Adiponectin resistance precedes the accumulation of skeletal muscle lipids and insulin resistance in high-fat-fed rats

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[1]*Department of Human Health and Nutritional Science, University of Guelph, Guelph, Ontario;* [3]*Alberta Diabetes Institute, University of Alberta, Edmonton, Alberta, Canada; and* [2]*Department of Physiology, Medical University of Bialystok, Bialystok, Poland*

Mullen KL, Pritchard J, Ritchie I, Snook LA, Chabowski A, Bonen A, Wright D, Dyck DJ. Adiponectin resistance precedes the accumulation of skeletal muscle lipids and insulin resistance in high-fat-fed rats. *Am J Physiol Regul Integr Comp Physiol* 296: R243–R251, 2009. First published December 10, 2008; doi:10.1152/ajpregu.90774.2008.—High-fat (HF) diets can induce insulin resistance (IR) by altering skeletal muscle lipid metabolism. An imbalance between fatty acid (FA) uptake and oxidation results in intramuscular lipid accumulation, which can impair the insulin-signaling cascade. Adiponectin (Ad) is an insulin-sensitizing adipokine known to stimulate skeletal muscle FA oxidation and reduce lipid accumulation. Evidence of Ad resistance has been shown in obesity and following chronic HF feeding and may contribute to lipid accumulation observed in these conditions. Whether Ad resistance precedes and is associated with the development of IR is unknown. We conducted a time course HF feeding trial for 3 days, 2 wk, or 4 wk to determine the onset of Ad resistance and identify the ensuing changes in lipid metabolism and insulin signaling leading to IR in skeletal muscle. Ad stimulated FA oxidation ($+28\%$, $P \leq 0.05$) and acetyl-CoA carboxylase phosphorylation ($+34\%$, $P \leq 0.05$) in control animals but failed to do so in any HF-fed group (i.e., as early as 3 days). By 2 wk, plasma membrane FA transporters and intramuscular diacylglycerol (DAG) and ceramide were increased, and insulin-stimulated phosphorylation of both protein kinase B and protein kinase B substrate 160 was blunted compared with control animals. After 4 wk of HF feeding, maximal insulin-stimulated glucose transport was impaired compared with control. Taken together, our results demonstrate that an early loss of Ad's stimulatory effect on FA oxidation precedes an increase in plasmalemmal FA transporters and the accumulation of intramuscular DAG and ceramide, blunted insulin signaling, and ultimately impaired maximal insulin-stimulated glucose transport in skeletal muscle induced by HF diets.

adipokines; adenosine 5′-monophosphate-activated protein kinase; acetyl-coenzyme A carboxylase; fatty acid translocase/CD36; insulin signaling AdipoR1; diacylglycerol; ceramide

CONSUMPTION OF A HIGH-FAT (HF) diet and obesity are well-known contributors to the development of insulin resistance (IR) and type 2 diabetes. HF diets may induce IR in part by altering lipid metabolism in skeletal muscle (19, 23, 39). Intramuscular lipid accumulation in untrained humans and rodents is consistently associated with skeletal muscle IR, although the cause of this accumulation is controversial. Elevated lipids may be attributable to increased fatty acid (FA) uptake (15), decreased oxidation (17, 18), increased esterification (19), or likely an imbalance among all of these. Impaired

rates of FA oxidation and oxidative capacity have been reported in skeletal muscle of obese, IR, and diabetic subjects (17, 18). Recently, increased FA uptake in the cell as a result of increased FA transporters [fatty acid translocase (FAT)/CD36, plasma membrane-bound fatty acid-binding protein (FABPpm)] at the plasma membrane has been suggested as an important cause of HF diet-induced intramuscular lipid accumulation (14, 35).

Despite controversies as to the method of accumulation, various lipids have been shown to interfere with insulin signal transduction and subsequent translocation of GLUT4 to the plasma membrane. Specifically, diacylglycerol (DAG) has been shown to activate protein kinase C, thereby impairing insulin receptor substrate (IRS)-1 and phosphatidylinositol 3-kinase (PI 3-kinase) activation (22), and ceramide has been shown to inhibit phosphorylation/activation of protein kinase B (Akt), thus diminishing Akt's stimulatory effect on GLUT4 translocation to the plasma membrane (10). Collectively, the imbalance between FA uptake and oxidation, resultant intramuscular lipid accumulation, diminished insulin signal transduction, and impaired glucose transport are hallmarks of skeletal muscle lipotoxicity, which can be induced by HF feeding. Adiponectin (Ad) is an insulin-sensitizing cytokine known to stimulate FA oxidation (5, 13, 23) and glucose uptake in skeletal muscle (8). More specifically, the globular head, gAd, which has been shown to circulate in small quantities, exerts these effects in skeletal muscle by binding to its receptor AdipoR1 (13, 20, 33). Globular Ad stimulates AMP-activated protein kinase (AMPK), which in turn phosphorylates and inactivates acetyl-CoA carboxylase (ACC), thereby decreasing malonyl-CoA formation and relieving inhibition on carnitine palmitoyltransferase (CPT)-1, allowing FA to enter the mitochondria to be oxidized (33, 40). Circulating Ad levels are decreased in obesity (1), and evidence of Ad resistance has also been shown in peripheral tissues of obese humans and animals. In genetically obese diabetic mice, the acute stimulatory effect of Ad on AMPK in muscle is lost. This may be due, at least in part, to an observed reduction in skeletal muscle AdipoR1 mRNA (34). In obese humans, the stimulatory effect of gAd on FA oxidation in skeletal muscle is also blunted (5, 11). Recently, we demonstrated that, following 4 wk of feeding rats a diet high in saturated or polyunsaturated fat, gAd no longer stimulated FA oxidation; that is, both HF diets induced Ad resistance (23). However, only the animals fed the saturated

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diet became insulin resistant by 4 wk, whereas polyunsaturated-fed animals were insulin responsive at this time point. Thus, although a HF diet can induce Ad resistance, it is unclear whether Ad resistance precedes intramuscular lipid accumulation and the development of IR.

Surprisingly, there has been virtually no examination of the early changes in muscle lipid metabolism and response to insulin-sensitizing adipokines during the development of diet-induced IR. Therefore, the purpose of the current study was to elucidate the time course over which diet-induced Ad resistance develops to determine if it precedes impairments in insulin response and identify ensuing changes in lipid metabolism and insulin signaling that may contribute to this relationship. We chose to employ a high saturated fat diet since we have previously shown this to produce both Ad resistance and impaired insulin response within a 4-wk period (23). We hypothesized that *1*) Ad resistance would occur first, attributable in part to decreased AdipoR1 content, followed by *2*) greater FA transporter abundance at the plasma membrane, leading to *3*) increased intramuscular DAG and ceramide content, *4*) impaired activation of the insulin-signaling cascade and, ultimately, *5*) impaired skeletal muscle glucose transport under maximal insulin-stimulated conditions.

METHODS

Animals and Diets

Upon arrival, female Sprague-Dawley rats (140–145 g; Charles River, Quebec, Canada) were assigned to individual cages in a controlled environment with a reverse 12:12-h light-dark cycle with ad libitum access to Purina standard rodent chow and water. Following a 3-day acclimation period, rats were randomly assigned to a control (12% kcal from fat, CON) or HF diet (60% kcal from lard) (Research Diets, New Brunswick, NJ) for 3 days, 2 wk, or 4 wk. HF-fed animals were pair-fed to CON rats (fed ad libitum) with respect to caloric intake on a daily basis, and body mass was recorded three times per week. After the predetermined length on the diets, the animals were overnight fasted before experimental procedures. All procedures were approved by the Animal Care Committee at the University of Guelph.

Muscle and Blood Sampling

Soleus muscle. Rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (6 mg/100 g body mass) and the soleus (Sol) muscle was carefully dissected into longitudinal strips from tendon to tendon using a 27-gauge needle. The two outside strips from each Sol were then incubated under various conditions for measurement of *1*) glucose transport, *2*) fat metabolism, or *3*) key signaling proteins. Because of the number of conditions in these experiments, one animal was used for metabolism measures (leg 1: glucose transport, leg 2: fat metabolism), and a second animal was used for assessment of signaling proteins (leg 1: insulin-stimulated signaling, leg 2: gAd-stimulated signaling). The remaining piece of Sol from each leg was immediately frozen in liquid N_2, without incubation, to determine the effects of the chronic dietary intervention on AdipoR1 protein levels or total muscle lipid content. For analysis of whole muscle lipid content, one Sol strip was subsequently freeze-dried, powdered, and cleaned of any visible connective tissue, and individual lipids (DAG and ceramide) were extracted and measured using gas-liquid chromatography as described previously (6).

Blood

Terminal blood collection was made at the completion of the treatment via cardiac puncture after first excising skeletal muscles for incubation. A glucometer reading (Elite XL; Bayer, Toronto, ON, Canada) of whole blood glucose was also made. All blood samples were collected in heparinized tubes after an overnight fast and centrifuged at 9,300 *g* for 5 min at 4°C, and the plasma removed for analyses of insulin (sensitive rat RIA kit; Linco, St. Charles, MS) and total Ad (mouse RIA kit; Linco).

Preparation of giant sarcolemmal vesicles. Red gastrocnemius (RG) and red tibialis anterior (RTA) muscles from each animal were pooled to prepare giant sarcolemmal vesicles as described previously (9). These representative oxidative muscles have previously been used as surrogates for the oxidative Sol when tissue is limited (26, 31). The vesicles were frozen at −80°C until analyzed for sarcolemmal membrane-associated FA transport protein expression (FAT/CD36, FABPpm).

FA metabolism. Sol strips were equilibrated in 2 ml of pregassed (95% O_2-5% CO_2) Krebs-Henseleit buffer (KHB; 4% BSA, 30°C) with 0.5 mM palmitate and 5 mM glucose for 30 min. Muscles were incubated for an additional 60 min with the addition of 0.5 µCi/ml [1-^{14}C]palmitate (Amersham, ON, Canada) and in the absence or presence of gAd (2.5 µg/ml; Peprotech, ON, Canada) to determine palmitate oxidation and incorporation into endogenous triacylglycerol (TAG) and DAG lipid pools as outlined previously (12).

After the incubations, muscles were blotted of excess liquid, trimmed of tendons, weighed, and treated to separate the aqueous and lipophilic phases. One milliliter of the aqueous phase was quantified by liquid scintillation counting to determine the amount of ^{14}C-labeled oxidation intermediates resulting from isotopic fixation. Muscle lipids were redissolved in 100 µl of 2:1 chloroform-methanol, spotted on to an oven-dried silica gel plate (Fisher Scientific Canada, Mississauga, ON, Canada), and placed in a sealed tank containing 60:40:3 heptane-isopropyl ether-acetic acid for 50 min. Plates were dried, sprayed with dichlorofluorescein dye (0.2% wt/vol in ethanol), and visualized under long-wave ultraviolet light. The individual lipid bands were scraped in vials for liquid scintillation counting. $^{14}CO_2$ accumulated in the buffer was released by transferring 1 ml of buffer in a sealed flask and acidifying with 1 ml of 1 M sulfuric acid and captured in benzethonium hydroxide. The trapped $^{14}CO_2$ were counted using standard liquid scintillation counting techniques. Total palmitate uptake was calculated by summing the incorporation of labeled palmitate in lipid pools plus oxidation.

Glucose Transport

Sol strips were equilibrated for 30 min in 2 ml of pregassed (95% O_2-5% CO_2) KHB (0.1% BSA, 30°C) containing 8 mM glucose and 32 mM mannitol, in the absence or presence of insulin (10 mU/ml, maintained in all subsequent steps), in a gentle shaking bath. Muscle strips were washed (2 × 10 min) with glucose-free KHB (4 mM pyruvate, 36 mM mannitol). Sol strips were then incubated for 20 min (insulin) or 40 min (basal) in KHB [4 mM pyruvate, 8 mM 3-*O*-[^{3}H]methyl-D-glucose (800 µCi/mmol), 28 mM [^{14}C]mannitol (60 µCi/mmol)]. Muscles were blotted, weighed, and digested (95°C, 10 min, 1 ml of NaOH). Glucose transport was calculated from a 200-µl aliquot of muscle digest to quantify intracellular 3-*O*-[^{3}H]methyl-D-glucose as described previously (37).

AMPK/ACC and insulin signaling proteins. Sol strips were incubated in KHB containing 4% FA-free BSA, 5 mM glucose, and 0.5 mM palmitate for either *1*) 30 min in the presence or absence of gAd (2.5 µg/ml) for the determination of total and phosphorylated AMPK and ACC protein content or *2*) for 10 min in the presence or absence of insulin (10 mU/ml) for the determination of total and phosphorylated insulin signaling proteins. After incubation, the strips were immediately frozen and stored in liquid N_2 until subsequent Western blot analyses.

Western Blot Analyses

Muscle tissue (~50 mg Sol) was homogenized (5,000 μl/g tissue, 1:5 dilution) in ice-cold buffer suitable for protein extraction and preserving phosphorylation states of proteins as described previously (30). Homogenates were centrifuged at 20,000 g for 20 min at 4°C, and the supernatant was removed, and protein content was determined using BSA as standards.

This whole tissue lysate protein (50 μg) or 10 μg of giant sarcolemmal vesicle protein were solubilized in 4× Laemmli's buffer, boiled (95°C, 5 min), resolved by SDS-PAGE, and wet-transferred to polyvinylidene difluoride membranes [1–1.5 h, 100 V; total and Ser[79]-phosphorylated ACC: 8–15 h, 25–40 V, 4°C]. The membranes were blocked for 1 h and then incubated overnight at 4°C with the specific primary antibodies for total and Thr[172] phosphorylated AMPK (tAMPK, pAMPK; Cell Signaling, Danvers, MA), total and Ser[79] phosphorylated ACC (tACC, Cell Signaling; pACC, Upstate, MA), sarcolemmal FABPpm (gift from Dr. J. Calles-Escandon, Wake Forest University School of Medicine), FAT/CD36 (gift from Dr. N. N. Tandon, Otsuka Maryland Medicinal Laboratories), AdipoR1 (AbCAM, Cambridge, MA), total (Upstate), Ser[473] (Santa Cruz Biotechnology, Santa Cruz, CA), and Thr[308] (Santa Cruz) phosphorylated Akt, total (Upstate) and Thr[642] phosphorylated (Medicorp, Montreal, QC, Canada) Akt substrate 160 (AS160), total IRS-1 (Upstate) and p85 subunit of PI 3-kinase (Upstate). After incubation with appropriate secondary antibody for 1 h, the immune complexes were detected using the enhanced chemiluminescence method (Syngene Chemigenius2; PerkinElmer, Waltham, MA) and quantified with densitometry (Gene Tools software; PerkinElmer). Equal loading was confirmed using nonspecific protein staining with Ponceau-S stain (Sigma Aldrich, Oakville, ON, Canada).

Calculations and Statistics

All data are reported as means ± SE. Results were analyzed using a randomized block-design two-way ANOVA, and a Student-Newman-Keul's post hoc test was used to test significant differences revealed by the ANOVA. For all muscle measurements, no differences were found between CON animals at the three time points; therefore, the CON groups were combined. Similarly, for Western blots of total protein content, no differences were found between basal and acute stimulated conditions; therefore, these groups were also combined. One-way ANOVA was used to analyze dietary and/or time effects when no additional treatment was present. Significance was accepted at $P \leq 0.05$.

RESULTS

Body Mass and Blood Measurements

There was no significant difference in pretrial or terminal body mass of CON and HF animals at any time point (Table 1). Fasting blood glucose and plasma insulin did not differ between CON and HF animals at any time point (Table 1). Fasting plasma Ad was significantly lower in HF animals compared with CON animals at each time point ($P \leq 0.05$, Table 1).

Lipid Metabolism

FA oxidation. Basal FA oxidation rate did not differ between CON and HF rats at any time point. Ad significantly increased FA oxidation in Sol from CON rats (+28%; $P \leq 0.05$), but it had no stimulatory effect on FA oxidation in HF animals at any time point (Fig. 1A).

FA esterification. FA esterification into TAG was greater in HF animals by 2 and 4 wk compared with CON (+23% and +34%, respectively, $P \leq 0.05$), but gAd did not have a significant acute effect within dietary treatments (Fig. 1B). FA esterification into DAG was not different between the dietary treatments at any time point and was unaffected by gAd (range, 13.1 ± 0.9 to 14.5 ± 0.5 nmol·g^{-1}·h^{-1}).

Total palmitate uptake. Total palmitate uptake was greater in HF-fed rats after 2 and 4 wk compared with CON (+13% for both, $P \leq 0.05$). Treatment with gAd did not change total FA uptake in any group (Fig. 1C).

Ad-Stimulated Signaling Proteins and Receptor

AMPK and ACC signaling. There was no significant effect of diet or acute gAd exposure on tAMPK, pAMPK (Fig. 2A) or tACC protein levels. Ad acutely increased pACC in CON rats (+34%; $P \leq 0.05$), but failed to do so in HF rats at any time point (Fig. 2B).

AdipoR1. There was no significant effect of diet or time on AdipoR1 protein content in Sol muscles (Fig. 3).

Sarcolemmal FA Transporters and Muscle Lipid Content

Transporters. FAT/CD36 (Fig. 4A) was significantly increased in the plasma membrane of red muscle (RG and RTA) of rats after 2 and 4 wk of chronic HF feeding compared with CON (+31% and +34%, respectively; $P \leq 0.05$). FABPpm (Fig. 4B) was elevated after 4 wk of HF feeding compared with CON (+57%; $P = 0.06$).

Muscle lipid. Total DAG and ceramide content was not different between 3 day HF and CON. However, total DAG (+19% to +25%; $P \leq 0.05$) and ceramide (+23% to +26%; $P \leq 0.05$) content was increased in Sol following 2 and 4 wk of HF feeding compared with CON (Fig. 4, C and D).

Insulin Signaling Proteins

Total proteins. HF feeding had no significant effect on the total amount of IRS-1, p85 PI 3-kinase, Akt, or AS160 protein in Sol muscle (data not shown).

Table 1. *Body mass and blood measurements*

	CON 3Days	HF 3Days	CON 2Wk	HF 2Wk	CON 4Wk	HF 4Wk
Pretrial body mass, g	184±2	186±3	195±2	191±3	191±2	188±2
Terminal body mass, g	188±3	189±2	234±3	237±3	265±5	272±4
Fasting blood glucose, mmol/l	7.5±0.3	8.1±0.2	7.5±0.3	8.8±0.3	7.6±0.5	8.2±0.4
Fasting plasma insulin, ng/ml	1.6±0.1	1.5±0.1	1.7±0.1	1.7±0.1	1.8±0.1	1.7±0.1
Fasting plasma Ad, μg/ml	4.3±0.6	2.9±0.4*	4.9±0.5	3.6±0.3*	4.6±0.4	3.6±0.2*

Data are means ± SE, $n = 8–12$ rats. CON, control diet; HF, high-fat diet; Ad, adiponectin. *$P \leq 0.05$, significantly different from control.
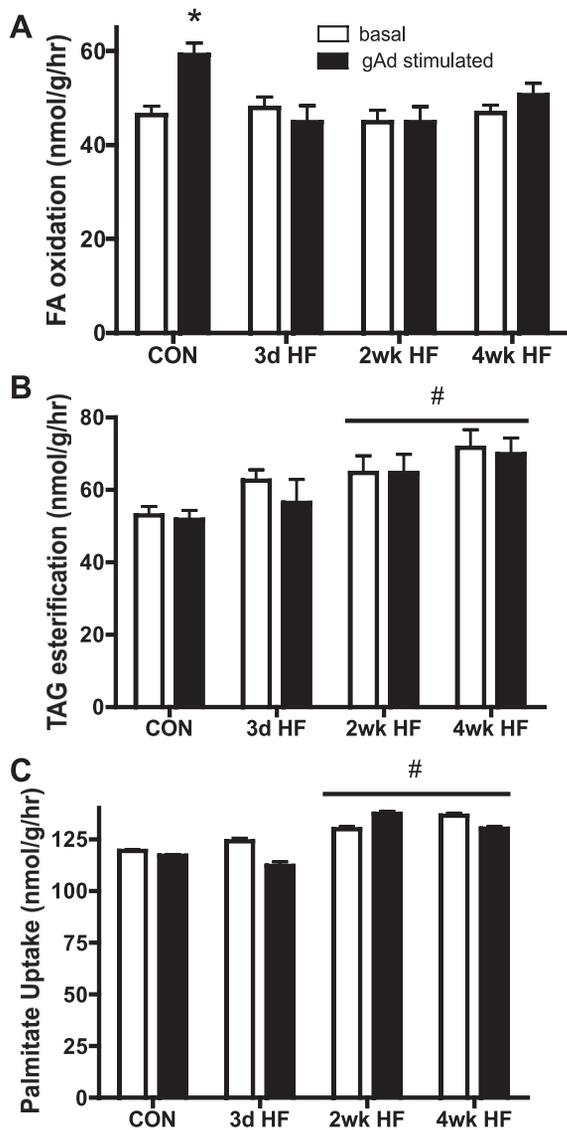
Fig. 1. Effect of globular head adiponectin (gAd) on skeletal muscle fatty acid (FA) metabolism. *A*: rate of FA oxidation. *B*: palmitate incorporation into triacylglycerol (TAG). *C*: rate of palmitate uptake calculated as palmitate oxidation plus diacylglycerol (DAG) and TAG esterification. Data are means ± SE, $n = 12$ rats. $P \leq 0.05$, significantly different from basal (*) and significantly different from control (CON) conditions (#). Open bars, basal; filled bars, gAd stimulated (2.5 μg/ml). 3d HF, 3-day high-fat (HF) diet; 2 wk HF, 2-wk HF diet; 4 wk HF, 4-wk HF diet.

Phosphorylated proteins. Insulin-stimulated phosphorylation of Akt Ser[473] and Thr[308] was blunted in HF animals compared with CON after only 3 days and remained blunted at 2 and 4 wk (Fig. 5, *A* and *B*). There was a trend ($P = 0.08$) for insulin-stimulated Thr[642] phosphorylation of AS160 to be blunted in HF animals at 2 and 4 wk compared with CON (Fig. 5*C*).

Skeletal Muscle Glucose Transport

Basal glucose transport was not different among the dietary groups at any time point. Insulin increased glucose transport above basal levels in all groups; this stimulation was significantly blunted only in the 4-wk HF compared with all other groups (Fig. 6).

DISCUSSION

Evidence of Ad resistance in skeletal muscle has been shown in diabetic mice (34), obese humans (5, 11) and following HF feeding in rats (23). However, whether Ad resistance precedes and potentially contributes to intramuscular lipid accumulation and IR is unknown. Therefore, we conducted a time course HF feeding trial in rats to determine the onset of Ad resistance and to identify the ensuing changes in lipid metabolism and insulin signaling leading to IR. Here we have shown that Ad resistance occurs very rapidly, after only 3 days of HF (saturated) feeding and is sustained, as determined by a failure of gAd to acutely stimulate FA oxidation and phosphorylate ACC in skeletal muscle of all HF-fed groups. However, this was not due to a decrease in AdipoR1 protein content. By 2 wk of HF feeding, we observed increased FAT/CD36 at the plasma membrane of skeletal muscle, accompanied by increased total FA uptake in
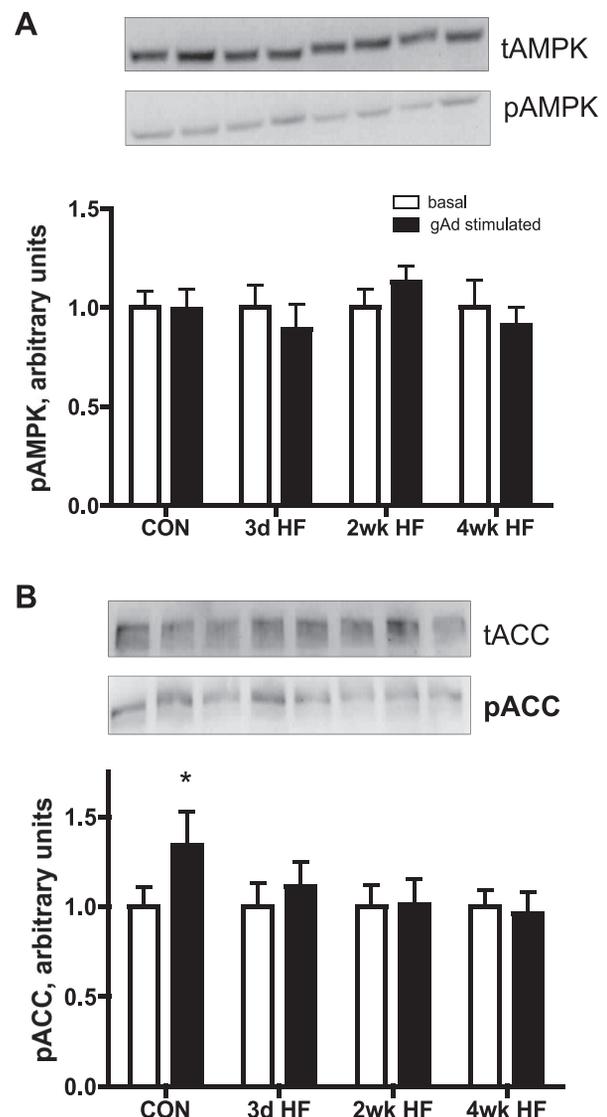

Fig. 2. Acute effect of 30 min gAd exposure on protein content of total (t) AMP-activated protein kinase (AMPK) and phosphorylated AMPK (pAMPK) (*A*) and total (t) acetyl-CoA carboxylase (ACC) and phosphorylated ACC (pACC) (*B*) in soleus muscle. Data are means ± SE, $n = 10$ rats. *$P \leq 0.05$, significantly different from basal. Open bars, basal; filled bars, gAd stimulated (2.5 μg/ml).
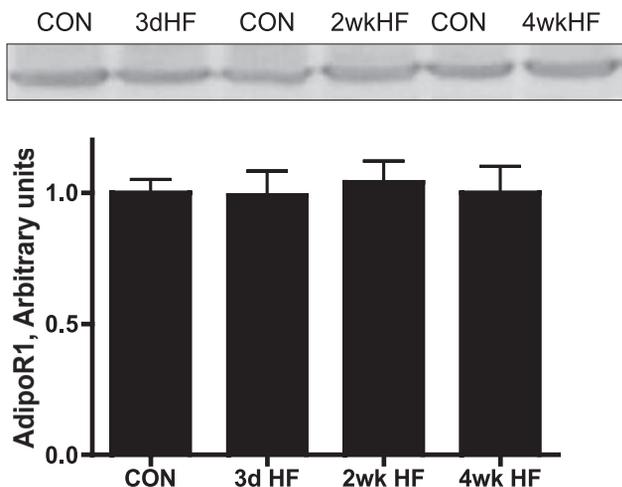
CON   3dHF   CON   2wkHF   CON   4wkHF

Fig. 3. Effect of chronic HF feeding on soleus muscle AdipoR1 protein content. Data are means ± SE, $n = 12$ rats.

muscle and intramuscular ceramide and DAG accumulation. Furthermore, blunted insulin-stimulated phosphorylation of both Akt and AS160 was apparent by 2 wk, whereas impaired maximally insulin-stimulated glucose transport did not occur until 4 wk (Fig. 7). Taken together, our results suggest that the early loss of gAd's stimulation of FA oxidation, coupled with a subsequent increase in FA transport, is associated with the

accumulation of reactive DAG and ceramide lipid species and impaired insulin response.

HF Feeding Induces Ad Resistance Before Increases in Intramuscular Lipid Content and Impaired Insulin Response

Similar to our previous study (23), we have shown that a 60% saturated fat diet can induce skeletal muscle Ad resistance, as evidenced by a failure of gAd to increase FA oxidation or phosphorylate ACC above basal levels in intact, isolated Sol muscles. However, we have now identified that this resistance occurs extremely rapidly, preceding intramuscular lipid accumulation and impairment of maximal insulin-stimulated glucose transport. Ad is known to increase FA oxidation in skeletal muscle by the inactivation of ACC, and reducing inhibition of CPT1 by malonyl-CoA, leading to increased FA uptake in the mitochondria (33, 40). Whether this requires the activation of AMPK is controversial (33). As with our previous study (23), we did not show an effect of 30 min gAd exposure on pAMPK in Sol muscle of any group but did observe a 30% increase in gAd-stimulated pACC in CON animals. This suggests that the loss of gAd-stimulated FA oxidation in HF animals may be attributable to a lack of phosphorylation of ACC, independent of changes in AMPK phosphorylation.

The decreased gAd response in HF-fed animals was not attributable to a decrease in receptor content. Several studies that have examined muscle AdipoR1 in response to HF feeding
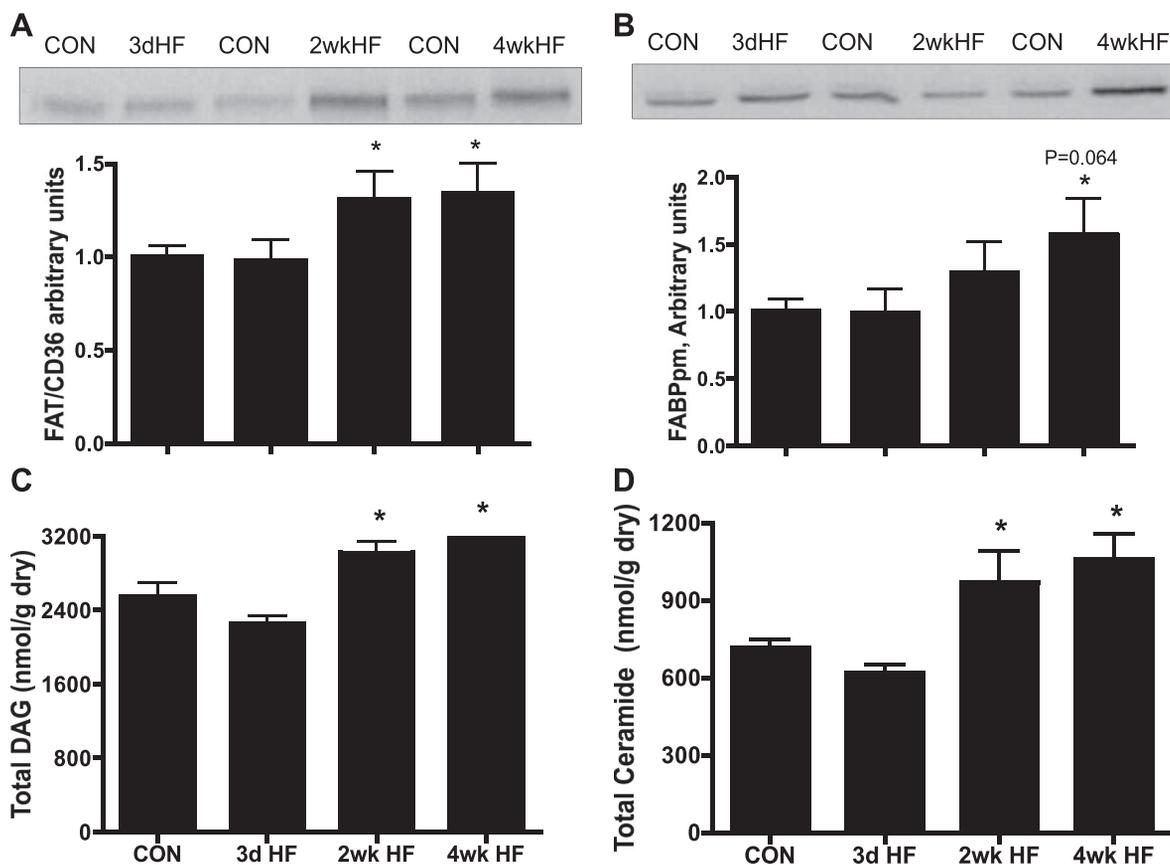

Fig. 4. Effect of chronic HF feeding on plasma membrane (PM)-associated FA transporter protein content and intramuscular lipid content. A and B: PM-associated fatty acid translocase (FAT)/CD36 (A) and PM-associated plasma membrane-bound fatty acid-binding protein (FABPpm) (B) measured in giant sarcolemmal vesicles. C and D: total DAG (C) and total ceramide (D) measured in soleus muscle. Data are means ± SE; $n = 12$ rats in A and B and 6–10 rats in C and D. *$P ≤ 0.05$, significantly different from CON, unless otherwise stated.
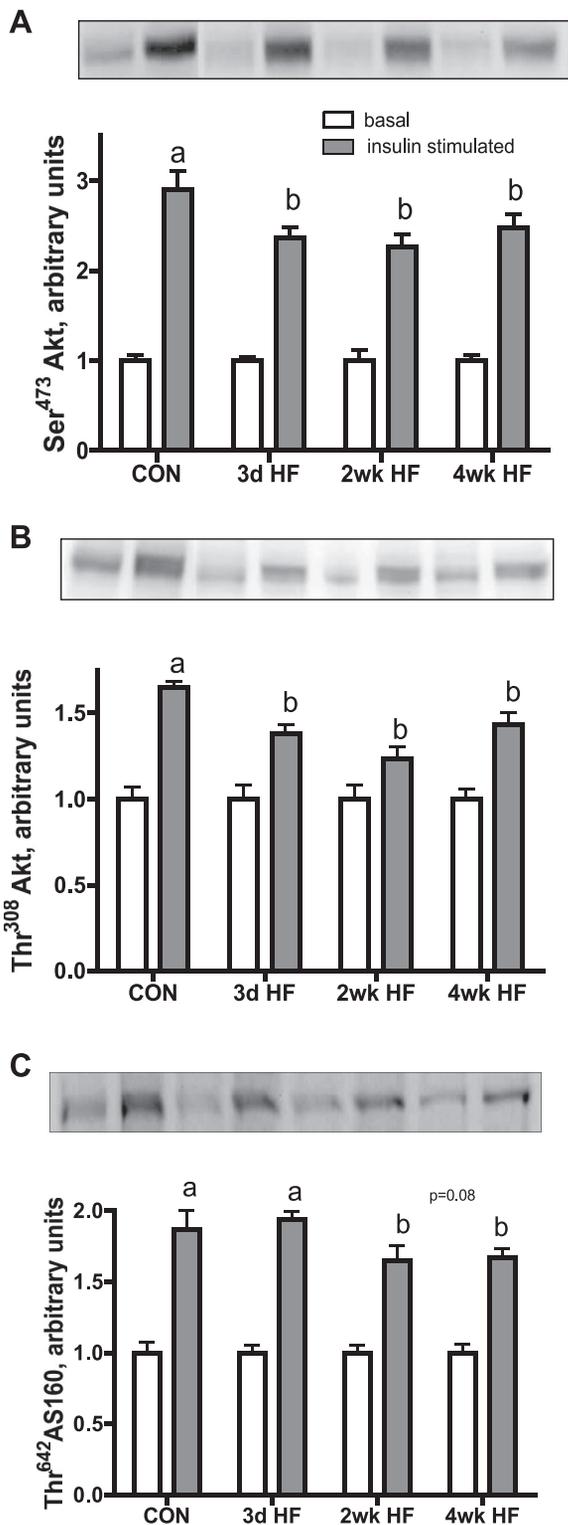
Fig. 5. Acute effect of 10 min insulin exposure on protein content of Ser[473] protein kinase B (Akt) (*A*), Thr[308] Akt (*B*), and Thr[642] Akt substrate 160 (AS160) (*C*) in soleus muscle. Data are means ± SE, $n = 10$ rats. Bars not sharing a letter are significantly different ($P \leq 0.05$), unless otherwise stated. Open bars, basal; filled bars, insulin-stimulated (10 mU).
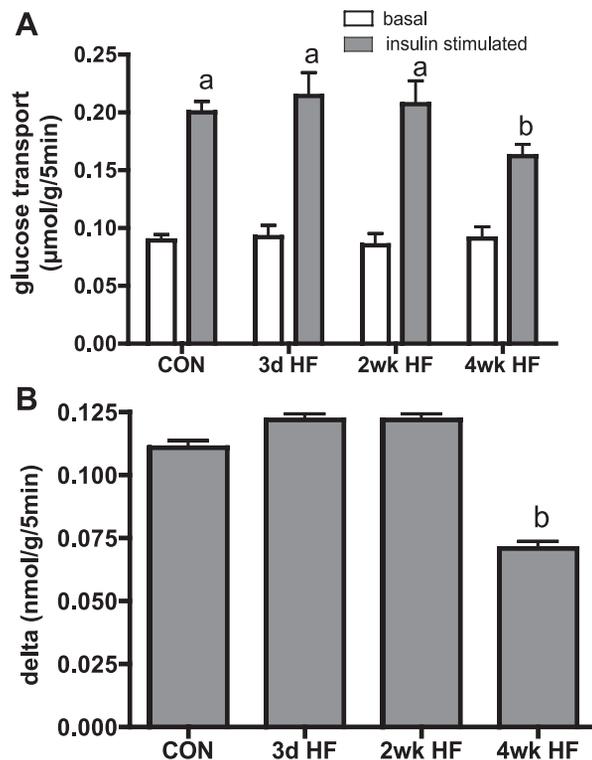
Fig. 6. *A*: basal and insulin-stimulated glucose transport in soleus muscle. *B*: change in glucose transport rate from basal in response to insulin. Data were calculated by subtracting respective rates of basal uptake from the insulin response. Data are means ± SE, $n = 12$ rats. Bars not sharing a letter are significantly different, $P \leq 0.05$. Open bars, basal; filled bars, insulin stimulated (10 mU).

creased in genetically obese diabetic mice (34). Interestingly, a recent study by Weigert et al. (38) reported increased AdipoR1 mRNA levels in monocytes from type 2 diabetic subjects, whereas AdipoR1 protein content was actually decreased, reinforcing the concern that changes in protein do not always parallel changes in mRNA. In the current study, we did not observe any changes in whole muscle AdipoR1 protein content. Therefore, a change in receptor protein content does not appear to be a likely cause of Ad resistance. However, we cannot rule out the possibility that AdipoR1 sensitivity, conformation, or association with the plasma membrane or other required molecules could have been altered by the HF diet and contributed to the observed resistance. Furthermore, the signaling events between gAd binding to AdipoR1 and AMPK-ACC phosphorylation are not well established. Suppressor of cytokine signaling 3 (SOCS3) has been shown to inhibit leptin activation of AMPK in cultured human myotubes and contribute to leptin resistance observed in obese subjects (32). Because leptin and Ad stimulate FA oxidation through similar mechanisms, it is possible that SOCS3 may interfere with

Fig. 7. Timeline of events occurring in skeletal muscle of HF-fed rats. All changes observed were maintained through subsequent time points.

have only examined mRNA levels and have reported no change (3, 4) or an increase (2, 7) following 1–5 mo of HF feeding. Moreover, AdipoR1 mRNA is reported to be de-
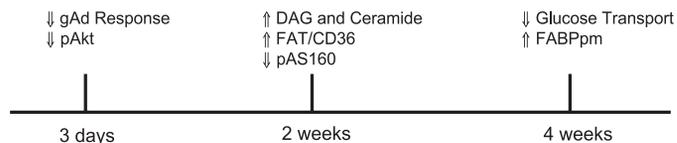
intracellular gAd signal transduction as well. Further research is required to determine the effects of SOCS3 on gAd signaling in skeletal muscle.

HF Feeding Increases FA Transporter and Intramuscular Lipid Content by 2 wk

Total palmitate uptake was increased above CON by 2 and 4 wk of HF feeding, as was the rate of esterification into TAG, and total muscle DAG and ceramide contents. A likely explanation for the increased palmitate uptake is a parallel increase in FA transporters at the plasma membrane. Specifically, plasma membrane-associated FAT/CD36 protein content was increased above CON in 2 and 4 wk HF, and FABPpm protein content was increased above CON in 4 wk HF. Thus, by 2 wk of HF feeding, membrane-associated FAT/CD36 and palmitate uptake in the muscle was increased, which, together with a diminished stimulation of FA oxidation by Ad, likely contributed to the observed accumulation of DAG and ceramide.

Clearly, the temporal data in this study cannot prove that the early development of Ad resistance is causative in the accumulation of intramuscular lipids and IR. Indeed, we (16) and others (14, 35) have recently questioned the notion that impaired mitochondrial FA oxidation is necessarily an initiating cause of lipid accumulation and the ensuing impairment of insulin-stimulated glucose transport. However, this certainly does not exclude FA oxidation as an important factor in these events. Thus it may be possible that a loss or blunted response of FA oxidation in vivo, to such adipokines as Ad and leptin, coupled with an increased capacity to take up and store FA, results in a relatively rapid accumulation of muscle lipids. Indeed, we have also noted a loss of leptin's ability to stimulate FA oxidation within 3 days of HF feeding (unpublished data).

HF Feeding Impairs Insulin Signaling Before Impairing Glucose Transport

Numerous studies have correlated an increase in intramuscular lipids with IR (36). Specifically, elevated TAG has been associated with IR, although, this is now recognized as a marker of elevated intramuscular lipids rather than a direct cause of IR. Here we have shown that accumulation of DAG and ceramide precede functional impairments in maximally insulin-stimulated glucose transport in muscle. Ceramide and DAG have both been shown to directly interfere with insulin signal transduction. Specifically, ceramide can impair phosphorylation of Akt, thereby preventing Akt's stimulatory effect on GLUT 4 translocation (10, 29). Blunted Akt phosphorylation has been observed in skeletal muscle of HF-fed animals (24, 27, 28). In the current study, we see early impairments of insulin-stimulated Akt phosphorylation, coinciding with increased ceramide accumulation by 2 and 4 wk in HF-fed animals. Somewhat surprisingly, insulin-stimulated Akt phosphorylation was also blunted at 3-day HF compared with CON although ceramide content was not yet elevated.

In comparison, impaired insulin-stimulated AS160 phosphorylation was not evident until 4 wk of HF feeding. Nascimento et al. (24) have reported blunted insulin stimulation of AS160 following 7 wk of a HF diet. Because AS160 is a downstream substrate of Akt, it is possible that impaired Akt activation does not translate into impaired AS160 activation until later on. To the best of our knowledge, it is unknown whether ceramide directly interferes with AS160 phosphorylation, independent of its effects on Akt.

Although intramuscular lipid accumulation may partially explain the blunted phosphorylation of Akt, it is interesting that this reduction in insulin signaling occurs well before the reduction in glucose transport. To the best of our knowledge, ours is one of the first papers to show this timeline. Akt is a known intermediate necessary for GLUT4 translocation (21). However, a recent study by Ng et al. (25) proposed the idea of Akt spareness; that is, minimal activation of Akt is sufficient to elicit a maximum effect on glucose transport and AS160 phosphorylation. Ng et al. showed that the dose response of insulin-stimulated 2-deoxyglucose uptake in 3T3-L1 adipocytes paralleled the dose-response activation of AS160 phosphorylation, but not that of Akt itself. Therefore, in the current study, although Akt signaling is impaired early on, it is possible that glucose transport can be maintained, and it is not until subsequent AS160 impairment that functional impairments in glucose transport is seen. Why AS160 phosphorylation is retained longer, and the physiological significance of early impairments of insulin-stimulated Akt phosphorylation remain to be determined.

Unlike the rapid development of Ad resistance, maximal insulin-stimulated glucose transport was not impaired until 4 wk of HF feeding. Although a 4-wk HF feeding period is still relatively brief, we have clearly demonstrated that several significant impairments in lipid metabolism and insulin signaling occur much earlier (i.e., after 3 days) and likely contribute to the development of lipid-induced IR. Although we cannot discount that impaired glucose transport may have developed before 4 wk, our results confirm that following 2 wk on a 60% high saturated diet, skeletal muscle is still fully responsive to a maximal dose of insulin. It is possible, however, that we may have observed decreased insulin sensitivity to a submaximal insulin dose before this.

Summary and Perspectives

The present study utilized a time course high saturated fat feeding model to determine the early metabolic events in skeletal muscle leading to IR. We propose that a rapid loss of gAd's stimulatory effect on FA oxidation leaves the muscle less able to adequately respond to the excess lipid it is exposed to during HF feeding. The actual cause of Ad resistance remains unknown, although a decrease in receptor number does not appear to be the cause. Regardless of the specific cause, it seems plausible that the rapid development of resistance to an insulin-sensitizing cytokine, such as Ad, may be a contributing factor to the ensuing development of IR. This inability to appropriately stimulate FA oxidation, coupled with increased FA transporters at the plasma membrane and increased rate of FA uptake, results in intramuscular lipid accumulation. Specifically, ceramide accumulation is likely involved in the observed impaired phosphorylation of Akt and AS160, eventually resulting in impaired glucose transport. It should be recognized that these findings were restricted to a high saturated fat diet, and may not represent the course of events with the feeding of a polyunsaturated fat diet. Future studies should determine the specific cause of Ad resistance to serve as a potential therapeutic target for the treatment of IR.

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