Skeletal Muscle Lipid Peroxidation and Insulin Resistance in Humans

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Objective: The relationships among skeletal muscle lipid peroxidation, intramyocellular lipid content (IMCL), and insulin sensitivity were evaluated in nine insulin-sensitive (IS), 13 insulin-resistant (IR), and 10 adults with type 2 diabetes (T2DM).

Design: Insulin sensitivity was assessed by hyperinsulinemic-euglycemic clamp [glucose disposal rate (GDR)]. Lipid peroxidation was assessed by 4-hydroxynonenal (HNE)-protein adducts and general oxidative stress by protein carbonyl content. All patients were sedentary.

Results: Protein-HNE adducts were elevated 1.6-fold in T2DM compared with IS adults, whereas IR showed intermediate levels of HNE-modified proteins. Protein-HNE adducts correlated with GDR, waist circumference, and body mass index. IMCL was increased by 4.0- and 1.9-fold in T2DM and IR patients, respectively, compared with IS, and was correlated with GDR and waist circumference but not BMI. Protein carbonyls were not different among groups and did not correlate with any of the measured variables. Correlations were detected between IMCL and protein-HNE.

Conclusion: Our data show for the first time that skeletal muscle protein-HNE adducts are related to the severity of insulin resistance in sedentary adults. These results suggest that muscle lipid peroxidation could be involved in the development of insulin resistance. (***J Clin Endocrinol Metab*** **97: E1182–E1186, 2012)**

Recent evidence indicates that reactive oxygen species generated under normal conditions by mitochondria play important roles in physiological regulation of glucose uptake (1). However, reactive oxygen species generated in excess may contribute to mitochondrial damage and dysfunction (2) and form lipid peroxidation products that are highly reactive with proteins, lipids, and DNA (3). Reactive aldehydes such as 4-hydroxynonenal (HNE) are biomarkers of lipid peroxidation and have been associated with intramyocellular lipid (IMCL) accumulation in sedentary individuals (4). It is widely postulated that lipid peroxides modify mitochondrial proteins and critical components of the insulin signaling pathway (5, 6), leading to impaired stimulation of glucose uptake, mitochondrial damage, and additional oxidative stress, thereby propagating a deleterious cycle (7). However, few data have been reported to substantiate this theory.

The current study investigated for the first time the relationships between IMCL and skeletal muscle lipid peroxidation as determinants of insulin resistance in humans. We hypothesized that skeletal muscle HNE is

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Abbreviations: BMI, Body mass index; GDR, glucose disposal rate; HNE, 4-hydroxynonenal; IMCL, intramyocellular lipid; IR, insulin resistant; IS, insulin sensitive; T2DM, type 2 diabetes mellitus.

elevated in insulin-resistant and diabetic patients compared with insulin-sensitive individuals and that HNE levels correlate with the severity of insulin resistance and IMCL accumulation.

Patients and Methods

Nine insulin-sensitive (IS), 13 insulin-resistant (IR), and 10 type 2 diabetes mellitus (T2DM) sedentary individuals were studied with a body mass index (BMI) of 22 kg/m^2 or greater and stable weight (±3%) for 3 months. Exclusion criteria included cardiovascular, renal, thyroid, or hepatic disease or the use of pharmacological agents that affect carbohydrate homeostasis, lipids, or body composition. T2DM patients were withdrawn from treatment for 2–3 wk before the study.

Subjects completed a three-day stay in the University of Alabama at Birmingham (Birmingham, AL) Clinical Research Unit, at which they received a eucaloric diet of 20% protein, 30% fat, and 50% carbohydrate calories. Race was determined by self-report. Protocols were approved by the University of Alabama at Birmingham Institutional Review Board, and informed consent was obtained from every subject.

Dual-energy x-ray absorptiometry was performed (DPX-L, software 1.33; Lunar Radiation, Madison, WI). Height, weight, and waist circumferences were assessed. Soleus IMCL was quantified using ^{1}H magnetic resonance spectroscopy (Philips 3T Medical System, Best, The Netherlands), as detailed (7). Voxels (1 cm^3) avoided gross marbling, facia, and vascular structures. Data are expressed as arbitrary units per pixel area relative to water.

Insulin resistance was determined by glucose disposal rate (GDR) via hyperinsulinemic-euglycemic clamp technique, as described (8). Regular insulin (Humulin; Eli Lilly, Indianapolis, IN) was administered at a rate of 200 mU/$^{-2}$ · min^{-1}, producing a steady-state insulin concentration of 3480 ± 138 pmol/liter, which is maximally effective for stimulating glucose uptake into skeletal muscle (8). Whole-body GDR was the glucose infusion corrected for glucose pool size (assuming 19% distribution volume and 0.65 pool fraction). IR was considered if the GDR was less than 12 mg/kg lean body mass per minute^{-1}, and T2DM was diagnosed when the fasting plasma glucose was 126 mg/dl or greater.

Vastus lateralis biopsies were performed after an overnight fast as described (7). Adipose tissue was removed and samples were divided, frozen in liquid nitrogen, and stored at −80 C. All tissue samples were carefully treated without reducing agents and with only two freeze-thaw cycles since collection (one after tissue collection and the other after preparing homogenate). Protein extraction, quantification, and carbonyl detection was performed as described (9). For protein-HNE analysis, proteins (15 μg) were loaded onto 10% polyacrylamide gel, electrophoresed at 90 V for approximately 3 h, and transferred to polyvinyl difluoride membranes overnight at 30 V. Membranes were blocked 2 h at room temperature with 5% milk and incubated with HNE polyclonal antibody (1:1000) (α Diagnostics International, San Antonio, TX) overnight at 4 C. After incubation with horseradish peroxidase-conjugated secondary antibody, bands were visualized (ECL Plus; GE Healthcare, Indianapolis, IN) and captured (Bio-Rad Imaging System, Hercules, CA). Whole-lane densitometry was performed (Quantity One, Discovery Series 4.6.5; Bio-Rad Imaging System). Membranes were stained with Amido Black for protein control.

Sample size was estimated using reported protein-HNE differences between lean and obese adults, normalized for IMCL (4). IMCL and fasting insulin were log transformed for normality. Univariate ANOVA and Bonferroni *post hoc* tests were used

TABLE 1. Descriptive characteristics of study participants

	IS	IR	T2DM
Sex	3 M/6 F	5 M/8 F	4 M/6 F
Race	4 EA/5 AA	6 EA/7 AA	5 EA/5 AA
Age (yr)	35.1 ± 12 (21–54)	37.2 ± 11 (24–58)	43.5 ± 11 (27–60)
BMI (kg/m^2)	26.1 ± 3.4 (22–34)	31.9 ± 5.7 (24–39)[a]	34.8 ± 5.5 (29–42)[b]
Waist circumference (cm)	88.3 ± 10 (74–104)	103.7 ± 14 (74–122)[a]	111.0 ± 10 (93–123)[b]
Body fat (%)	36.3 ± 10.4 (18–50)	38.8 ± 8.0 (28–52)	38.2 ± 9.8 (24–55)[c]
Lean body mass (kg)	45.3 ± 8.6 (36–57)	56.1 ± 10.6 (43–73)[a]	57.1 ± 9.5 (44–73)[a,c]
GDR (mg/kg LBM per minute)	16.6 ± 2.6 (13–20)[d]	9.32 ± 1.6 (6–11)[b]	6.69 ± 2.1 (4–11)[b,d,e]
IMCL (AU)	2.03 ± 1.0 (0.8–3.7)[d]	3.80 ± 2.8 (0.9–10.9)[f]	8.06 ± 3.8 (4.7–14.0)[b,e,g]
Protein-HNE (AU)	609 ± 170 (436–877)	812 ± 239 (496–1232)	971 ± 436 (415–1692)[a]
Carbonyl (AU)	1481 ± 470 (971–2017)	1595 ± 579 (695–2769)	1407 ± 516 (820–2269)
Insulin (μU/ml)	9.31 ± 3.5 (5–17)	21.4 ± 14 (6–50)[f]	17.2 ± 14 (8–48)[c]
Glucose (mg/dl)	89.6 ± 6.3 (82–101)	95.4 ± 12 (78–119)	173.0 ± 45 (110–258)[a,c,h]

Data are expressed as mean ± SD (range). M, Male; F, female; EA, European-American; AA, African-American; LBM, lean body mass; AU, arbitrary unit.

[a] Different from IS ($P < 0.05$).

[b] Different from IS ($P < 0.01$).

[c] n = 9.

[d] n = 8.

[e] Different from IR ($P < 0.05$).

[f] n = 12.

[g] n = 5.

[h] Different from IR ($P < 0.01$; n = 9, 13, and 10 for IS, IR, and T2DM, respectively), unless otherwise indicated.

to identify mean differences. Relationships among protein-HNE, IMCL, and GDR were examined with correlations controlled for the influence of age, overall body fat, and oxidative stress (control excluded when analyzing carbonyls). Unstandardized residuals of protein-HNE, IMCL, and GDR adjusted for age, body fat, and carbonyls were calculated. Analyses were performed with SPSS 19.0 (SPSS Inc., Chicago, IL).

Results

Groups were similar regarding age, race, gender, and body fat. Waist circumference, body weight, BMI, and lean body mass were lower in IS than in both IR and T2DM. Missing were GDR data from three subjects: two who experienced difficulties with iv access and one who withdrew before completing dual-energy x-ray absorptiometry (Table 1). IMCL data were missing from two subjects who withdrew and from five subjects who were studied before IMCL measurements were added to the protocol. Even so, IMCL in T2DM was significantly higher than in IS and IR (Table 1). A nonparametric comparison of means verified that the five T2DM missing IMCL data were not different from the subjects with IMCL measures regarding any of the other study parameters.

Protein-HNE in T2DM was higher than IS but not IR (Table 1). Protein-HNE and IMCL correlated negatively with GDR (Fig. 1), and these correlations persisted when controlling for age and BMI ($r = -0.39$, $P = 0.04$ and $r = -0.47$, $P = 0.03$). Furthermore, when T2DM data were removed, the negative relationship between GDR and protein-HNE persisted (Table 1). Protein carbonyl content did not differ among groups (Table 1) and was not correlated with GDR (Fig. 1), protein-HNE ($r = -0.34$, $P = 0.10$), or IMCL ($r = 0.21$, $P = 0.35$).

The correlation between protein-HNE and IMCL in all subjects was only borderline significant (Fig. 1); however, an extreme outlier (greater than 3.0 times the interquartile range beyond the third quartile) for IMCL (T2DM female) may have skewed the results. A sensitivity analysis excluding the outlier was performed. IMCL remained higher in T2DM than in IS and IR and a significant correlation between HNE and IMCL emerged (Fig. 1).

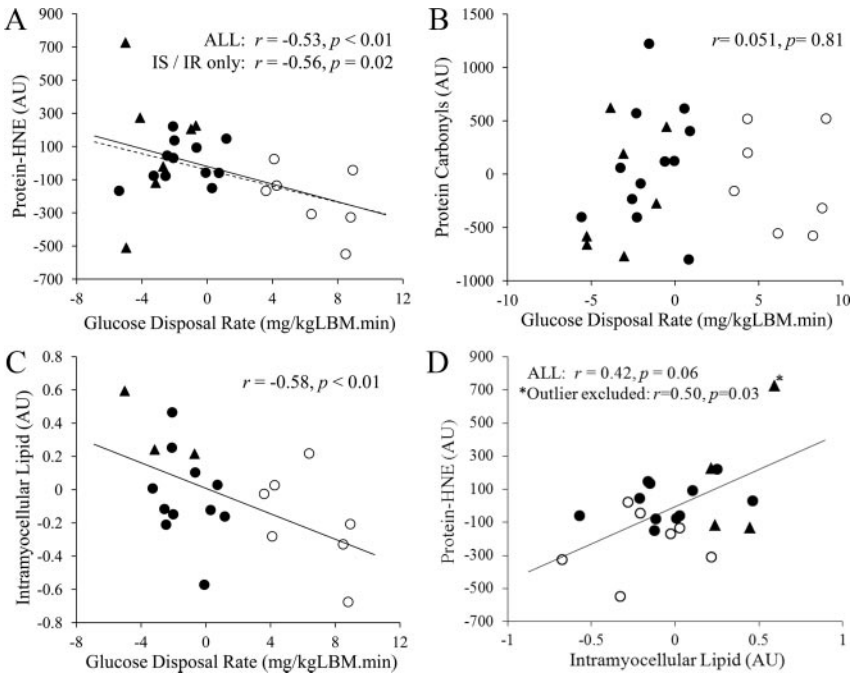

FIG. 1. A–D, Relationships between glucose disposal rate and skeletal muscle protein-HNE (A), skeletal muscle protein carbonyl content (B), and intramyocellular lipid content (C) in IS (*open circles*), IR (*filled circles*), and T2DM (*triangles*) patients. D, Relationship between IMCL content and skeletal muscle protein-HNE. X-axis values reflect unstandardized residual values adjusted for age, percent body fat, and protein carbonyls, except for B, which is adjusted for only age and percent body fat. Partial correlations are reported in all subjects (ALL; *continuous line*) and in A with T2DM removed (IS/IR only, *dashed line*).

Discussion

We tested the hypothesis that skeletal muscle lipid peroxidation and IMCL are related to each other and to the severity of insulin resistance in sedentary individuals. Here we report that IMCL and protein-HNE are significantly elevated in T2DM patients compared with IS subjects and that both of these indices correlate with insulin resistance. Our findings are consistent with others linking IMCL with insulin sensitivity, independent of general adiposity (10). Although others have investigated skeletal muscle HNE as it relates to mitochondrial function and diabetes in rodents (11, 12) and to IMCL accumulation (4) in humans, this is the first study to examine the interrelationships among IMCL, skeletal muscle protein-HNE, and insulin sensitivity in humans.

Protein-HNE modifications are symptomatic of oxidative stress and are detected in numerous pathological conditions (12, 13). Our data show a negative relationship between protein-HNE and GDR that persisted when data from diabetic subjects were excluded. This suggests that lipid peroxidation and the accumulation of protein-HNE adducts characterizes insulin resistance independent of diabetes in prediabetic individuals. However, another measurement commonly used to detect oxidative stress, protein carbonyls, did not correlate with peripheral insulin resistance. A possible explanation is that carbonylation can be introduced

into proteins independent of lipid peroxidation (14). Other measurements of oxidative stress may be similarly difficult to use under chronic or oscillating conditions of oxidative stress. For example, glutathione is up-regulated under conditions of oxidative stress but is rapidly extruded from cells (15). Moreover, the high reactivity of most free radicals paradoxically limits their ability to incite tissue injury (16) and makes them difficult to measure accurately in human biopsies. Conversely, protein-HNE modifications are relatively long-lived footprints of lipid peroxidation and are easily measured through immunological techniques (17). Hence, the use of specific and metastable protein-aldehyde adducts makes them useful biomarkers of oxidative stress.

Contrary to our results, a recent study reported no difference in skeletal muscle protein-HNE content between BMI-matched, obese, insulin-sensitive adults and those with type 2 diabetes (11). Although we cannot be certain why the current data are discrepant with this previous report, there are a few possible explanations. The BMI of the IS group in the previous study was higher than the average BMI of our IS group (32 ± 1 *vs.* 26.1 ± 3 kg/m^2), and our subgroups differed in BMI and waist circumference; nevertheless, the relationship we observed between protein-HNE and GDR was independent of both BMI and age. Differences in sample preparation impact protein-HNE detection, *e.g.* freeze-thaw cycles and the absence or presence of reducing agents, and may have resulted in our ability to discern group differences in protein-HNE adducts.

The results provide evidence of a relationship between skeletal muscle lipid peroxidation and IMCL. Both are related to the severity of insulin resistance in sedentary individuals. This finding is consistent with another study that found a relationship between HNE and IMCL in sedentary individuals (4). Because these are correlative studies, we cannot conclude that a causal relationship exists. However, we propose that accumulating IMCL serves as a substrate for lipid peroxidation and that reactive lipid aldehydes alter the function of proteins critical to metabolism, such as mitochondrial proteins, glucose transporter 4 (GLUT4), and/or insulin receptor substrate-1/2 (IRS 1 or 2). Schrauwen (18) proposed a similar model of lipotoxicity in which interactions of lipid-derived aldehydes lead to mitochondrial damage, the propagation of oxidative stress, and insulin resistance, thereby perpetuating a catastrophic cycle. The present study did not assess mitochondrial damage; however, reduced mitochondrial respiration was previously shown in type 2 diabetes (11).

Two study limitations must be addressed. First, IMCL measurements were missing on seven subjects, five of whom were T2DM. Because T2DM exhibited the highest levels of IMCL, an underestimation of relationships between IMCL and other variables may have occurred. The observed relationships involving IMCL are likely conservative; however, there is the potential of a skewed relationship resulting from the missing data. Second, a relationship between soleus IMCL and vastus lateralis protein-HNE appeared, despite being measured from different mixed-fiber muscle groups. However, IMCL accumulation is independent of fiber type in obese and type 2 diabetic patients (19). Therefore, it is logical to expect IMCL accumulation to be uniformly proportional in the soleus and vastus lateralis.

In conclusion, these data show that protein-HNE and IMCL in skeletal muscle increase as a function of insulin resistance, whereas protein carbonyls are independent of insulin sensitivity. Furthermore, these data provide evidence of a relationship between skeletal muscle protein-HNE and IMCL. The data support the contention that lipid peroxidation products constitute a group of reactive species that may be pathogenic in insulin resistance. Further research is needed to delineate the role of lipid peroxidation in the etiology of insulin resistance.

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