130 kDa and particulate ~135 kDa) in stimulated macrophages with each variant markedly contributing to the total iNOS activity (31). Due to this existing evidence, we believe that it is appropriate and necessary to represent skeletal muscle iNOS protein content as the aggregate signal of the observed doublet. Therefore, all results are presented with total iNOS protein represented as the sum of the signal of both iNOS bands. Additionally, analysis of the data using either of the bands, instead of the sum of the signal from both, resulted in the same statistical outcomes.

**Adipokine multiplexed analysis.** Circulating adipokine concentrations were analyzed to assess the inflammatory status of the subjects using two commercially available multiplexed bead-based assays (Millipore, Billerica, MA). These assays are antibody sandwich assays using capture and detector (secondary) antibodies and streptavidin-phycoerythrin (SA-PE) as the reporter signal. The median fluorescent intensity (MFI) of SA-PE from a minimum of 50 beads/events is
reported and converted to a concentration using five-parameter logistics and modeling software. All samples were analyzed on a Luminex 200 System (Luminex, Austin, TX) using Masterplex QT 2010 software (MiraiBio, Hitachi Solutions America, South San Francisco, CA). The assays used were the human cytokine/chemokine (MPXH-CYTO-60K) for IL-1β, IL-1α, IL-6, MCP-1, and TNF-α, and human serum adipokine (Panel A; HADK1-61K-A) for adiponectin and resistin. Assays for IL-1 β, IL-1α, IL-6, MCP-1, and TNF-α, had reported sensitivities of 0.4, 2.9, 0.3, 0.9, and 0.1 pg/ml, respectively, whereas adiponectin and resistin had sensitivities of 145.4 and 6.7 pg/ml, respectively. Overnight-fasted plasma samples drawn before the start of the IVGTT were run in duplicate and within one plate for each. Basal plasma samples for IL-1α and IL-6 did not meet the assay sensitivity and, therefore, were excluded from the analysis.

Statistical analysis. The software package Statview 5.0.1 (SAS Institute, Cary, NC) was used for statistical analyses. Values are expressed as means ± SE. Subject characteristics and skeletal muscle variables were compared with unpaired Student’s t-tests. Stepwise linear regression analysis was performed to predict SI from potential independent variables. Alpha level was set at 0.05 for all analyses.

RESULTS

Subject characteristics are listed in Table 1. Body mass, time to exhaustion, and $V\dot{O}_2$ peak were significantly different between the nonobese and obese groups. There were trends for both low-density lipoprotein ($P = 0.13$) and fasting insulin ($P = 0.10$) to be higher in the obese compared with the nonobese group. The obese group had a significantly lower SI than the nonobese group, indicating that they were more insulin resistant (Fig. 1). No significant differences in blood plasma adipo- and resistin, which was higher in the high tertile SI group ($P < 0.05$), were observed between the groups established based on SI (see Fig. 10). No significant association ($r = 0.08; P = 0.68$) was found between total skeletal muscle iNOS protein and total 3-nitrotyrosine protein.

Skeletal muscle eNOS protein was significantly greater in the nonobese than in the obese group (Fig. 5C), but no differences were observed between the groups established based on SI (Fig. 6). The nonobese and obese groups did not vary in skeletal muscle nNOS protein content (Fig. 7B); however, the skeletal muscle nNOS protein tended ($P = 0.054$) to be less in the low compared with high tertile SI group (Fig. 8). Correlation analysis (Pearson product) revealed a significant relationship ($r = 0.49; P < 0.05$) between SI and nNOS protein.

Table 1. Subject characteristics

<table>
<thead>
<tr>
<th>Low Tertile SI</th>
<th>High Tertile SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (obese/nonobese)</td>
<td>7/2</td>
</tr>
<tr>
<td>n (men/women)</td>
<td>7/2</td>
</tr>
<tr>
<td>Age, yr</td>
<td>48.6 ± 2.1</td>
</tr>
<tr>
<td>Height, cm</td>
<td>166.7 ± 7.8</td>
</tr>
<tr>
<td>Mass, kg</td>
<td>101.6 ± 5.3</td>
</tr>
<tr>
<td>Body mass index, kg/m$^2$</td>
<td>33.5 ± 1.5</td>
</tr>
<tr>
<td>$V\dot{O}_2$ peak, liter/min</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>Insulin, μU/ml</td>
<td>14.0 ± 3.4</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>93.0 ± 6.1</td>
</tr>
<tr>
<td>$S_i$, μU · liter$^{-1}$ · min$^{-1}$</td>
<td>2.0 ± 0.4</td>
</tr>
<tr>
<td>IL-1β, pg/ml</td>
<td>9.6 ± 3.8</td>
</tr>
<tr>
<td>MCP-1, pg/ml</td>
<td>232.6 ± 17.1</td>
</tr>
<tr>
<td>TNF-α, pg/ml</td>
<td>4.8 ± 9.8</td>
</tr>
<tr>
<td>Resistin, ng/ml</td>
<td>49.0 ± 3.6</td>
</tr>
<tr>
<td>Adiponectin, μg/ml</td>
<td>15.9 ± 1.9</td>
</tr>
</tbody>
</table>

Table 2. Subject characteristics for groups generated according to each subject’s SI, high vs. low tertile

<table>
<thead>
<tr>
<th>SI</th>
<th>Nonobese</th>
<th>Obese</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (men/women)</td>
<td>7/6</td>
<td>8/6</td>
</tr>
<tr>
<td>Age, yr</td>
<td>51.2 ± 1.7</td>
<td>50.9 ± 1.5</td>
</tr>
<tr>
<td>Height, cm</td>
<td>171.5 ± 2.0</td>
<td>163.3 ± 5.1</td>
</tr>
<tr>
<td>Mass, kg</td>
<td>75.1 ± 2.2</td>
<td>101.5 ± 3.2*</td>
</tr>
<tr>
<td>Body mass index, kg/m$^2$</td>
<td>25.5 ± 0.3</td>
<td>35.6 ± 0.4*</td>
</tr>
<tr>
<td>Cholesterol, mg/dl</td>
<td>178.5 ± 11.2</td>
<td>190.0 ± 7.4</td>
</tr>
<tr>
<td>HDL-C, mg/dl</td>
<td>50.8 ± 4.6</td>
<td>46.4 ± 3.8</td>
</tr>
<tr>
<td>LDL-C, mg/dl</td>
<td>110.9 ± 6.9</td>
<td>125.1 ± 6.0</td>
</tr>
<tr>
<td>Triglyceride, mg/dl</td>
<td>99.2 ± 8.7</td>
<td>119.4 ± 12.7</td>
</tr>
<tr>
<td>Insulin, μU/ml</td>
<td>7.3 ± 1.1</td>
<td>11.9 ± 2.4</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>86.3 ± 2.0</td>
<td>93.3 ± 4.0</td>
</tr>
<tr>
<td>Time to exhaustion, s</td>
<td>862.2 ± 61.7</td>
<td>618.7 ± 45.2*</td>
</tr>
<tr>
<td>$V\dot{O}_2$ peak, liter/min</td>
<td>2.4 ± 0.2</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>$V\dot{O}_2$ peak, ml · kg$^{-1}$ · min$^{-1}$</td>
<td>30.9 ± 1.7</td>
<td>24.6 ± 1.3*</td>
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</tbody>
</table>

Subject characteristics for groups generated according to each subject’s insulin sensitivity index (SI), high vs. low tertile ($n = 9$). For the multiplexed analytes analyzed from blood plasma, sample sizes were $n = 8$ /group, except for adiponectin analysis (high tertile SI, $n = 7$; low tertile SI, $n = 8$). Data are means ± SE. *Significant difference between high and low tertile SI groups ($P < 0.05$).
but not between SI and eNOS protein \( (r = 0.04; P = 0.83) \).

Stepwise linear regression analysis among numerous variables (age, body mass, BMI, time to exhaustion, \( \dot{V}O_2 \) peak, total cholesterol, HDL-C, LDL-C, triglycerides, fasting glucose, fasting insulin, and skeletal muscle nNOS, eNOS, and iNOS protein content) revealed that only body mass followed by skeletal muscle nNOS protein were independent predictors of SI, accounting for 29% and 25% of the variability in SI, respectively.

**DISCUSSION**

The most prominent finding in this investigation was that there was not significant elevation in skeletal muscle iNOS protein content despite evidence for insulin resistance in obese individuals. Additionally, there was no evidence for an association between iNOS content and insulin action in human skeletal muscle when we partitioned our population into tertiles based on relative insulin sensitivity. Together, these data show that human insulin resistance is not associated with an elevation in skeletal muscle iNOS protein in middle-aged individuals under fasting conditions. Skeletal muscle iNOS is therefore not necessarily increased in all states of insulin resistance.

The present iNOS and insulin sensitivity results contradict previous reports that showed that insulin resistance was related to an elevation in skeletal muscle iNOS expression (11, 12, 21, 43, 53, 55, 58). It is possible that methodological differences might explain this discrepancy since our study design contrasts sharply with that of these previous reports (12, 43, 52, 55, 58).
The present study utilized obese, yet otherwise healthy, sedentary men and women, whereas the previous studies employed the use of rodents with a genetic dominance for Type 2 diabetes, 4- and 8-wk high-fat diet interventions on rodents, and humans with Type 2 diabetes (12, 43, 52, 55, 58). Torres et al. conducted the most relevant research for comparison to the present investigation but utilized a population of subjects with uncontrolled Type 2 diabetes, as indicated by a mean glycosylated hemoglobin level (HbA1c) of 11.2% (55). The elevated skeletal muscle iNOS protein content of these Type 2 diabetic patients might be explained by chronically elevated circulating glucose levels. In critically ill patients, Langouche et al. found that prevention of hyperglycemia with intensive insulin therapy resulted in significantly less skeletal muscle iNOS gene expression compared with patients who received typical insulin therapy (33). Although such a result fails to support a direct cause-and-effect relationship between hyperglycemia and the upregulation of iNOS expression, a follow-up study in a rabbit model of critical illness conclusively demonstrated that hyperglycemia was responsible for the elevation in iNOS gene expression in skeletal muscle (18). Thus the experimental design of these past investigations more likely depicts uncontrolled Type 2 diabetes rather than insulin resistance, and possibly any elevation in skeletal muscle iNOS protein could therefore be explained as a consequence rather than an etiological factor of Type 2 diabetes.

The present study utilized obese, yet otherwise healthy, sedentary men and women, whereas the previous studies employed the use of rodents with a genetic dominance for Type 2 diabetes, 4- and 8-wk high-fat diet interventions on rodents, and humans with Type 2 diabetes (12, 43, 52, 55, 58). Torres et al. conducted the most relevant research for comparison to
the results from the present study, but this would be purely speculative since no hepatic tissue was collected for analysis. The present investigation in humans extends the previous recent findings in animal models that have demonstrated that skeletal muscle iNOS is not associated with insulin resistance.

Total 3-nitrotyrosine protein content is an index of NO exposure (5) and has been shown to mirror iNOS expression in skeletal muscle (5, 24, 55). When iNOS protein is present within skeletal muscle, it is believed to be responsible for a large volume of NO production, both because it is predominately a transcriptionally regulated enzyme and because the specific activity of iNOS (~800 nmol·min⁻¹·mg⁻¹) is significantly greater than eNOS (~130 nmol·min⁻¹·mg⁻¹) or nNOS (~400 nmol·min⁻¹·mg⁻¹) (22, 23, 41, 48). In the present study, we did not find total skeletal muscle 3-nitrotyrosine to be different between any of the groups. It is possible that no difference in 3-nitrotyrosine was found because the iNOS protein content difference between any two groups was insufficient to result in an appreciable difference in total 3-nitrotyrosine content. By comparison, Torres et al. reported a significant difference in total skeletal muscle 3-nitrotyrosine protein between Type 2 diabetics and control subjects, but the magnitude elevation in skeletal muscle iNOS protein (~400%) suggested to be responsible for the elevation in 3-nitrotyrosine was far greater than in the present investigation (55).

Circulating plasma levels of various adipokines were measured to further describe the subjects. The adiponectin results are compatible with existing literature, which has found a positive relationship between insulin sensitivity and circulating adiponectin (46). Both IL-1β and TNF-α were chosen as targets because the expression of iNOS has been shown to increase in a variety of cultured skeletal muscle cells when exposed to a mixture of cytokines that included IL-1β and TNF-α (1, 42, 57). Similar to the skeletal muscle iNOS results, no difference in plasma IL-1β or TNF-α was found between the subset of the low tertile and high tertile SI groups. This parallel relationship of both TNF-α and IL-1β with iNOS is consistent with previous investigations, which found that elevated skeletal muscle IL-1β in chronic heart failure...

An alternative analysis was performed to describe the relationship between whole-body insulin action and skeletal muscle iNOS protein content irrespective of obesity. For this, the population was arbitrarily divided into tertile groups based on SI. The difference in SI between the high and the low tertile SI groups was considerable, with group means ± SE of 11.2 ± 1.1 and 2.0 ± 0.4 mU·liter⁻¹·min⁻¹, respectively, and a 3.03 mU·liter⁻¹·min⁻¹ magnitude of SI separation between the two closest points of the high and low tertile SI groups. This magnitude difference in SI is at least as great as previous studies that utilized an intravenous glucose tolerance test with subsequent minimal model analysis, which also reported a statistically significant difference in SI between cross-sectional studies (16, 20, 37). For example, Fonseca et al. reported an SI of 2.6 ± 1.6 and 4.3 ± 2.0 mU·liter⁻¹·min⁻¹ of normal weight subjects, respectively (20). These comparisons with the present study give us confidence to assert that the subjects within the high and low tertile SI groups represent two unequivocally different populations based on insulin sensitivity. The result of the analysis from these strictly defined SI groups did not support that iNOS protein is elevated in the skeletal muscle of insulin-resistant subjects.

Recent investigations in animal models have also demonstrated a similar disconnect between skeletal muscle iNOS and insulin resistance (13, 14, 35). In mice, a 6-h intralipid infusion induced significant impairments in peripheral glucose disposal but failed to significantly increase skeletal muscle iNOS protein content (14). Lu and colleagues found that, compared with chow-fed mice, mice consuming a 60% high-fat diet for 18 wk had a significantly reduced glucose infusion rate during euglycemic hyperinsulinemic clamps; however, no difference in skeletal muscle iNOS gene expression was found between the chow-fed and high-fat-fed mice (35). In both of these examples, hepatic iNOS expression was significantly elevated compared with the control condition, and it was this hepatic iNOS expression that was purported to be responsible for the insulin-resistant state. It is possible that this mechanism might explain...
patients (1) and TNF-α in Type 2 diabetics (55) were associated with elevated skeletal muscle iNOS protein expression. The present data indicate that insulin resistance can occur in the absence of classic markers of inflammation (i.e., iNOS, TNF-α, IL-1β).

The present skeletal muscle eNOS data (Fig. 5C) confirm past findings from our laboratory observing that obese subjects have significantly less skeletal muscle eNOS protein than nonobese subjects (26). The endothelial cells of capillaries are the predominate cellular location for eNOS protein expression within human skeletal muscle (49), and since obese humans have a significantly lower capillary density (29), it would be logical to suggest this as an explanation as to why the skeletal muscle homogenates of obese subjects possessed significantly less eNOS protein. Given that skeletal muscle capillarization, a primary source of eNOS, and insulin sensitivity have a strong relationship (34), it was somewhat of a surprise that skeletal muscle eNOS protein and SI were not related and that the high insulin-sensitive tertile did not have significantly more eNOS protein than the low insulin-sensitive tertile (Fig. 6). This finding, however, is in agreement with a past report where skeletal muscle eNOS protein was not different between patients with Type 2 diabetes and control subjects (55). Although eNOS is necessary for normal systemic glucose homeostasis and insulin action in rodents (17), the available data in humans indicate that skeletal muscle eNOS protein may not play a meaningful role in peripheral insulin sensitivity.

Another novel finding from the present investigation was the positive linear relationship (r = 0.49; P < 0.05) between skeletal muscle nNOS protein and SI (Fig. 9). Stepwise linear regression analysis indicated that skeletal muscle nNOS was a significant independent predictor accounting for 25% of the variability in SI. Furthermore, nNOS protein tended (P = 0.054) to be less in the low compared with the high tertile SI group (Fig. 8). These results complement those reported by Bradley et al., where skeletal muscle nNOS expression in humans with impaired glucose homeostasis was found to be significantly lower than that of healthy subjects (8). However, a clear role for skeletal muscle nNOS in insulin action remains in question because Bradley et al. also found that the subjects with impaired glucose homeostasis significantly improved homeostasis model assessment (HOMA) scores after 4 wk of exercise training without a simultaneous increase in skeletal muscle nNOS (8). In mice, the importance of nNOS for proper insulin action was illustrated by significantly lower (~16%) steady-state glucose infusion rates during hyperinsulinemic-euglycemic clamps in nNOS knockout mice compared with wild-type mice (50). Although skeletal muscle nNOS is certainly associated with and required for optimal insulin action, its exact role in this process is unclear.

In conclusion, we found that human insulin resistance was not associated with an elevation in skeletal muscle iNOS protein in a population of healthy, middle-aged men and women under the fasted condition. As expected, skeletal muscle eNOS protein was significantly higher in the nonobese compared with the obese subjects, although eNOS protein content was not correlated with insulin sensitivity. An additional novel finding was the positive relationship between skeletal muscle nNOS protein and whole-body insulin action; the explanation for this relationship is not yet evident and requires further research.

GRANTS
This study was supported in part by National Institutes of Health Grants R01 HL-57354 and R21 AG-19209-02.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES
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