Metabolic, Endocrine, and Genitourinary Pathobiology

Adiponectin and Skeletal Muscle

Pathophysiological Implications in Metabolic Stress

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Upregulation of muscular adiponectin could act as a local protective mechanism to counteract cellular damage in obesity by weakening inflammation, oxidative stress, and apoptosis. To test this hypothesis, adiponectin-knockout (KO) and wild-type (WT) mice were fed a Western diet (WD). WT mice under WD conditions displayed 63% higher adiponectin expression in myocytes than those under standard laboratory diet (SLD) conditions ($P = 0.011$). WD-fed KO mice exhibited approximately threefold larger myocyte degeneration than WT mice ($P = 0.003$). Even under SLD conditions, myotubes of KO mice displayed already moderate immunolabeling for markers of oxidative stress (peroxiredoxin-3/5) and for a lipid peroxidation product (hydroxynonenal). Expression of tumor necrosis factor- α (TNF- α) and caspase-6, a marker of apoptosis, was also present. After WD challenge, immunoreactivity for these markers was strong in muscle of KO mice, although it was detected to a lesser extent in WT mice. Activation of NF- κ B and caspase-6 doubled in myocytes of WD-fed KO mice when compared to WT mice ($P < 0.001$). Furthermore, muscle electrotransfer of the adiponectin gene prevented these abnormalities in WD-fed KO mice. Finally, gene abrogation of the adiponectin receptor 1 (AdipoR1) by siRNA recapitulated a pro-inflammatory state in C2C12 myotubes. Thus, upregulation of muscular adiponectin may be triggered by obesity and be crucial locally to counteract oxidative stress, inflammation, and apoptosis. These effects operate in an autocrine/paracrine manner via AdipoR1 and down-regulation

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Adiponectin (ADIPOQ, so named for its structural homology with complement factor C1q¹) is a hormone abundantly secreted by adipocytes, the circulating levels of which are decreased in obese individuals and in patients who meet criteria for the metabolic syndrome. Adiponectin can exhibit insulin-sensitizing, fat-burning, and anti-inflammatory properties as well as a modulatory effect on oxidative stress, thereby thwarting simultaneously several facets of the metabolic syndrome.^{2,3}

Adiponectin receptors (AdipoR)–1 and AdipoR2 serve as major receptors for adiponectin and have important roles in the regulation of glucose and lipid metabolism, inflammation and oxidative stress *in vivo*.⁴ AdipoR1 is abundantly expressed in muscle, whereas AdipoR2 is mainly expressed in liver.⁵ In skeletal muscle, adiponectin enhances insulin sensitivity by stimulation of AMP-activated protein kinase, which in turn activates IRS-1.⁶ Decreased muscular triglyceride content and reduced inflammation and oxidative stress may contribute to adiponectin insulin-sensitizing properties as well.^{6,7} The decreased muscular triglyceride content may be ascribed to upregulation of mitochondrial biogenesis and fatty acid oxidation through activation of a master regulator PGC-1 α (peroxisome proliferator-activated receptor γ coactivator-1 α).⁶ As mentioned earlier, adiponectin is also known to attenuate inflammation and oxidative responses to multiple stimuli by modulating different signaling pathways in a variety of cell types.⁸

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Skeletal muscle has been recognized as an endocrine organ that secretes cytokines/hormones that are collectively referred to as myokines.⁹ Although adiponectin is secreted exclusively by adipocytes under normal conditions, emerging studies point out its presence in nonadipose tissues,¹⁰ such as skeletal muscle,^{11–17} where the adipokine has been detected within myofibers.^{11,12,18}

We have shown that adiponectin was upregulated in skeletal muscle after an acute inflammation caused by lipopolysaccharide (LPS) injection or after a metabolic stress caused by genetic obesity and diabetes in *ob/ob* mice.^{11,12} Likewise, adiponectin was upregulated in C2C12 myotubes challenged by pro-inflammatory cytokines,¹¹ lipoperoxidation products, or reactive oxygen producers.¹² The hypothesis that upregulation of adiponectin may be a local protective mechanism was raised and further supported by our data in adiponectin-KO mice acutely challenged by ip LPS. When compared to wild-type (WT) mice, muscle of adiponectin-KO mice displayed increased oxidative stress, inflammation, and apoptosis after LPS injection. Muscle electrotransfer of the adiponectin gene prevented all of these abnormalities, thereby suggesting that the presence of adiponectin is crucial to counterbalance an acute inflammation in murine muscle.¹⁸ Whether adiponectin is also induced by chronic and low-grade metabolic inflammation such as that typically observed in the common form of obesity³ and whether this local production of adiponectin is also needed to counteract potential muscular damage is still unclear.

The aim of the present work was to address these questions. To this end, we first assessed whether adiponectin is actually induced in muscle of WT mice fed a Western diet (WD), an acquired model of obesity, which is highly relevant in the context of obesity pandemic. Second, we examined whether muscles of KO mice exhibit higher degree of inflammation, oxidative stress and apoptosis than those of WT mice when challenged by WD and whether these abnormalities may be corrected by local administration of adiponectin in an autocrine/paracrine manner. Finally, we evaluated *in vitro* whether silencing adiponectin or its receptors may recapitulate in C2C12 myotubes some of the *in vivo* findings.

Materials and Methods

Animals

The University of Louvain Animal Care Committee approved all procedures. Adiponectin-knockout (KO) mice, which are characterized by a generalized lack of adiponectin, were obtained from Maeda et al⁷ and maintained on a C57BL/6J background. Wild-type (WT) controls were C57BL/6J mice that were raised together with KO mice (but were not their littermates). KO and WT mice were housed at a constant temperature (22°C) with a fixed 12-hour light, 12-hour dark cycle. We used only male mice.

At 7 weeks of age, KO mice and WT mice received *ad libitum* either WD (high fat/sucrose diet) (RD Western Diet,

Research Diets, Inc., New Brunswick, NJ) or a standard laboratory diet (SLD) (Rat and Mouse no.3 Breeding, Special Diets Services, Witham, UK) for 8 weeks. Four groups of mice ($n = 6/\text{group}$) were studied: wild-type mice fed with SLD (WT + SLD), KO mice fed with SLD (KO + SLD), wild-type mice fed with WD (WT + WD), and KO mice fed with a WD (KO + WD). All of these mice underwent an oral glucose tolerance test (OGTT) after an overnight fast 1 week before the end of the study. The test started at 8 AM. Glucose (30% in water) was introduced into the stomach through a fine gastric catheter at a dose of 2 g/kg body weight. Mice were gently wrapped in a towel to restrain them during blood collections.¹⁹ At the end of the 8-week diet administration, mice were sacrificed by decapitation (between 9 and 10:30 AM). Blood samples were saved. Pairs of *tibialis anterior* muscles and inguinal fat were dissected, weighted, frozen in liquid nitrogen and stored at -80°C . Not all mice were used for all measurements/analyses, mainly those dealing with muscle immunochemistry; the exact number of mice used is provided in the appropriate section.

In an additional experiment, after 4 weeks on WD, muscle transfer of adiponectin/*ADIPOQ* gene was performed in anesthetized KO mice ($n = 4$) by injection of *ADIPOQ* cDNA containing-plasmid followed by electroporation in one *tibialis anterior*, the contralateral muscle being electroporated by a control plasmid. Mice were maintained on the WD for another 4 weeks. Adiponectin was overexpressed only in the muscle injected with *ADIPOQ* cDNA without any rise in circulating levels of the adipokine.¹⁸

Electrotransfer of Adiponectin Gene into Muscle

Expression Plasmids and DNA Preparation

Plasmid pcDNA 3.1-*ADIPOQ* was constructed by inserting the mouse full-length adiponectin cDNA²⁰ into the *KpnI*-*AgeI* sites, between the cytomegalovirus promoter and a 3' flanking sequence of bovine GH polyadenylation signal, in the pcDNA 3.1-V5-His A expression vector (Invitrogen, Life Technologies, Belgium). Plasmids were amplified in *Escherichia coli* top 10 F' (Invitrogen), purified with an EndoFree plasmid giga kit (Qiagen, Hilden, Germany), and then were stocked at -80°C .

DNA Injection and Electroporation

A 30- μL quantity of plasmid solution (2 $\mu\text{g}/\mu\text{L}$) was injected into each *tibialis anterior*. Muscles were then electroporated by using transcutaneous electric pulses (8 square-wave pulses of 200V/cm and 20 milliseconds per pulse at 2 Hz) that were applied by two 4-mm-spaced electrodes. Pulses were delivered by a Cliniporator system (Cliniporator, IGEA, Carpi, Italy), as described.^{18,21}

Quantification of Circulating Parameters

Blood glucose was measured using a glucometer (Medisense Precision Xtra Plus, Abbott-Medisense, Louvain-la-Neuve, Belgium). Plasma adiponectin concentrations were

determined by a commercially available kit (RIA mouse adiponectin kit; Linco Research, St. Charles, MO). Plasma insulin, total cholesterol, NEFA and triglyceride levels were quantified, as reported previously.¹²

Light Microscopy, Immunohistochemistry, and Morphometry

Muscle samples were fixed in 10% formaldehyde for 24 hours and embedded in paraffin. Sections (5 μ m thick) were stained with hematoxylin-eosin-safran (HES). For quantification of myocyte degeneration, the percentage of ghost cells was counted on more than 250 cells per slide section. For immunohistochemistry, sections were processed as previously described^{22,23} using rabbit polyclonal antibodies directed against adiponectin (Chemicon, Biognot, Heule, Belgium), Caspase-6 (Tebu-bio, Baeckout, Belgium), Peroxiredoxin 3 (PRDX3), Peroxiredoxin 5 (PRDX5) (gifts from B. Knoop, University of Louvain, Brussels, Belgium²⁴), 4-hydroxy-2-nonenal (HNE) (Calbiochem, Darmstadt, Germany), TNF- α (Abcam, Cambridge, UK), and NF- κ B p65 (Abcam). We also used antibodies directed against two macrophage markers, mouse monoclonal F4/80 antibody (AbD, Serotec, Dusseldorf, Germany; dilution 1:100 for 24 hours) and rat monoclonal CD68 antibody (Abcam, Cambridge, UK; dilution 1:40 for 24 hours). Other antibody concentrations and incubation times were similar to those previously reported.¹⁸ Sections used for adiponectin, caspase-6, HNE, NF- κ B, F4/80, and CD 68 were pretreated in a microwave oven in Tris-citrate buffer (pH 6.5) for one cycle of 3 minutes at 750 W and three cycles of 3.5 minutes at 350 W. Binding of antibodies was detected by applying for 30 minutes at room temperature a second antibody, which was a goat anti-rabbit, a rat anti-mouse, or a rabbit anti-rat immunoglobulin conjugated to peroxidase labeled polymer (En Vision +, Dako, Copenhagen, Denmark). Peroxidase activity was revealed with 3-amino-9-ethylcarbazole substrate (AEC; Dako, Copenhagen, Denmark), which produces a red stain. Immunohistochemical controls were performed by omission of the first antibody or of the first and second antibodies, by using preimmune serum, and by incubation with an irrelevant antibody (anti-thyroglobulin). For quantification of caspase-6 and NF- κ B, the percentage of immunolabeled nuclei was counted on magnification $\times 400$. The other immunohistochemistry data were qualitative: the intensity of staining was classified by two independent researchers into four categories defined as no/low/moderate/strong signal intensity for each mouse. An example of such scoring is provided online (see Supplemental Figure S1 at <http://ajp.amjpathol.org>). There was no discordance between the two classifications of the researchers.

Oil Red O Staining

Muscle samples were embedded in tissue-Tek and rapidly frozen in isopentane cooled in liquid nitrogen to generate 5- μ m-thick cryostat sections.¹² Frozen sections were then fixed in 10% formaldehyde and stained for 10 minutes with Oil Red O solution (Sigma-Aldrich, Bornem, Belgium).

Western Blot

Skeletal muscle was homogenized in a lysis buffer (Cell Signaling Technology, BIOKE, Leiden, the Netherlands) supplemented with NaF and protease inhibitor cocktail as described.²⁵ Immunoblotting was performed as reported²⁵ by using a rabbit polyclonal antibody directed against murine adiponectin (BioVendor Laboratory Medicine, Heidelberg, Germany). Signals were revealed by enhanced chemiluminescence, quantified, and normalized to those of actin.

Cell Culture and Small Inhibitory RNA Experiments

C2C12 myoblasts were cultured as previously described.¹¹ Briefly, after proliferation, cells were cultured in basal medium (high-glucose DMEM + 2% heat-inactivated equine serum) for 3 days to induce myogenic differentiation. Myotubes were used at this stage and transfected for 24 hours with DharmaFECT2 (Thermoscientific, Lafayette, IN) and 160 nmol/L small Inhibitory RNA (siRNA) targeting adiponectin or 60 nmol/L siRNA directed against AdipoR1 in antibiotic-free medium, according to the manufacturer's instructions (Thermoscientific). We used negative control (nontargeting) siRNA (Thermoscientific) in each experiment. In some tests, 21 hours after initiation of transfection, myotubes were challenged by 10 ng/mL TNF- α (Biovendor) for 3 hours. At the end of the culture, cells were washed in ice-cold PBS before RNA extraction. The exact number of experiments for each protocol ($n = 7$ to 11) is indicated in the appropriate section.

RNA Extraction and RT-Quantitative PCR

RNA was isolated from cultured cells and from mouse muscles with TriPure reagent (Roche Diagnostics, Vilvoorde, Belgium). A 2- μ g quantity of total RNA was reverse transcribed, as described previously.¹¹ Real-time quantitative PCR (qPCR) primers were designed (Primer Express Software version 2.0; Applied Biosystems, Carlsbad, CA) for PGC-1 α (sense, 5'-ACGCTTCGCTGCTCTTGAG-3'; antisense, 5'-GTGGAAGCAGGGTCAAAATCG-3') and for cytochrome c (sense, 5'-AAAAGGGAGGCAAGCATAAGAC-3'; antisense, 5'-TCTCCAAATACTCCATCAGGGTATC-3'); the other sets of primers for mouse AdipoR1, AdipoR2, adiponectin, cyclophilin, TNF- α , interleukin-6 (IL-6), and inhibitory κ B (I κ B)- α were similar to those previously reported.^{12,18,19} In all, 120 ng total RNA equivalents were amplified using an iCycler iQ real-time PCR detection system (Bio-Rad Laboratories, Nazareth, Belgium).¹¹ The threshold cycles (Ct) were measured in separate tubes and in duplicate. The identity and purity of the amplified product were checked by electrophoresis on agarose minigels, and analysis of the melting curve was performed at the end of the amplification. To ensure the quality of the measurements, each plate included a negative control for each gene.

Table 1. Body Weight, Organ Weights, and Circulating Parameters in Four Groups of Six Mice Each

	WT + SLD	KO + SLD	WT + WD	KO + WD
Body weight	28.0 ± 0.5	26.7 ± 1.3	31.0 ± 0.8 ^{††}	33.3 ± 2.5 ^{*†††}
Δ (g)	4.8 ± 0.4	4.6 ± 0.6	8.0 ± 1.2 ^{†††}	10.8 ± 1.9 ^{***†††}
Adipose tissue				
Weight (mg)	266 ± 29	284 ± 35	762 ± 85 ^{†††}	1061 ± 294 ^{*†††}
Skeletal muscle				
Weight (mg)	98 ± 6	97 ± 5	100 ± 5	100 ± 8
Circulating parameters				
Glucose homeostasis				
Blood glucose (mg/dL)	92 ± 4	105 ± 17	98 ± 11	107 ± 14
Plasma insulin (ng/mL)	0.014 ± 0.018	0.02 ± 0.02	0.13 ± 0.16	0.83 ± 0.64 ^{***†††}
Insulin resistance index [I (ng/mL) × G (mg/dL)]	1.25 ± 1.53	1.85 ± 1.78	11.65 ± 13.39	85.44 ± 63.73 ^{***†††}
Plasma lipid levels				
Triglycerides (mg/dL)	47.9 ± 8.9	70.1 ± 13.6 ^{**}	62.2 ± 10.4	71.9 ± 13.7
Cholesterol (mg/dL)	74.8 ± 9.5	92.1 ± 12.8 [*]	141.0 ± 12.4 ^{†††}	143.6 ± 14.3 ^{†††}
NEFA (mg/dL)	58.0 ± 9.2	67.7 ± 5.5	56.8 ± 14.3	60.3 ± 11.8
Adiponectin levels				
Plasma adiponectin (μg/mL)	8.2 ± 0.64	ND	10.1 ± 0.69 ^{†††}	ND
Plasma adiponectin/g inguinal adipose tissue	29.8 ± 4.4		13.3 ± 1.5 ^{†††}	

KO mice were challenged with WD for 8 weeks. Four groups of mice were studied: wild-type mice fed a standard laboratory diet (WT+SLD), KO fed a standard diet (KO+SLD), wild-type mice fed WD (WT + WD), and KO mice fed a Western diet (KO + WD). All circulating parameters were collected at 9 AM in fed mice at the end of the study except for those of glucose homeostasis, which were obtained after an overnight fast (time 0 of the OGTT) 1 week before the completion of the study. The insulin resistance index is calculated as I (ng/mL) × G (mg/dL) (19). Organ weights refer to pairs of *Tibialis anterior* muscles or inguinal fat pads. Δ Body weight, changes in weight over the 8 weeks of the study. ND, not detectable. Data are means ± SD. Analysis of variance was performed on a log scale for plasma insulin and insulin resistance index because of an increase in SD with the mean. ^{†††}*P* < 0.01, ^{††††}*P* < 0.001 for the effect of diet; ^{*}*P* < 0.05, ^{**}*P* < 0.01, ^{***}*P* < 0.001 for the effect of genotype.

Statistical Analysis

Results are means ± SD. Because the main message of the *in vivo* study was to show that adiponectin protects against muscle damage and inflammation, we selected two parameters considered as two major outcomes of the present work (changes in myocyte degeneration and muscular NFκB activation). Power analysis and sample sizes were based *a priori* on previous measurements of these parameters in KO versus WT mice after LPS challenge.¹⁸ Sample sizes of three or four mice per group were found to achieve a 80% or 90% power to detect a twofold difference in the means of the two groups with known group standard deviations (2 and 2¹⁸) and with a significance level (α) of 0.025 using a two-sided two-sample *t*-test with statistical and power analysis software (PASS, Kaysville, UT). This statement has been validated *a posteriori* for every parameter measured in the present work. Ranges for gene expression levels were presented as 2^{-(ΔΔCt ± SD)}, in which SD is calculated from the ΔΔCt values.¹¹ The integrated glucose and insulin responses during the OGTT were calculated as the areas under the curves and above-zero levels.²⁶

Comparisons between two conditions (two groups of mice, effects of electrotransfer with adiponectin plasmid versus empty plasmid, or *in vitro* siRNA experiments targeting or not targeting a selected RNA) were made using two-tailed paired Student's *t*-test. When the four groups of mice were compared, the influence of genotype and that of diet were assessed by two-way analysis of variance with F test, followed by *post hoc* 2 × 2 comparisons with Bonferroni correction for multiple comparisons (Prism 4; Graphpad Software, San Diego, CA). Because of an increase in SD with the mean, analysis of variance was performed on a log scale for plasma insulin and for insulin resistance index. Likewise, in *in vitro* experiments, in

which siRNA directed against AdipoR1 and addition of TNF-α were combined, the influence of siRNA and that of TNF-α were analyzed by two-way analysis of variance. Differences were considered to be statistically significant when *P* values were less than 0.05.

Results

Body Weight, Body Composition, and Circulating Parameters in KO Mice Challenged with a Western Diet

WD induced an increase in body weight in mice of both genotypes compared to the SLD, or normal laboratory chow. Yet, after 8-week on WD, both final body weight and body weight gain were higher in KO mice than in WT mice (*P* = 0.021 and *P* < 0.001, respectively; Table 1). Accordingly, after WD diet challenge, adiposity was increased in both genotypes (*P* < 0.001), but this increase was ~40% higher in KO mice (*P* = 0.012; Table 1). There were no significant differences in muscle weights among the four groups of mice (Table 1).

Blood was sampled at the end of the study except for parameters of glucose homeostasis, which were obtained 1 week earlier [before and during an OGTT performed after an overnight fast (time 0 of the test)]. As shown in Table 1, blood glucose levels measured in the fasted state were not different among the four groups, whereas fasted insulin levels were higher in KO mice under WD (*P* = 0.004 versus WT+WD). Accordingly, the insulin resistance indices were, strikingly, approximately sevenfold larger in KO than in WT mice under WD (*P* = 0.003; Table 1). After the glucose load, there was an elevation in glucose and insulin responses (areas under the curves) in WD-fed mice, with no differences between

the genotypes for either glucose responses (WT + SLD versus KO + SLD, $P = 0.26$ and WT+WD versus KO+WD, $P = 0.86$; $n = 6$ per group) or insulin responses (WT + SLD versus KO + SLD, $P = 0.96$ and WT+WD versus KO+WD, $P = 0.26$; $n = 6$).

Compared with WT mice fed with the SLD, plasma levels of triglycerides and cholesterol were increased in KO mice under the same diet (by ~45% and ~25%, respectively, $P = 0.008$ and $P = 0.03$; Table 1), in line with the reductions previously reported in mice overexpressing adiponectin.¹⁹ After the WD challenge, plasma cholesterol levels increased in both genotypes, likely because this diet was enriched in saturated fat and contained cholesterol. There were no differences in plasma NEFA levels among the four groups of mice (Table 1).

As expected, plasma adiponectin was undetectable in KO mice. In WT mice, plasma adiponectin rose on WD (Table 1). However, when normalized for fatness (ie, expressed “per secretion unit”), adiponectin levels were halved after WD challenge ($P < 0.001$; Table 1), in agreement with the well-known reduction of circulating adiponectin in obesity and insulin resistance.³

Induction of Adiponectin in Tibialis Anterior of WT Mice After WD Challenge

A faint immunolabeling for adiponectin/ADIPOQ was detected under the sarcolemma of muscle fibers in normal mice fed with a SLD. After challenge with a WD, ADIPOQ was more abundantly produced under the sarcolemma, whereas a weak labeling was also observed in the cytoplasm (Figure 1A). The overexpression of ADIPOQ in skeletal muscle under WD was confirmed by Western blot analysis and was more than 60% higher than under normal chow consumption (relative expression: 1.63 ± 0.17 versus 1 ± 0.21 , $P = 0.011$; $n =$ four mice per group).

As expected, there was no immunolabeling for adiponectin/ADIPOQ in KO mice under any diet (not shown).

Evaluation of WD-Induced Muscular Oxidative Stress, Inflammation, Ectopic Lipid Deposit, and Apoptosis

To investigate the functional significance of WD-induced muscular adiponectin expression in normal mice, we tested *a contrario* the hypothesis that skeletal muscle of KO mice would be more susceptible to a metabolic stress. As assumed, muscles of KO mice exhibited some myocyte degeneration when compared with WT mice after WD challenge: muscle fibers were somewhat disorganized, and ghost cells were present in these degenerating areas (Figure 1B). Indeed, the percentage of ghost cells was approximately threefold higher in muscles of KO mice than in WT mice after WD challenge ($6.3 \pm 1.9\%$ versus $2.2 \pm 1.5\%$; $P = 0.003$; $n = 6$ mice/group).

The mechanisms potentially involved in this degeneration were explored by searching for markers of oxidative stress, inflammation, ectopic lipid deposit, and apoptosis.

Figure 1. Immunodetection of adiponectin/ADIPOQ production in *tibialis anterior* of WT mice (A) and HES staining in muscle sections of the four groups of mice (B). (A) WT mice were fed with a WD or a SLD for 8 weeks. Next, mice were sacrificed and *tibialis anterior* muscles were collected. Myofibers of WT mice challenged with WD showed marked adiponectin labeling under the sarcolemma compared to those of control mice fed with the standard diet. Scale bar = 20 μ m. **Insets:** Higher magnification of immunohistochemistry images. Scale bar = 20 μ m. Representative sections for three mice per group are shown. (B) ADIPOQ-KO mice were compared to WT mice after either SLD or WD diet. *Tibialis anterior* muscles were sampled as described above. Muscle sections of KO mice displayed degenerating myotubes mainly after WD. Scale bar = 100 μ m. Representative sections for six mice per group are shown.

Oxidative Stress and Inflammation

Even in basal conditions (ie, under SLD), myotubes of KO mice already displayed a low to moderate labeling for peroxiredoxins (PRDX) 3 and 5, two markers of oxidative stress, when compared with those of WT mice (Figure 2 and Table 2). After WD challenge, immunoreactivity for these proteins was also detectable in WT mice at a low to moderate degree, whereas signal intensity was strong in KO mice (Figure 2 and Table 2). A similar immunoreactivity pattern was observed for hydroxynonenal (HNE), a product of lipid peroxidation, and for the pro-inflammatory cytokine, TNF- α (Figure 3 and Table 2).

This pro-inflammatory context originates from myocytes themselves and not from other participating cells such as macrophages. There were no differences in immunolabeling for the macrophage markers, F4/80, and CD 68 in any of the four groups studied (no detectable signal; $n = 3$ per group; not shown). This argues against a role for macrophage infiltration in inducing or maintaining inflammation within the skeletal muscle of WD-fed KO mice.

Ectopic Lipid Deposit

Using Oil Red O coloration, we researched for ectopic lipid infiltration induced by WD in cryocut sections of *tibialis anterior* muscles. However, there was a lack of immunolabeling in the four groups of mice ($n = 3$ /group). In line with these data, mRNA levels of PGC-1 α , a regu-

Figure 2. Immunodetection of PDRX3 and PDRX5 in muscle sections of the four groups of mice. *Tibialis anterior* muscles were sampled from KO and WT mice after SLD or WD. Specific antibodies against two oxidative stress markers (PDRX3, PDRX5) were used. Following SLD(basal state), immunolabeling was more pronounced in myotubes of KO mice than in those of WT mice. After WD, some labeling was observed in WT mice, but immunoreactivity for these markers was further amplified in KO mice. Scale bar = 20 μ m. **Insets:** Higher magnification. Scale bar = 20 μ m. Representative sections for three mice per group are shown.

lator of mitochondrial biogenesis and fatty acid oxidation and of cytochrome *c*, a mitochondrial marker were not different in KO and WT mice after WD challenge [relative expression for PGC-1 α : 1 ± 0.28 (WT+WD) versus 1.22 ± 0.34 (KO+WD), $P = 0.59$; $n = 6$ per group and for cytochrome *c*: 1 ± 0.34 (WT+WD) versus 1.16 ± 0.38 (KO+WD), $P = 0.46$; $n = 6$ per group).

Figure 3. Immunodetection of HNE and TNF- α in muscle sections of the four groups of mice. Specific antibodies against a lipid peroxidation product (HNE) and a pro-inflammatory cytokine (TNF- α) were used. Following SLD (basal state), immunolabeling was stronger in myotubes of KO mice than in those of WT mice. Following WD, some labeling was observed in WT mice, but immunoreactivity for these markers was further amplified in KO mice (Bar = 20 μ m). **Insets:** Higher magnification. Scale bar = 20 μ m. Representative sections for three mice per group are shown.

Apoptosis

The activation of caspase-6, an executioner caspase, which is up-regulated in several conditions of apoptosis in skeletal muscle,¹⁸ was not detectable in WT mice fed with the SLD (Figure 4 and Table 2). There was a slight activation of caspase-6, as shown by red nuclear labeling, in myofibers of KO mice studied in the basal state or

Table 2. Immunodetection in Muscle Sections of the Four Groups of Mice

	Oxidative stress			Inflammation TNF- α	Apoptosis Caspase-6	Nuclear transcription factor NF- κ B
	PRDX3	PRDX5	HNE			
WT mice fed						
SLD	2/1/0/0	2/1/0/0	0/3/0/0	3/0/0/0	0	3.4 ± 1.0
WD	0/1/2/0	0/0/3/0	0/0/2/1	0/3/0/0	$10.6 \pm 0.9^{++}$	$6.4 \pm 3.1^{\dagger}$
KO mice fed						
SLD	0/1/2/0	0/0/3/0	0/0/3/0	0/3/0/0	$11.1 \pm 0.9^{**}$	$7.8 \pm 1.6^{**}$
WD	0/0/1/2	0/0/0/3	0/0/0/3	0/0/2/1	$23.7 \pm 4.7^{***+++}$	$11.2 \pm 1.6^{***\dagger}$

Tibialis anterior muscles were sampled from KO and WT mice fed SLD or WD. Specific antibodies against three oxidative stress markers (PDRX3, PDRX5, and HNE), a pro-inflammatory cytokine (TNF- α), a marker of apoptosis (caspase-6), and a nuclear transcription factor induced by cellular stress (NF- κ B) were used. For markers of oxidative stress and inflammation, the intensity of staining was classified into four categories defined as no/low/moderate/strong signal for each mouse ($n = 3$ per group). For caspase-6 and NF- κ B, data were quantified, and the percentage of immunolabelled nuclei was counted. Results are means $\pm$ SD for three (caspase -6) and six (NF- κ B) mice/group. $^{\dagger}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$ for the effect of diet; $^{**}P < 0.01$, $^{***}P < 0.001$ for the effect of genotype.

Figure 4. Immunodetection of caspase-6 in muscle sections of the four groups of mice. **A:** Following SLD (basal state), nuclear labeling for caspase-6, a marker of apoptosis (indicated by **arrows**) was more elevated in myotubes of KO mice than in those of WT mice. Following WD, some nuclear labeling was observed in WT mice, but nuclear immunoreactivity was higher in KO mice. Scale bar = 20 μ m. **Insets:** Higher magnification. Scale bar = 20 μ m. **B:** Quantification of caspase-6 immunolabeling in myofiber nuclei (expressed as % total nuclei). Data are means $\pm$ SD for three mice/group. $**P < 0.01$, $***P < 0.001$ for the effect of genotype; $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ for the effect of treatment.

in WT mice under WD. This immunoreactivity became much stronger in KO mice on WD and the percentage of labeled nuclei was approximately twofold higher in KO than WT mice after WD challenge ($P < 0.001$) (Figure 4, A and B and Table 2). Taken together, these data suggest that the degenerating cells, previously identified by HES staining in muscles of KO mice after WD challenge, were apoptotic as a consequence of the pronounced oxidative stress and inflammation.

Effects of Muscle Electrotransfer of Adiponectin Gene on Oxidative Stress and Inflammation in KO Mice

In a second set of experiments, we directly tested the hypothesis that local administration of adiponectin/ADIPOQ could protect KO mice against WD diet-induced muscular damage. After 4 weeks on WD, mice were submitted to electrotransfer of the ADIPOQ gene: one *tibialis anterior* muscle was injected with a plasmid containing the ADIPOQ sequence and the contralateral muscle was injected with an empty plasmid, muscles were then elec-

troporated. Mice were maintained on WD for an additional 4-week period. We have previously shown that muscular ADIPOQ was expressed because of electrotransfer up to 10 days after injection. Here, we showed that, 4 weeks after the electrotransfer, there was still a local expression of ADIPOQ in the target muscle of KO mice (Figure 5). Concomitantly, there was no expression of the adipokine in the contralateral muscle and no rise of adiponectin concentrations in the plasma, in which the levels remained undetectable (not shown). Muscle electrotransfer of the adiponectin/ADIPOQ gene reduced the expression of oxidative stress markers (PRDX3/5) when compared with the contralateral untreated muscle, and also reduced the expression of HNE and TNF- α (Figure 5).

Figure 5. Effects of adiponectin/ADIPOQ gene electrotransfer on ADIPOQ and stress marker expression in muscles of KO mice challenged with WD. After 4 weeks on WD, muscle transfer of ADIPOQ gene was performed in anesthetized KO mice by injection of ADIPOQ cDNA containing-plasmid followed by electroporation in one *tibialis anterior*, the contralateral muscle being electroporated by a control plasmid. Mice were maintained on the WD for 4 additional weeks. ADIPOQ protein was detected under the sarcolemma in the muscle injected/electroporated with ADIPOQ cDNA, whereas no expression was detected in the muscle injected/electroporated with the control plasmid. **Insets:** Higher magnification. Scale bar = 20 μ m. Immunoreactivity for oxidative stress markers (PRDX3/5, HNE) and TNF- α was attenuated by adiponectin gene electrotransfer. Scale bar = 20 μ m. Representative sections for four mice are shown.

Potential Involvement of NF- κ B

NF- κ B is a transcription factor that controls several genes involved in stress, inflammation, and apoptosis. To attempt to elucidate the molecular mechanisms responsible for the protective effects of adiponectin, we measured NF- κ B activation (nuclear translocation) in our two sets of *in vivo* experiments.

In the first set of experiments, the percentage of NF- κ B immunolabeled nuclei in myocytes was compared among the four groups of mice (KO and WT mice under either SLD or WD). In the basal state (SLD), the percentage of NF- κ B immunolabeled nuclei was low in WT mice and was approximately twofold higher in KO mice ($P = 0.003$; $n = 6$ per group; Figure 6A and Table 2). After WD challenge, the percentage of immunolabeled nuclei increased in both genotypes but remained approximately

twofold larger in KO than in WT mice ($P < 0.001$; $n = 6$ /group; Figure 6A and Table 2).

In the second set of experiments dealing with electrotransfer, NF- κ B activation was markedly reduced by muscular transfection of adiponectin in WD-challenged KO mice. The percentage of labeled nuclei in the muscle transfected by the ADIPOQ cDNA-containing plasmid was approximately threefold lower than in the contralateral untreated muscle ($P < 0.001$; $n = 4$; Figure 6, B and C) and was almost the same to that counted in “normal” muscle of WT [compare Figure 6B versus 6A (WT muscle)].

Anti-Inflammatory Protection by Endogenous Adiponectin in C2C12 Myotubes and a Role for AdipoR1

Abrogation of ADIPOQ in C2C12 Myotubes

Basal adiponectin/ADIPOQ gene expression was rather low and was similar in mouse *tibialis anterior* muscles and in C2C12 myotubes (Ct values: ~ 29 in both cases for an input of 120 ng total RNA equivalents). To enlighten the role of basal levels of endogenous ADIPOQ in directly controlling the immune/inflammatory homeostasis in muscle cells, we abrogated ADIPOQ gene expression in C2C12 myotubes by using siRNAs. When compared with control conditions (ie, cells transfected with unrelated siRNA), silencing ADIPOQ resulted in altered expression of its known target genes in myotubes.¹⁸ Indeed, mRNA abundance of several pro-inflammatory markers was enhanced (TNF- α , IL-6, and I κ B- α ; Figure 7). The quantification of I κ B- α mRNAs, a target gene of NF- κ B, is a sensitive method to assess the transactivation of NF- κ B.²⁷ Therefore, basal adiponectin in muscle, although weakly expressed, is a necessary factor to shift the immune/inflammatory balance toward an anti-inflammatory pattern. We next explored which adiponectin receptor mediated this protection afforded by adiponectin into muscle.

Abrogation of AdipoR1 in C2C12 Myotubes

Gene expression of adiponectin receptors, AdipoR1 and AdipoR2, was similar in *tibialis anterior* muscle and in C2C12 myotubes, with far greater ($\sim 10,000$ -fold) mRNA abundance of AdipoR1 in both cases (Figure 8), in agreement with other reports.²⁸ It should be mentioned that there were no differences in our *in vivo* experiments in AdipoR1 mRNA abundance among *tibialis* of the four groups of mice as well as between *tibialis* electroporated with adiponectin gene or with an empty plasmid (not shown). Based on the much greater abundance of AdipoR1, we decided to abrogate the expression of this adiponectin receptor isoform in C2C12 cells by using siRNA. Because TNF α was elevated in muscles of KO mice under WD, we also challenged the myotubes by TNF- α , which was added to culture medium 3 hours before the end of the experiments. At baseline (without any pro-inflammatory treatment), mere silencing of AdipoR1 gene resulted in enhanced expression of genes coding for TNF- α , IL-6 and I κ B- α , thereby mimicking the

Figure 6. Immunodetection of muscular NF- κ B in the four groups of mice or in KO mice after adiponectin/ADIPOQ gene electrotransfer. **A** and **B**: Quantification of nuclear NF- κ B immunolabeling in muscles of the four groups of mice (**A**) or in muscles of KO mice transfected or not transfected with the ADIPOQ gene during WD challenge (**B**). Quantification was performed by analyzing sections (as for those shown in **C**). Data are mean $\pm$ SD for six mice/group (**A**) and for four mice (the contralateral muscle being used as control in this case) (**B**). $^{**}P < 0.01$, $^{***}P < 0.001$ for the effect of genotype; $^{++}P < 0.01$, $^{+++}P < 0.001$ for the effect of treatment [WD versus SLD (**A**) or electrotransfer of ADIPOQ cDNA containing-plasmid versus empty plasmid (**B**)]. **C**: Nuclear labeling for the transcription factor NF- κ B was attenuated by electrotransfer of the ADIPOQ gene in KO mice. Scale bar = 20 μ m. Some positive (brown) nuclei are indicated by arrows. Some negative nuclei (blue) are indicated by asterisks.

Figure 7. Effects of silencing adiponectin/ADIPOQ mRNA in C2C12 cells. Myotubes were transfected with 160 nmol/L siRNA directed against ADIPOQ or with nonfunctional siRNA (unrelated) in antibiotic-free medium. Cells were then washed in ice-cold PBS before RNA extraction. mRNA levels of ADIPOQ, IL-6, TNF- α , and I κ B- α were quantified by qPCR, normalized to the level of cyclophilin and presented as relative expression compared with unrelated siRNA cells. Results are expressed as means $\pm$ SD for seven experiments. * $P < 0.05$, *** $P < 0.001$.

silencing of adiponectin gene ($P < 0.001$ for three genes; $n = 11$ experiments; Figure 9, compare the first two histograms of each panel). TNF- α alone increased its own expression and that of the other two inflammatory markers ($P < 0.001$ for each gene; Figure 9). This increase was further amplified when cells were treated with siRNA against AdipoR1 ($P < 0.001$ for each gene; Figure 9, compare the last two histograms).

Discussion

We first showed that adiponectin may be up-regulated in skeletal myocytes in response to a metabolic stress in mice rendered obese by WD. These data are in agreement with our previous finding in *ob/ob* mice¹² and with

some,¹⁶ but not all^{13,15} reports in high fat diet-induced obesity in rodents. Although adiponectin-KO mice did not display a florid metabolic syndrome in basal conditions (no obesity, no systemic insulin resistance, only lipid profile abnormalities under a SLD²⁷), they already exhibited local signs of inflammation in muscle similar to those observed in WD-fed WT mice. After WD challenge, signs of inflammation, oxidative stress and apoptosis were further increased in muscle of KO mice with doubling activation of NF- κ B, an orchestrator of these processes. Muscle electrotransfer of the adiponectin gene prevented all of these abnormalities, whereas this experimental maneuver did not induce any rise in circulating adiponectin. Mere local restoration of adiponectin was thus sufficient to alleviate oxidative stress/inflammation in skeletal muscle, thereby suggesting that adiponectin may exert some of its effects in an autocrine/paracrine manner. Yet, our work was mainly based on supraphysiological or pharmacological tools (use of KO mice, electrotransfer of the deficient gene), and further studies are needed to unequivocally assess the protective role of endogenous adiponectin on muscle *in vivo*. Our hypothesis was, however, supported *in vitro*: silencing the adiponectin gene in C2C12 myotubes recapitulated a pro-inflammatory state, similar to that observed in obesity. These anti-inflammatory effects of adiponectin were mediated through AdipoR1.

The low-grade inflammation associated with obesity is now considered as the common soil for the pathogenesis of insulin resistance and the metabolic syndrome.³ Our data suggest that the production of muscular adiponectin may be a crucial mechanism to locally counterbalance inflammation/oxidative stress and to cope with the metabolic stress in muscle resulting from diet-induced obesity. In our work, adiponectin suppressed diet-induced TNF- α overproduction and down-regulated NF- κ B signaling in skeletal muscle. It is well known that TNF- α plays a determinant role in the pathogenesis of the metabolic syndrome³ and that down-regulation of muscular NF- κ B exerts protective effects in murine muscle against injury²⁹ or inflammation.¹⁸ When compared with WT mice, muscles of KO mice exhibited higher expression of TNF- α and higher degrees of nuclear translocation of NF- κ B

Figure 8. Expression of AdipoR1 and AdipoR2 in the *tibialis anterior* muscle of mice and in C2C12 cells. AdipoR1 mRNA levels far exceed those of AdipoR2 both in *tibialis anterior* muscles of mice and in C2C12 cells. mRNA levels were quantified by qPCR and presented as relative expression compared with AdipoR2 levels in C2C12 cells. Results are expressed as means $\pm$ SD for six muscles and six cultures of differentiated C2C12 myotubes.

Figure 9. Effects of silencing AdipoR1 in C2C12 cells challenged or not challenged by TNF- α . Myotubes were transfected with 60 nmol/L siRNA directed against AdipoR1 or with nonfunctional (unrelated) siRNA in antibiotic-free medium. After 21 hours, myotubes were challenged or not challenged with TNF- α (10 ng/mL) for 3 hours. mRNA levels of AdipoR1, IL-6, TNF- α , and I κ B- α were quantified by qPCR, normalized to the level of cyclophilin, and presented as relative expression compared with controls (ie, not treated either by siRNA against AdipoR1 or by TNF- α). Results are expressed as means $\pm$ SD for 11 experiments. *** P < 0.001 for the effect of siRNA directed against AdipoR1; ††† P < 0.001 for the effect of TNF- α .

after WD challenge, and these disorders were specifically attenuated by electrotransfer of the adiponectin gene. Likewise, silencing the adiponectin gene *in vitro* resulted in higher expression of TNF- α mRNA and activation of NF- κ B. These data fit with the concept that adiponectin and TNF- α exert negative reciprocal interactions on their local production and functions.⁷ This also corroborates our previous finding that adiponectin sup-

presses LPS-induced TNF- α production in skeletal muscle.¹⁸ Adiponectin suppresses oxidative stress in skeletal muscle as well. Several markers of oxidative stress (two peroxiredoxins, 3 and 5, and a lipid peroxidation product, HNE) were increased in muscle of KO mice challenged by WD, and these abnormalities were again prevented by muscular electrotransfer of the adiponectin gene. Inflammation and oxidative stress could contribute in concert to the presence of apoptotic myocytes in adiponectin-KO mice, especially after WD. Both processes could also worsen the insulin-resistant state usually observed in adiponectin-KO mice under WD.⁷ Hence, besides direct beneficial effects of adiponectin on insulin action through AMPK activation in muscle, indirect mechanisms such as alleviation of oxidative stress and inflammation are also likely to contribute.⁶

Increased lipid delivery triggered by WD may produce ectopic lipid storage in nonadipose tissues such as muscle. The ensuing lipotoxicity and lipoapoptosis could explain the enhanced production of lipid peroxidation by-products (HNE) and inflammatory markers³⁰ and could contribute to myocyte apoptosis and insulin resistance as well. However, at variance with other study findings,^{7,14} there were no major differences in circulating lipids and/or ectopic muscular fat deposits in our KO mice submitted to lipid oversupply when compared with WT mice under the same diet. Likewise, there were no differences between the two genotypes in the muscular expression of cytochrome *c*, a mitochondrial marker and of PGC-1 α , known to stimulate mitochondrial biogenesis and regulate genes involved in fatty acid catabolism.³¹ Yet, adiponectin has been shown to stimulate these molecules and impaired adiponectin action to have opposite effects.⁶ Differences in strains of KO mice, in diet composition or time of diet administration, as well as potential compensatory mechanisms, may explain these apparent discrepancies.

Our *in vivo* experiments suggest that adiponectin defends the muscle against metabolic stress mainly through its anti-inflammatory and anti-oxidative properties. An important issue of this work was then to enlighten the role of endogenous adiponectin in directly controlling the immune/inflammatory homeostasis in muscle cells. In basal conditions, the expression of adiponectin (mRNA or protein) was rather low in C2C12 myotubes as in skeletal muscle (Figures 1A and 8). Albeit low, this basal expression still played an important role in the cellular immune balance. Silencing adiponectin gene expression resulted in a pro-inflammatory phenotype of C2C12 cells, as shown by enhanced expression of TNF- α , IL-6, and I κ B- α mRNAs. Taken together, these data reinforce the concept that adiponectin is a true myokine that potently acts on the myocyte in an autocrine and paracrine manner. The anti-inflammatory action of adiponectin may partly result from reduced activation of NF- κ B in muscle, as mentioned above. This is in line with our previous work, which described anti-inflammatory properties of recombinant adiponectin in C2C12 cells challenged by LPS.¹⁸

We next explored which adiponectin receptor mediates this protection afforded by adiponectin into muscle. Silencing AdipoR1, the most abundant receptor in the

skeletal muscle, did recapitulate the proinflammatory phenotype brought about by silencing adiponectin in C2C12 cells. This suggests that AdipoR1 mediates the anti-inflammatory effects of adiponectin in muscle. An elegant study has recently shown that muscle-specific AdipoR1-knockout mice developed decreased endurance capacity, mitochondrial dysfunction, and insulin resistance, features seen in obesity and diabetes.⁶ However, this work did not focus on adiponectin production by muscle.⁶ Herein, we extend those data by strengthening the role of adiponectin as a myokine, which likely acts in autocrine/paracrine manner. We further demonstrate that AdipoR1 is also required for the anti-inflammatory effects of adiponectin and subsequent reduced activation of NF- κ B in muscle.

Besides protection against a metabolic aggression, inflammation, and oxidative stress, adiponectin production could also exert other beneficial effects on muscle. Adiponectin has been shown to activate muscle stem cells to proliferate³² and to promote the differentiation of myoblasts into myotubes.³³ Adiponectin could therefore act as a pleiotropic factor for muscle regeneration.

In conclusion, adiponectin production is enhanced in skeletal muscle in response to a metabolic challenge such as that caused by diet-induced obesity. Adiponectin production seems, in turn, to be crucial to locally counteract the ensuing inflammatory damage, oxidative stress, and apoptosis. Adiponectin is therefore critical in maintaining the inflammatory/immune balance of myocyte. These effects operate in an autocrine/paracrine manner via AdipoR1 and subsequent down-regulation of NF- κ B signaling.

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