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Obesity, insulin resistance, and skeletal muscle nitric oxide synthase

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Kraus RM, Houmard JA, Kraus WE, Tanner CJ, Pierce JR, Choi MD, Hickner RC. 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 (NO) 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 (12, 58). This S-nitrosation-mediated 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 \( (n = 13; \text{BMI of } <22.7 \text{ kg/m}^2) \) and an obese group \( (n = 14; \text{BMI of } >33.5 \text{ kg/m}^2) \). 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, respiratory exchange ratio of 1.15, and 3-nitrotyrosine) there were no significant differences in total protein content as the aggregate signal of the observed doublet. Western blot analyses. Muscle homogenates were mixed (1:1) with \( \times 2 \) lamelli buffer: 4% SDS, 20% glycerol, 20% β-mercaptoethanol, 0.004% bromphenol blue, and 0.125 M Tris-HCl, pH of \( \sim 6.8 \) 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 \( \sim 1 \) h. For 3-nitrotyrosine analysis, proteins were separated by 4–20% SDS-PAGE at 175 V for \( \sim 75 \) 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 \( \text{SI} \). 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 \( \sim 2 \) 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 blocking buffer. Primary 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 concentration of 0.5 µg/ml for 3-nitrotyrosine (Upstate Cell Signaling Solutions). Then, after successive washes in TBS-T, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Millipore, Billerica, MA) in blocking buffer for 1 h at room temperature followed by washes with TBS-T. Immunogenic detection was accomplished with enhanced chemiluminescent substrate (Pierce Biotechnology), and bands were visualized by autoradiography. 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 content between the obese and nonobese groups as well as the low tertile \( S_i \) and high tertile \( S_j \) 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 (cytosolic \( \sim 130 \) kDa and particulate \( \sim 135 \) 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-phycocerythrin (SA-PE) as the reporter signal. The median fluorescent intensity (MFI) of SA-PE from a minimum of 50 beads/events is

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Significant relationship (independent variables. Alpha level was set at 0.05 for all analyses. No significant differences in blood plasma adiponectin and resistin (Fig. 1). No significant differences in blood plasma adiponectin and resistin had 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 each plate. Basal plasma samples for IL-1ra 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 variables. Alpha level was set at 0.05 for all analyses.

RESULTS

Subject characteristics are listed in Table 1. Body mass, age, height, 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 had a significantly lower SI than the nonobese group, indicating that they were more insulin resistant (Fig. 1). No significant differences in blood plasma adipokines emerged between a subset of the high tertile SI and low tertile S\(I\) groups (Table 2), with the exception of adiponectin, which was higher in the high tertile S\(I\) group (P < 0.05).

No significant difference was found when the skeletal muscle iNOS protein of nonobese and obese groups were compared (P = 0.25; Fig. 2C). Linear regression analyses revealed no significant relationship (r = 0.13; P = 0.51) between S\(I\) and skeletal muscle iNOS protein content (Fig. 3A); however, upon closer inspection and analysis of the data, a significant positive correlation between skeletal muscle iNOS protein content and S\(I\) existed among only the obese subjects (n = 14; r = 0.63; P < 0.05; Fig. 3B). Comparisons of skeletal muscle iNOS protein content were also made between reconfigured groups generated according to each subject’s S\(I\). The high and low tertiles according to S\(I\) (n = 9/group) had S\(I\) means ± SE of 11.2 ± 1.1 and 2.0 ± 0.4 mU·liter\(^{-1}\)·min\(^{-1}\), respectively, and skeletal muscle iNOS protein was not statistically different in the high compared with the low tertile S\(I\) group (P = 0.17; Fig. 4).

Individual 3-nitrotyrosine bands were quantified with no difference in total 3-nitrotyrosine protein found between the nonobese and obese groups (1.02 ± 0.05 vs. 0.98 ± 0.05; P = 0.60), nor were there differences in 3-nitrotyrosine protein content between the groups generated based on S\(I\) (see Fig. 10). No significant association (r = 0.08; P = 0.68) was found between total skeletal muscle eNOS 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 S\(I\) (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 S\(I\) group (Fig. 8). Correlation analysis (Pearson product) revealed a significant relationship (r = 0.49; P < 0.05) between S\(I\) and nNOS protein levels.

Table 1. Subject characteristics

<table>
<thead>
<tr>
<th></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>163.9 ± 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>99.9 ± 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>VO(\dot{2}) peak, liter/min</td>
<td>2.4 ± 0.2</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>VO(\dot{2}) peak, ml · kg(^{-1}) · min(^{-1})</td>
<td>30.9 ± 1.7</td>
<td>24.6 ± 1.3*</td>
</tr>
</tbody>
</table>

Data are means ± SE. *Significant difference between nonobese and obese groups (P < 0.05).

Table 2. Subject characteristics for groups generated according to each subject’s S\(I\), high vs. low tertile

<table>
<thead>
<tr>
<th></th>
<th>Low Tertile S(I)</th>
<th>High Tertile S(I)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (obese/nonobese)</td>
<td>7/2</td>
<td>4/5</td>
</tr>
<tr>
<td>n (men/women)</td>
<td>7/2</td>
<td>3/6</td>
</tr>
<tr>
<td>Age, yr</td>
<td>48.6 ± 2.1</td>
<td>52.9 ± 1.6</td>
</tr>
<tr>
<td>Height, cm</td>
<td>166.7 ± 7.8</td>
<td>166.4 ± 2.2</td>
</tr>
<tr>
<td>Mass, kg</td>
<td>101.6 ± 5.3</td>
<td>82.2 ± 4.1*</td>
</tr>
<tr>
<td>Body mass index, kg/m(^2)</td>
<td>33.5 ± 1.5</td>
<td>29.9 ± 2.0</td>
</tr>
<tr>
<td>VO(\dot{2}) peak, liter/min</td>
<td>2.7 ± 0.2</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>VO(\dot{2}) peak, ml · km(^{-1}) · min(^{-1})</td>
<td>26.6 ± 2.1</td>
<td>26.9 ± 2.3</td>
</tr>
<tr>
<td>Insulin, µU/ml</td>
<td>14.0 ± 3.4</td>
<td>6.3 ± 1.3</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>93.0 ± 6.1</td>
<td>87.4 ± 2.6</td>
</tr>
<tr>
<td>S(I), mU · liter(^{-1}) · min(^{-1})</td>
<td>2.0 ± 0.4</td>
<td>11.2 ± 1.1*</td>
</tr>
<tr>
<td>IL-1β, pg/ml</td>
<td>9.6 ± 3.8</td>
<td>5.0 ± 2.9</td>
</tr>
<tr>
<td>MCP-1, pg/ml</td>
<td>232.6 ± 17.1</td>
<td>314.3 ± 58.0</td>
</tr>
<tr>
<td>TNF-α, pg/ml</td>
<td>4.8 ± 0.9</td>
<td>5.2 ± 0.7</td>
</tr>
<tr>
<td>Resistin, ng/ml</td>
<td>49.0 ± 3.6</td>
<td>58.6 ± 0.8</td>
</tr>
<tr>
<td>Adiponectin, µg/ml</td>
<td>15.9 ± 1.9</td>
<td>30.4 ± 3.9*</td>
</tr>
</tbody>
</table>

Subject characteristics for groups generated according to each subject’s insulin sensitivity index (S\(I\)), high vs. low tertile (n = 9/group). For the multiplexed analytes analyzed from blood plasma, sample sizes were n = 8/group, except for adiponectin analysis (high tertile S\(I\), n = 7; low tertile S\(I\), n = 8). Data are means ± SE. *Significant difference between high and low tertile S\(I\) groups (P < 0.05).
(Fig. 9), but not between $S_i$ 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 $S_i$, accounting for 29% and 25% of the variability in $S_i$, 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.

Fig. 5. Skeletal muscle endothelial NO synthase (eNOS) protein content determined by Western blot analyses from the vastus lateralis muscle of nonobese (n = 13) and obese (n = 14) sedentary subjects. Representative eNOS Western blot (A), representative Ponceau S stained blot for total protein quantification (B), and comparison between nonobese and obese skeletal muscle eNOS protein content (C). *Significant difference between nonobese and obese (P < 0.05).

Fig. 6. Comparison of skeletal muscle eNOS protein content between groups generated according to each subject’s SI, high vs. low tertile (n = 9/group). No statistically significant difference was observed.

Fig. 7. Skeletal muscle neuronal NO synthase (nNOS) protein content determined by Western blot analyses from the vastus lateralis muscle of nonobese (n = 13) and obese (n = 14) sedentary subjects. Representative nNOS Western blot (A), and comparison between nonobese and obese skeletal muscle nNOS protein content (B). The representative eNOS and nNOS blots share the same Ponceau S blot because commercial stripper was utilized so that membranes could be reprobed. No statistically significant difference was observed.

Fig. 8. Comparison of skeletal muscle nNOS protein content between groups generated according to each subject’s SI, high vs. low tertile (n = 9/group). No statistically significant difference was observed.
that the subjects within the high and low tertile SI groups comparisons with the present study give us confidence to assert statistically significant difference in SI between cross-sectional subsequent minimal model analysis, which also reported a studies that utilized an intravenous glucose tolerance test with magnitude difference in SI is at least as great as previous
groups was considerable, with group means of 2.6 and 1.1 and 2.0, respectively, and a 3.03 mU·liter⁻¹·min⁻¹ magnitude of S₁ separation between the two closest points of the high and low tertile S₁ groups. This magnitude difference in S₁ 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 S₁ between cross-sectional groups (16, 20, 37). For example, Fonseca et al. reported an S₁ of 2.6 ± 1.6 and 4.3 ± 2.0 mU·liter⁻¹·min⁻¹ (means ± SE) for obese and 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 S₁ groups represent two unequivocally different populations based on insulin sensitivity. The result of the analysis from these strictly defined S₁ 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 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 (−40%) 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 S₁ 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

![Graph](http://example.com/graph.png)

**Fig. 9.** Linear regression between S₁ and skeletal muscle nNOS protein content among nonobese (n = 13) and obese (n = 14) sedentary subjects. There was a significant positive association between S₁ and skeletal muscle nNOS protein content.

**Fig. 10.** Skeletal muscle 3-nitrotyrosine protein content determined by Western blot analyses from the vastus lateralis muscle of nonobese (n = 13) and obese (n = 14) sedentary subjects. Representative 3-nitrotyrosine Western blot. No statistically significant difference was observed.
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 S1 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 S1 (Fig. 9). Stepwise linear regression analysis indicated that skeletal muscle nNOS was a significant independent predictor accounting for 25% of the variability in S1. Furthermore, nNOS protein tended (P = 0.054) to be less in the low compared with the high tertile S1 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 clamp 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.

### REFERENCES


### DISCLOSURES

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### AUTHOR CONTRIBUTIONS


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