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## Induction of adiponectin in skeletal muscle of type 2 diabetic mice: in vivo and in vitro studies

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**Abstract** *Aims/hypothesis:* Adiponectin is an adipokine that exhibits insulin-sensitising, fat-burning and anti-inflammatory properties as well as modulatory effects on oxidative stress. We examined whether adiponectin could be induced in a non-adipose tissue, skeletal muscle, in response to metabolic or oxidative aggression both in vivo (in a murine model of type 2 diabetes) and in vitro. *Methods:* Obese and diabetic *ob/ob* mice were used and compared with lean littermates. Some obese mice were treated with the antioxidant probucol for 3 weeks. At the end of the experiment, blood was sampled and tibialis anterior muscles were collected for mRNA measurement and immunohistochemistry. Additional in vitro experiments were performed on C2C12 myotubes cultured for up to 48 h. *Results:* In spite of hypo adiponectinaemia, *Adipoq* mRNA levels were markedly increased in the skeletal muscle of *ob/ob* mice and correlated with systemic oxidative stress. *Adipoq* upregulation was shown in laser-microdissected myocytes of obese mice. Concomitantly, immunoreactivity for adiponectin was enhanced in obese muscle fibres together with lipid infiltration and local markers of oxidative stress. In cultured C2C12 myotubes, a triglyceride mix and reactive oxygen species producers ( $H_2O_2$  or a lipoperoxidation end-product) upregulated *Adipoq* expression and adiponectin production. This effect was reversed by an antioxidant. Finally, treatment of obese mice with probucol also attenuated upregulation in muscle.

*Conclusions/interpretation:* The paradoxical upregulation of adiponectin in muscle of obese and diabetic mice may result from lipotoxicity and related oxidative stress. This unexpected finding could be viewed as a local protection to counteract ectopic fat deposition and oxidative damage.

**Keywords** Adiponectin · Obesity · Oxidative stress · Skeletal muscle · Type 2 diabetes

**Abbreviations** Adipor: adiponectin receptor · HES: haematoxylin–eosin–safran · HNE: 4-hydroxy-2-nonenal · LPS: lipopolysaccharide · NAC: *N*-acetylcysteine · PPAR $\gamma$ : peroxisome proliferator-activated receptor  $\gamma$  · PRDX3/5: peroxiredoxin 3/5 · ROS: reactive oxygen species · RTQ-PCR: real-time quantitative PCR · TBARS: thiobarbituric acid reactive substance

### Introduction

Adiponectin (whose gene is referred to as *Adipoq*) is an adipokine that plays a fundamental role in energy homeostasis and inflammation [1]. It is composed of an N-terminal collagenous domain and a C-terminal globular domain. The latter fragment, globular adiponectin, generated by proteolysis, may exert some biological effects by itself and often proves to be far more potent than full-length adiponectin on muscle [2, 3]. This may be explained by the relative abundance of the two types of adiponectin receptor in this tissue. Adiponectin receptor 1 (Adipor1), which is a high-affinity receptor for globular adiponectin, is most abundantly expressed in skeletal muscle, while adiponectin receptor 2 (Adipor2), which serves as a moderate-affinity receptor for both forms of adiponectin, is predominant in liver [4].

Adiponectin exhibits insulin-sensitising properties on liver and muscle in vivo and in vitro [5, 6]. It increases glucose uptake by C2C12 myocytes or isolated mouse muscle [3, 7]. Adiponectin also alters lipid metabolism, by stimulating fatty acid oxidation in several tissues including muscle, thereby accelerating the clearance of plasma NEFA

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[2, 3, 7, 8]. Most of these effects are mediated by stimulation of AMP kinase and peroxisome proliferator-activated receptor (PPAR)- $\alpha$  ligand activities [4, 9]. Eventually, adiponectin exerts anti-atherogenic and anti-inflammatory properties as well as a protective role against oxidative stress [10–13]. It inhibits the inflammatory response to TNF- $\alpha$  and superoxide generation in endothelial cells [11, 14].

Further support for the metabolic and anti-atherogenic effects of adiponectin comes from clinical studies. Thus, plasma adiponectin is decreased in human subjects with obesity, type 2 diabetes or cardiovascular disease [11, 15–17]. Circulating adiponectin is also negatively correlated with systemic oxidative stress, independently of C-reactive protein, adiposity and glucose-tolerance status [13, 18]. This is of importance since oxidative stress may be involved in type 2 diabetes by promoting insulin resistance and decreasing insulin secretion, as well as in cardiovascular disease and hypertension by directly affecting vascular cell walls [18]. Circulating adiponectin is now proposed as a biomarker of the metabolic syndrome [19] and oxidative stress as a critical instigator of this syndrome [18].

To date, adiponectin regulation has been nearly exclusively studied in the adipocyte, the unique site of its production under normal conditions [20]. However, we have recently shown that adiponectin can be induced in mouse skeletal muscle and cultured myotubes in response to lipopolysaccharide (LPS) or a pro-inflammatory cytokine combination [21].

The aim of the present work was to examine whether adiponectin could also be induced in muscle in response to metabolic and/or oxidative aggression both in vivo, in a murine model of type 2 diabetes, and in vitro, in cultured myotubes. We used leptin-deficient *ob/ob* mice as a model of obesity and type 2 diabetes.

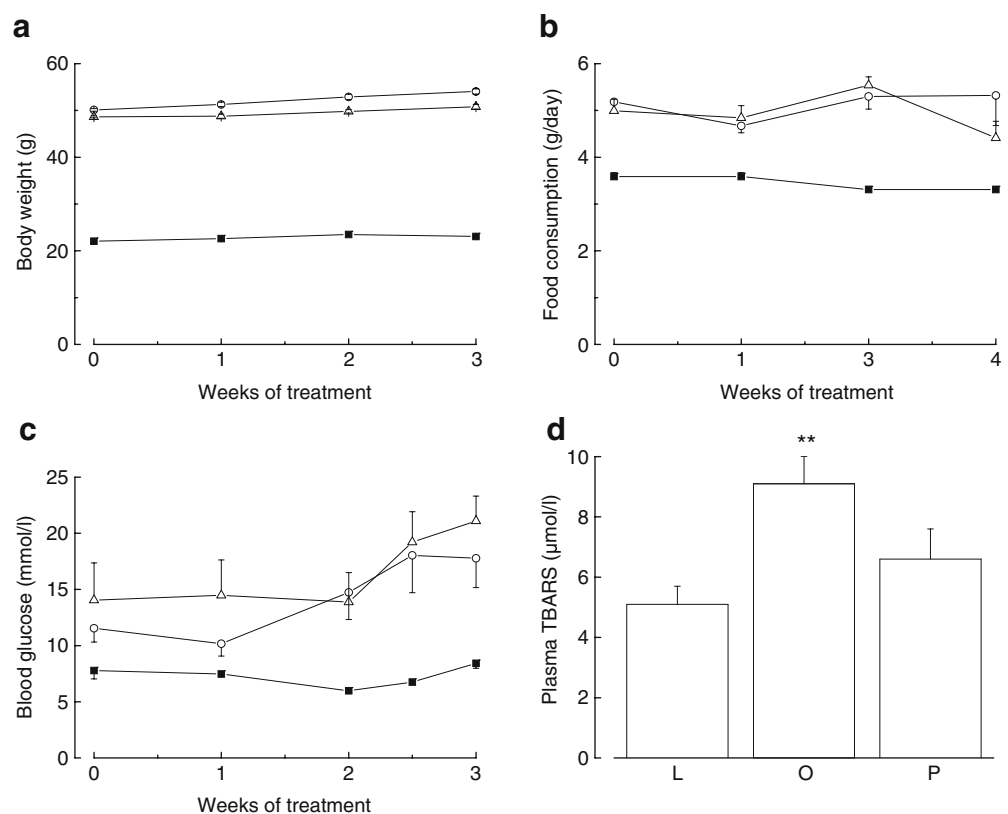
## Materials and methods

**Animals** The University Animal Care Committee approved all procedures. Female C57BL/6J obese (*ob/ob*) and lean littermate (+/?) mice were purchased from Charles River Laboratories (Brussels, Belgium) and studied from the age of 13 weeks. They received a free supply of common laboratory chow in powdered form (R04-10; UAR, Villemoisson-sur-Orge, France).

The animals were divided into three experimental groups: lean mice ( $n=10$ ), untreated obese mice ( $n=5$ ) and obese mice treated with probucol ( $n=5$ ). Probucol (MP Biomedicals, Eschwege, Germany) was homogeneously added to food (0.5% w/w) as described [22] and administered for 3 weeks. At the beginning of the study, the two groups of *ob/ob* mice were matched for body weight and post-feeding blood glucose (Fig. 1a,c).

Body weight and food intake were measured daily. On several occasions, tail vein blood was collected from fed mice (between 08.00 and 09.00 h). At the end of the experiment, the mice were killed by decapitation (between 09.00 and 10.30 h), and larger blood samples were saved.

**Fig. 1** Body weight (a), food consumption (b), blood glucose (c) and plasma TBARS levels (d). Three groups of mice were studied: lean (L, filled squares); untreated *ob/ob* (O, open circles); and *ob/ob* treated with probucol (P, open triangles) for 3 weeks. All measurements or samplings were made between 08.00 and 09.00 h at the indicated period. Plasma was sampled at the end of the study (3 weeks), and TBARS, a marker of oxidative stress, was measured by a colorimetric method. Values are means $\pm$ SEM for ten, five and five mice in the respective groups. \*\* $p<0.01$  vs L mice



Tibialis anterior muscle and inguinal fat were dissected, weighed, frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

**Light microscopy and immunohistochemistry** Muscle samples were fixed in 10% formaldehyde for 24 h and embedded in paraffin. Five-micron-thick sections were stained with haematoxylin–eosin–safran (HES). For immunohistochemistry, sections were processed as previously described [23], using rabbit polyclonal antibodies directed against adiponectin (Chemicon, Biognast, Hevle, Belgium), PPAR $\gamma$  or caspase-6 (both from Tebu-Bio, Baeckout, Belgium), peroxiredoxin 3 (PRDX3) or peroxiredoxin 5 (PRDX5) (gifts from B. Knoop, University of Louvain, Brussels, Belgium [24]) or a mouse monoclonal antibody directed against myosin (DSMZ, Braunschweig, Germany). Antibody concentrations and incubation times were 1  $\mu\text{g}/\text{ml}$  for 48 h (adiponectin), 4  $\mu\text{g}/\text{ml}$  for 48 h (PPAR $\gamma$ ), 10  $\mu\text{g}/\text{ml}$  overnight (caspase-6 and myosin), 5  $\mu\text{g}/\text{ml}$  for 1 h (PRDX3) and 2  $\mu\text{g}/\text{ml}$  for 1 h (PRDX5). The sections used for adiponectin, PPAR $\gamma$  and caspase-6 were pretreated in a microwave oven in Tris–citrate buffer (pH 6.5) for one cycle of 3 min at 750 W and three cycles of 3.5 min at 350 W. Binding of antibodies was detected by applying for 30 min at room temperature a second antibody, which was a goat anti-rabbit or anti-mouse immunoglobulin conjugated to peroxidase-labelled polymer (En Vision +; Dako, Copenhagen, Denmark). Peroxidase activity was revealed with 3-amino-9-ethylcarbazole substrate (Dako), which produces a red stain. Immunohistochemical controls were performed by omission of the first antibody or of the first and second antibodies, by using pre-immune serum, or by incubation with an irrelevant antibody (anti-thyroglobulin).

**Morphometry** The mean relative proportion of myocyte and adipocyte areas was estimated by a point-counting technique [25] at  $\times 50$  magnification on paraffin sections of tibialis anterior muscle.

**Oil Red O staining** Muscle samples were embedded in Tissue-Tek and rapidly frozen in isopentane cooled in liquid nitrogen to generate 5- $\mu\text{m}$ -thick cryostat sections. Unfixed frozen sections were then treated as described [26].

**Laser microdissection and pressure catapulting** Microdissection was used to selectively capture myocytes from frozen muscle. Seven-micron-thick sections were performed on muscle samples of two lean and two obese mice and laid on PEN membrane-coated slides. Immediately after dehydration, areas of intact myocytes (700,000  $\mu\text{m}^2$  for each mouse) were microdissected with a PALM Microlaser system (Bernried, Germany) equipped with a pulsed laser (nitrogen, 337 nm) and the PALM Robot Software Version 2.2. This system was coupled to an Axiovert 200 microscope and a Plan Neofluar 20X (Zeiss, Oberkochen, Belgium). Then, myocytes were catapulted into an RNase-free microtube cap and frozen until RNA extraction.

**Cell culture** C2C12 myoblasts were cultured as previously described [21]. At the time-zero point (96 h differentiation), different agents were added to the basal medium (high-glucose DMEM+2% heat-inactivated horse serum) alone or in combination for up to 48 h. We prepared AGE as described [27], and purchased 4-hydroxy-2-nonenal (HNE) (Calbiochem, VWR International, Zaventem, Belgium), recombinant murine leptin (Peprotech, London, UK), *N*-acetylcysteine (NAC) (Merck, VWR International), Structolipid 20% (structured triglycerides containing randomly esterified medium and long chain fatty acids; Fresenius Kabi, Bad Homburg, Germany), and other high-grade agents. The concentrations of the agents used were similar to those reported by others and devoid of overt cytotoxicity [27, 28]. HNE and cholesterol were dissolved in ethanol. In this case, the control conditions included the same amount of ethanol. At the end of the culture, aliquots of medium were saved and stored at  $-20^{\circ}\text{C}$  and the cells rinsed twice in PBS before RNA isolation.

**RNA extraction and real-time quantitative PCR (RTQ-PCR)** RNA was isolated from mouse tissues and cultured cells with TriPure (Roche Diagnostics, Vilvoorde, Belgium) or from laser-microdissected samples with an RNAqueous-Micro isolation kit (Ambion, Huntingdon, UK). Two micrograms of total RNA (or the whole amount extracted from microdissected samples) were reverse transcribed as described previously [21]. RTQ-PCR primers were designed (Primer Express Software; Applied Biosystems) for mouse adipocyte P2 (*Fabp4*), *Adipoq*, *Adipor1*, *Adipor2*, cyclophi-

**Table 1** Murine gene sequences used as forward and reverse primers for RTQ-PCR

Gene	Sense primer	Reverse primer	Length of amplicon (bp)
<i>Adipoq</i>	GCA GAG ATG GCA CTC CTG GA	CCC TTC AGC TCC TGT CAT TCC	101
<i>Adipor1</i>	AAC GGG CCA TCC ATT TTT G	TTA GCC GGG CTA CAT CAA GG	101
<i>Adipor2</i>	AGT GTT TTC AGC ACG CCC TC	GCT GAG CTC CAC GGA TTC TT	151
<i>Fabp4</i>	TGG GAG TGG GCT TTG	TGT CGT CTG CGG TGA T	165
<i>Ppar<math>\gamma</math></i>	TTG ACC CAG AGC ATG GTG C	GAA GTT GGT GGG CCA GAA TG	101
<i>Ppia</i>	AAC CCC ACC GTG TTC TTC	TGC CTT CTT TCA CCT TCC C	364
<i>Prdx3</i>	GAC ATA CTG TGG TCT GCC TCT GC	CCT TTA AAA TAG GGC GCG TG	101
<i>Prdx5</i>	ACA CCT GGC TGT TCT AAG ACC C	TCA TTA ACG CTC AGA CAG GCC	101
<i>Tnf</i>	GCC ACC ACG CTC TTC TGT CT	GTC TGG GCC ATG GAA CTG AT	101

lin (*Ppia*), *Ppar $\gamma$* , *Prdx3*, *Prdx5* and *Tnf* (Table 1). One hundred and twenty nanograms of total RNA equivalents (or the whole amount obtained for microdissected samples) were amplified using an iCycler iQ Real Time PCR System (Bio-Rad Laboratories, Belgium) [21].

**Quantification of adiponectin and circulating parameters** Adiponectin concentrations were measured with an RIA (kit from Linco Research, St Charles, MO, USA) in mouse plasma and homogenates of C2C12 myotubes, as reported previously [21]. Lipid peroxidation was assessed by measuring thiobarbituric acid reactive substance (TBARS) formation [29] and plasma 8-isoprostanes by an enzyme immunoassay kit (Cayman Chemical Company, Ann Arbor, MI, USA). The correlation between these two methods was good ( $r=0.5842$ ,  $p<0.01$ ,  $n=20$ ). Blood glucose and plasma insulin, corticosterone, total cholesterol, NEFA and triglyceride levels were determined as reported previously [28, 30].

**Presentation of results and statistical analysis** Results are the means $\pm$ SEM for the indicated number of individual mice (in vivo study) or separate experiments (in vitro studies). Unless otherwise indicated, ranges for gene expression levels are presented in each Figure or Table as  $2^{-\Delta\Delta Ct\pm SEM}$ , where SEM is calculated from the  $\Delta\Delta Ct$  values [21].

Comparisons between two conditions were made using a two-tailed unpaired Student's *t* test. Comparisons of at least three conditions were carried out by ordinary ANOVA followed by a Newman-Keuls test. Statistical analysis for gene expression levels was performed on the  $\Delta\Delta Ct$  values. The correlation analysis was performed using Pearson's test. Differences were considered statistically significant at  $p<0.05$ .

## Results

Body weight, food consumption and plasma parameters of obese mice

Body weights and daily food consumption of *ob/ob* mice were about twofold higher than those of lean mice (Fig. 1a, b). Obese mice were also hyperglycaemic and hyperinsulinaemic as compared with lean mice (Fig. 1c and Table 2) [31]. All these parameters remained rather stable throughout the 3 weeks of the study and were not significantly modified by probucol treatment.

Obese mice were also markedly hypercholesterolaemic as compared with lean mice. As expected from a drug exerting cholesterol-lowering effects [22], probucol fully reversed this abnormality (Table 2). Other plasma lipids did not differ significantly between lean and obese mice, and were not altered by the treatment (Table 2).

Oxidative stress plays a critical role in the pathogenesis of various diseases including diabetes and obesity [18]. Accordingly, obese mice exhibited a two-fold rise in plasma levels of TBARS, a marker of lipid peroxidation and systemic oxidative stress. P, which is mainly a reactive oxygen species (ROS) scavenger, reduced this oxidative stress as demonstrated by the normalisation of plasma TBARS in treated mice (Fig. 1d). Qualitatively comparable results were obtained with plasma measurement of 8-isoprostanes, another marker of lipid peroxidation and oxidative stress (not shown).

Besides type 2 diabetes and leptin deficiency, other endocrine disorders characterise *ob/ob* mice. As described [28], our obese mice displayed augmented plasma corticosterone levels while their plasma adiponectin concentrations were markedly reduced. Probucol did not correct these abnormalities (Table 2).

**Table 2** Plasma parameters of the three groups of mice: tail vein blood was collected at the end of the study

	L (n=10)	O (n=5)	P (n=5)
Circulating glucose/insulin			
Fed blood glucose (mmol/l)	8.4 $\pm$ 0.4	17.8 $\pm$ 2.6**	21.1 $\pm$ 2.2***
Plasma insulin (nmol/l)	0.19 $\pm$ 0.02	24.63 $\pm$ 5.14***	16.94 $\pm$ 5.59**
Plasma lipids			
Plasma total cholesterol (mmol/l)	2.90 $\pm$ 0.09	6.94 $\pm$ 0.71***	2.58 $\pm$ 0.32+++
Plasma triglycerides (mmol/l)	1.13 $\pm$ 0.10	1.02 $\pm$ 0.12	0.78 $\pm$ 0.12
Plasma NEFA (mmol/l)	0.54 $\pm$ 0.07	0.54 $\pm$ 0.05	0.51 $\pm$ 0.11
Others			
Plasma adiponectin ( $\mu$ g/ml)	9.9 $\pm$ 1.5	5.4 $\pm$ 0.4*	5.7 $\pm$ 0.5*
Plasma corticosterone (nmol/l)	111.6 $\pm$ 16.6	179.8 $\pm$ 7.7*	166.1 $\pm$ 14.3*

Data are shown as means $\pm$ SEM. Three groups of mice were studied: lean mice (L), untreated *ob/ob* mice (O) and *ob/ob* mice treated with probucol for 3 weeks (P)

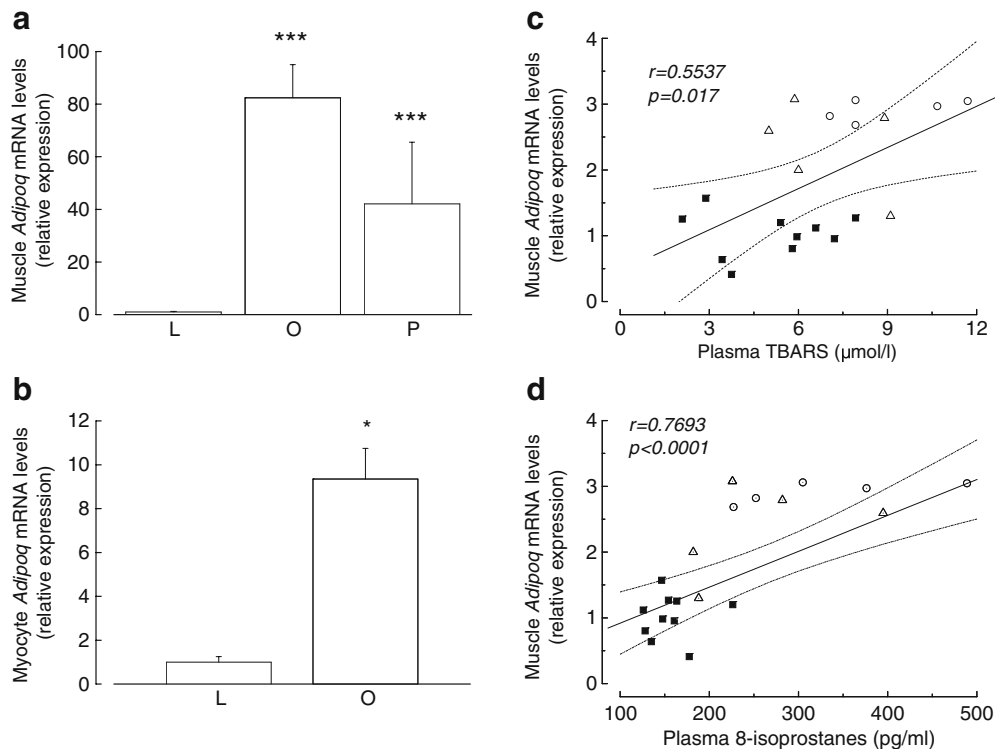
\* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$  vs L mice; +++ $p<0.001$  vs O mice

## Characteristics of obese muscle

**Induction of *Adipoq* expression and adiponectin production in the tibialis anterior** Expression of the gene for adiponectin (*Adipoq* mRNA levels) was measured by RTQ-PCR in mouse tibialis anterior muscle and compared with the values obtained in subcutaneous (inguinal) fat (Fig. 2a and Table 3). As expected, muscle of lean mice expressed only very low levels of *Adipoq* mRNA, as compared with fat mice (Table 3). In obese mice, muscle *Adipoq* mRNA abundance was ~80-fold higher than in lean littermates and amounted to one-tenth of that detected in fat (Fig. 2a and Table 3, left columns). In contrast to the downregulation observed in visceral fat, *Adipoq* mRNA was not markedly modified in inguinal fat of obese mice (Table 3), in agreement with a previous report [28]. Probucol treatment of *ob/ob* mice did not fully restore muscle *Adipoq* mRNA expression to values measured in lean mice. Nevertheless, Fig. 2a indicates a clear tendency for tibialis anterior *Adipoq* mRNA levels of probucol-treated mice to be lessened (by about 50%) when compared with untreated obese mice. Muscle *Adipoq* mRNA levels of the three groups of mice were positively correlated with the two distinct markers of oxidative stress,

namely plasma TBARS and 8-isoprostanes (Fig. 2c,d). Because RNA yield (total RNA/mg wet tissue) depends on tissue type (higher in muscle than in fat) and on the *ob* mutation (dramatically lower in fat of obese or probucol-treated obese mice than in that of lean ones, with no intergroup differences for muscle), *Adipoq* mRNA data were also expressed per mg tissue. As shown in Table 3 (right columns), 1 mg muscle tissue of obese mice exhibited only two to three times less *Adipoq* mRNA than 1 mg fat.

In agreement with mRNA data, immunohistochemistry clearly demonstrated that adiponectin (protein) content was increased in skeletal muscle of obese mice (Fig. 3). Immunostaining of muscle sections showed that adiponectin was localised near to the sarcolemma of muscle fibres in lean mice (Fig. 3a,b) [32]. In obese mice, adiponectin was much more abundantly produced under the sarcolemma, while a faint labelling was also observed in the cytoplasm (Fig. 3c,d). Muscle fibres of obese mice were somewhat disorganised when compared with those of lean mice, a finding confirmed by HES coloration (compare Fig. 4a vs b). Between intact fibres, we found large areas with intense adiponectin staining, which were identified as degenerating myocytes by HES coloration and myosin immunostaining (Fig. 4a–c). The latter



**Fig. 2** Relationships between the level of *Adipoq* mRNA in tibialis anterior muscle and plasma concentrations of TBARS or 8-isoprostanes. At the end of the study, tibialis anterior muscles and blood were sampled from lean (L, filled squares) mice; untreated *ob/ob* mice (O, open circles); and *ob/ob* mice treated with probucol (P, open triangles). TBARS was measured in plasma by a colorimetric method, and 8-isoprostanes by enzyme immunoassay. *Adipoq* mRNA levels were extracted from whole muscle (a–d) or from laser-microdissected areas of intact

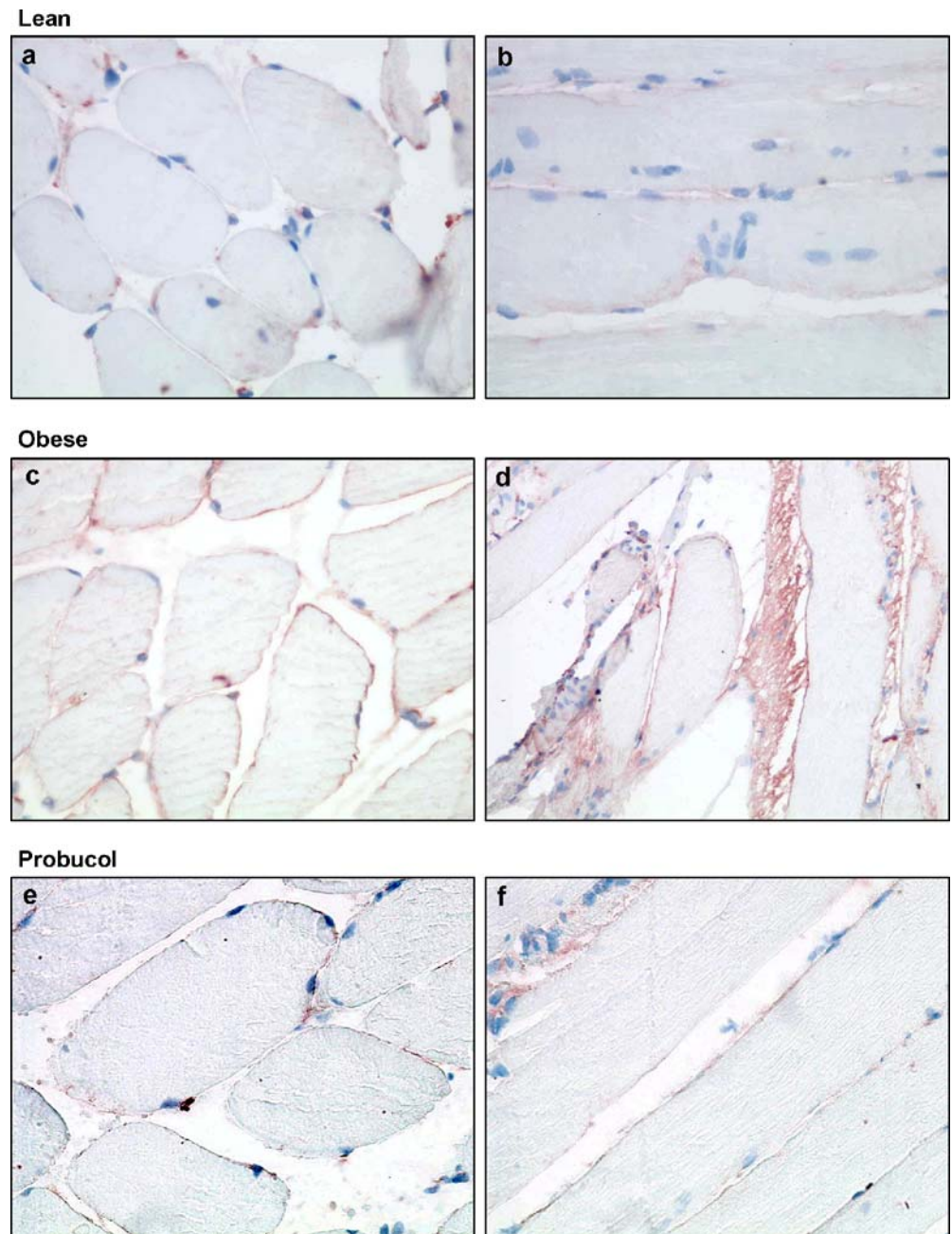
myotubes (b), then quantified by RTQ-PCR. Data are presented as relative expression compared with L mice values without (a, b) or with a logarithmic transformation (c, d). Results are normalised to the level of cyclophilin and expressed as means  $\pm$  SEM for ten L, five O and five P mice (a) or two representative L and two representative O mice (b) with \*\*\* $p$ <0.001 and \* $p$ <0.05 vs L mice. The correlation analysis shown in c and d was performed in all the mice. Dotted lines correspond to the 95% CI for the  $r$  value

**Table 3** *Adipoq* mRNA levels in skeletal muscle and inguinal adipose tissue of the three groups of mice

Mice	<i>Adipoq</i> mRNA levels (relative to those in L mice)			
	$2^{-\Delta\Delta Ct}$		$2^{-\Delta\Delta Ct}/\text{mg tissue}$	
	Tibialis anterior	Inguinal adipose tissue	Tibialis anterior	Inguinal adipose tissue
L ( $n=10$ )	1 (0.8–1.3)	501.9 (411.3–612.5)	1 (0.8–1.3)	472.0 (376.7–590.6)
O ( $n=5$ )	82.4 (69.8–97.2)***	797.9 (616.2–1,033.1)	77.2 (65.4–91.2)***	191.5 (147.8–247.8) <sup>o</sup>
P ( $n=5$ )	42.1 (18.5–65.7)***	685.0 (492.6–952.7)	39.4 (17.3–61.5)***	164.4 (117.6–228.6) <sup>o</sup>

Data are shown as means (ranges). Three groups of mice were studied: lean mice (*L*), untreated *ob/ob* mice (*O*) and *ob/ob* mice treated with probucol for 3 weeks (*P*). Tibialis anterior muscles and inguinal adipose tissue were sampled at the end of the study. *Adipoq* mRNA levels were quantified by RTQ-PCR. Data are normalised to the level of cyclophilin and expressed or not on a per mg wet tissue basis <sup>o</sup> $p=0.07$ , \*\*\* $p<0.001$  vs L mice. All differences between muscle and fat within a given group of mice are statistically significant

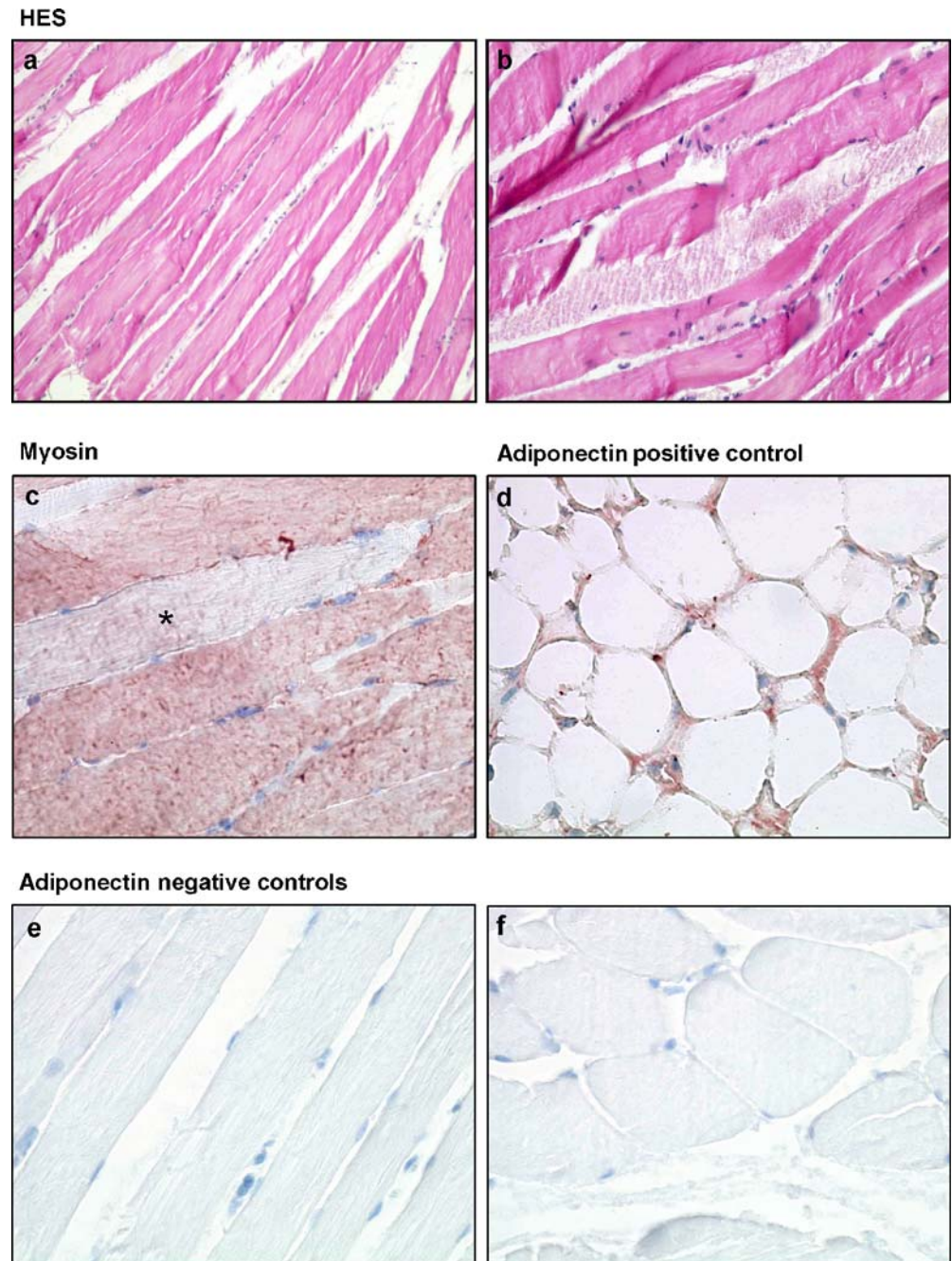
**Fig. 3** Immunodetection of adiponectin production in muscle sections of *ob/ob* mice. Cross ( $\times 165$ , **a**, **c**, **e**) and longitudinal ( $\times 165$ , **b**, **d**, **f**) sections of tibialis anterior muscle sampled from lean (**a**, **b**), untreated obese (**c**, **d**) or probucol-treated obese mice (**e**, **f**). Compared with lean mice, muscle fibres of untreated obese mice showed marked labelling under the sarcolemma and faint cytoplasmic immunoreactivity. A strong labelling was also observed between fibres, which was likely to correspond to degenerating myocytes. Muscle of probucol-treated obese mice was only moderately immunostained. Representative sections are shown



labelling was intense in intact myofibres and faint in degenerating cells, in which the typical multinucleated and cylindrical morphology of myocytes was preserved. Adiponectin staining was specific as there was no labelling when muscle was incubated with pre-immune serum (Fig. 4e,f) or with an irrelevant anti-thyroglobulin antibody used as control (not shown). A positive control for adiponectin immunostaining obtained from adipose tissue of lean mice (intense cytoplasmic labelling in adipocytes) is also shown in Fig. 4d. Probuco-treated obese mice displayed substantially lower muscle adiponectin labelling than obese mice, a finding consistent with *Adipoq* mRNA results (Fig. 3e,f).

Adipocytes, if any, were as scarce in muscle sections of obese mice as in those of lean mice. This observation was checked by a morphometric semi-quantitative method of area assessment. The mean relative proportion of myocyte and adipocyte areas was similarly low ( $<0.01$ ) in tibialis anterior sections of the three groups of mice. This virtual absence of adipocytes allows us to ascertain that our observations did actually originate from *Adipoq* upregulation in myocytes and did not result from contamination of muscle with adipose tissue. To further support this affirmation, we also measured mRNA levels of *Fabp4*, an adipose-specific fatty acid transporter. As shown in Table 4, *Fabp4* mRNA levels were similar in the three groups of mice. Eventually, we laser microdissected areas

**Fig. 4** HES and myosin staining in muscle of obese mice and positive and negative controls for adiponectin immunodetection. HES staining of tibialis anterior sections from lean (**a**,  $\times 40$ ) and obese mice (**b**,  $\times 103$ ). Immunodetection of myosin in obese mice (**c**,  $\times 165$ ). Degenerating myotubes indicated by the asterisk display weaker immunostaining when compared with intact ones. **d** Adiponectin immunostaining in lean mouse adipose tissue sections used as a positive control (cytoplasmic labelling in adipocytes). **e**, **f** No adiponectin labelling in muscle incubated with pre-immune serum used as negative controls for lean (**e**) and obese (**f**) mice. Representative sections are shown



of intact myocytes, avoiding all other structures, in sections of two representative lean and obese mice. We then quantified *Adipoq* mRNA abundance in these samples. As shown in Fig. 2b, *Adipoq* remained upregulated in myocytes from obese mice. It should be noted that in these two lean and two obese samples, *Fabp4* was not detectable (not shown).

**Intracellular lipids** Obese mice accumulate lipids in non-adipose tissues such as liver and muscle [33]. We thus looked for ectopic lipid storage in cryocut sections of the tibialis anterior muscle using Oil Red O coloration (Fig. 5a,b). There were almost no lipids in lean muscle. By contrast, in obese mice, lipids were readily detected as intracellular storage in small droplets within myocytes, as well as extracellular deposits. There was no clear difference in ectopic lipids between obese and probucol-treated obese mice (not shown).

In this context, we analysed the expression of PPAR $\gamma$ , a transcription factor involved in lipid metabolism and adipocyte differentiation. *Ppar $\gamma$*  was upregulated in skeletal muscle of obese mice as compared with lean mice (Table 4). This activation was further confirmed by immunohistochemistry. Myotubes of obese mice showed strong labelling for PPAR $\gamma$  (red) (Fig. 5c vs d). Probuco-treated obese mice had intermediate values for these parameters as compared with those of obese and lean mice (Table 4 for mRNAs, and data not shown for immunochemistry).

**Oxidative stress and apoptosis** Besides systemic oxidative stress, peripheral tissues of obese mice such as skeletal muscle showed some evidence of local stress as well. Figure 5e–h display the immunohistochemical analysis of PRDX3/5 production. These two peroxidases, which are upregulated during oxidative stress [24], were abundantly present in muscle of obese mice and produced at low levels in those of lean mice (Fig. 5e–h). Again, staining in samples from probucol-treated obese mice was intermediate (not

shown). The regulation of PRDX3 and PRDX5 occurred at a translational rather than transcriptional level as confirmed by the lack of mRNA upregulation in obese and probucol-treated obese mice (Table 4).

Moreover, caspase-6, implicated in apoptosis, was activated in myotubes of obese mice, but not in those of lean mice. This activation was confirmed by the intense nuclear labelling visible in Fig. 5j. Thus, the degenerating cells, previously identified by HES staining in muscle of obese mice turned out to be apoptotic, rather than simply necrotic, as either a potential consequence of the pronounced oxidative stress or of another unknown mechanism.

Lastly, the expression of *Tnf*, which encodes TNF- $\alpha$ , a pro-inflammatory cytokine that has been associated with insulin resistance, oxidative stress and apoptosis, was also slightly upregulated in the tibialis anterior of obese mice as compared with lean mice (Table 4). Again, probucol-treated obese mice showed intermediate values for both *Tnf* expression (Table 4) and caspase 6 levels (data not shown).

**Receptors** Because the major finding of this study was the upregulation of adiponectin, we next investigated whether adiponectin receptors were also regulated in type 2 diabetes. Unlike previously published data [34], we did not find any modification in *Adipor1* and *Adipor2* mRNA levels in obese and probucol-treated obese mice compared with lean ones (Table 4).

#### Potential mechanisms underlying adiponectin induction in C2C12 cells

We subsequently attempted to identify the mechanisms underlying muscle adiponectin induction in vitro. Hence, we tested on cultured myotubes the effects of glucose/lipid overload, high insulin and ROS to mimic the in vivo diabetic situation. Besides type 2 diabetes, we also

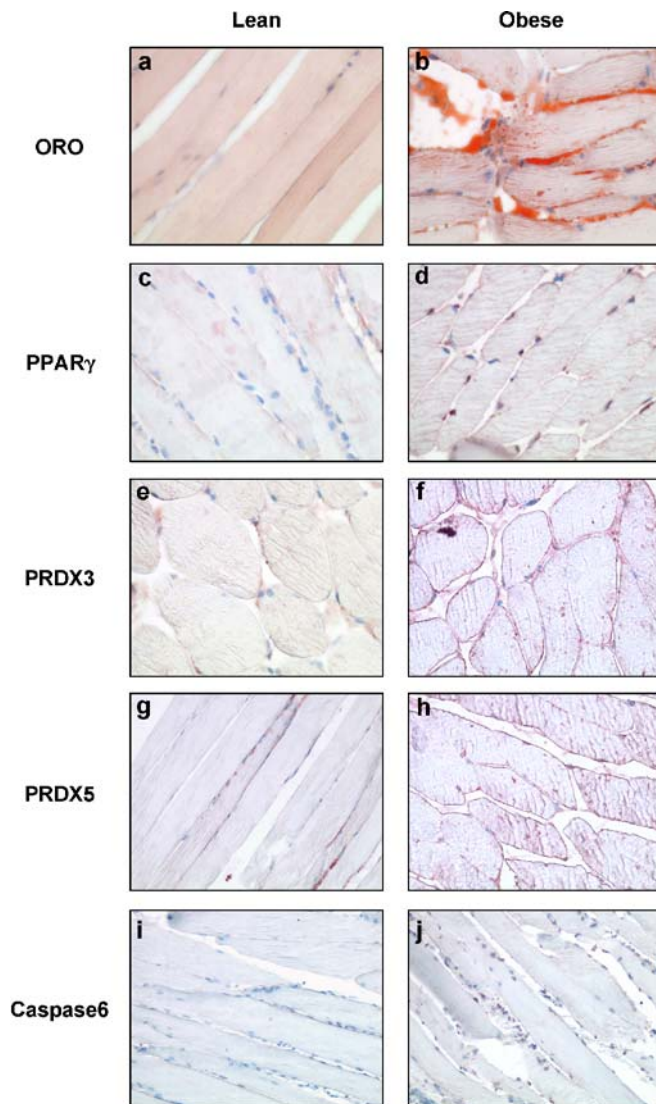
**Table 4** mRNA levels of proteins involved in lipid metabolism and oxidative stress and of adiponectin receptors in the tibialis anterior muscle of the three groups of mice

	L (n=10)	O (n=5)	P (n=5)
<b>Lipid metabolism</b>			
<i>Fabp4</i>	1 (0.76–1.32)	1.31 (0.93–1.86)	0.80 (0.53–1.20)
<i>Ppar<math>\gamma</math></i>	1 (0.77–1.30)	32.15 (25.02–41.30)***	20.63 (11.94–35.65)***
<b>Oxidative stress</b>			
<i>Prdx3</i>	1 (0.94–1.06)	0.97 (0.94–1.03)	1.03 (0.95–1.11)
<i>Prdx5</i>	1 (0.95–1.06)	0.82 (0.74–0.90)	0.84 (0.77–0.91)
<i>Tnf</i>	1 (0.78–1.29)	1.8 (1.34–2.43) <sup>o</sup>	1.26 (0.83–1.92)
<b>Adiponectin receptors</b>			
<i>Adipor1</i>	1 (0.83–1.20)	1.33 (0.84–2.09)	0.83 (0.62–1.10)
<i>Adipor2</i>	1 (0.70–1.43)	1.75 (1.36–2.26)	1.41 (1.28–1.54)

Data are for relative expression compared with that in L mice, presented as means (ranges). Three groups of mice were studied: lean mice (L), untreated *ob/ob* mice (O) and *ob/ob* mice treated with probucol for 3 weeks (P). Tibialis anterior muscles were sampled at the end of the study. mRNA levels were quantified by RTQ-PCR. Results are normalised to the level of cyclophilin

\*\*\* $p < 0.001$ , <sup>o</sup> $p = 0.079$  vs L mice





**Fig. 5** Ectopic lipid storage and immunodetection of PPAR $\gamma$ , PRDX3, PRDX5 and caspase-6 in muscle sections of *ob/ob* mice. At the end of the study, tibialis anterior muscles were sampled from lean or untreated obese mice, then processed for (immuno) histochemistry. Lipids were stained with Oil Red O (ORO,  $\times 165$ , **a, b**). Ectopic lipids are stored within and between muscle fibres of obese mice. Immunodetection was performed with specific antibodies against PPAR $\gamma$  ( $\times 165$ , **c, d**), PRDX3 ( $\times 165$ , **e, f**), PRDX5 ( $\times 165$ , **g, h**) and caspase-6 ( $\times 103$ , **i, j**). When compared with lean mice, immunoreactivity for PPAR $\gamma$ , PRDX3, PRDX5 and caspase-6 was more pronounced in obese mice, with strong nuclear and, in some cases (PPAR $\gamma$ , PRDX), faint cytoplasmic labelling. Representative sections are shown

examined the potential repercussions of other endocrine disorders, which are characteristic of *ob/ob* mice, such as hypercortisosteronaemia and hypoleptinaemia.

We first tested the effects of glucose over a wide range of concentrations (5.5–25 mmol/l) and we more specifically addressed the repercussions of glucotoxicity (using glucosamine or AGE) (Table 5). These experimental conditions did not affect *Adipoq* mRNA levels in C2C12 myotubes. Similarly, insulin, dexamethasone, leptin or cholesterol were without effect (Table 5).

We next examined the potential role of lipotoxicity/ectopic lipid accumulation and oxidative stress on adiponectin induction. To this end, we cultured C2C12 cells with a mixture of triglycerides (Structolipid) for 48 h. In those conditions, *Adipoq* mRNA levels rose by  $\sim 225\%$ . This effect was almost totally reversed when the antioxidant, NAC, was added to the medium (Fig. 6a). These results suggest a role of ectopic muscular lipids in the adiponectin upregulation observed in the tibialis anterior of *ob/ob* mice.

To investigate the contribution of oxidative stress we cultured C2C12 myotubes with ROS producers, such as H<sub>2</sub>O<sub>2</sub> or the active end-product of lipid peroxidation, HNE. When compared with control values, both conditions increased levels of *Adipoq* mRNA ( $\sim 2.6$ -fold and  $\sim 7$ -fold, respectively). Again, NAC abrogated this effect of the ROS (Fig. 6b,c).

All these in vitro data provide evidence for a role of increased oxidative stress and ROS production in adiponectin upregulation. In this context, we measured the production of adiponectin protein in homogenates of C2C12 cells incubated with ROS producers, HNE and H<sub>2</sub>O<sub>2</sub> (Fig. 7a,b). Adiponectin levels rose in both conditions (by 200 and 150%, respectively).

Lastly, we measured *Ppar $\gamma$*  mRNA levels in all the experiments described in Fig. 5. Concurrently with the upregulation of *Adipoq* expression, levels of *Ppar $\gamma$*  mRNA, present at low levels in C2C12 myotubes, were increased by two- to four-fold in the presence of Structolipid, H<sub>2</sub>O<sub>2</sub> or HNE ( $p < 0.05$  for each condition). This increase was again prevented by the addition of NAC (not shown).

## Discussion

In this study, we showed that *Adipoq* expression and adiponectin production were paradoxically upregulated in the skeletal muscle of obese and diabetic *ob/ob* mice, while they decreased in some adipose tissue sites [28], with a decrease in circulating levels as a consequence. This upregulation of adiponectin did occur in myocytes, as shown by laser microdissection experiments, and was associated with ectopic lipid storage, markers of oxidative stress and apoptosis.

The cellular mechanisms responsible for adiponectin upregulation were further characterised in cultured murine myotubes. We attempted to isolate each pathophysiological feature of the *ob/ob* mice that could trigger muscle adiponectin induction and then evaluated their repercussions in vitro. Several conditions were tested. Firstly, glucotoxicity. Culturing myotubes in the presence of high glucose, glucosamine or AGE did not result in any significant changes in *Adipoq* mRNA. Secondly, hyperinsulinaemia/insulin resistance and other endocrine disorders such as leptin deficiency or hypercortisosteronaemia. These potential contributors were excluded, as insulin, leptin or dexamethasone did not affect *Adipoq* mRNA. Thirdly, hyperlipidaemia/lipid overload and oxidative stress. Although the role of cholesterol was ruled

**Table 5** Role of metabolic and hormonal factors on *Adipoq* mRNA levels in C2C12 myocytes

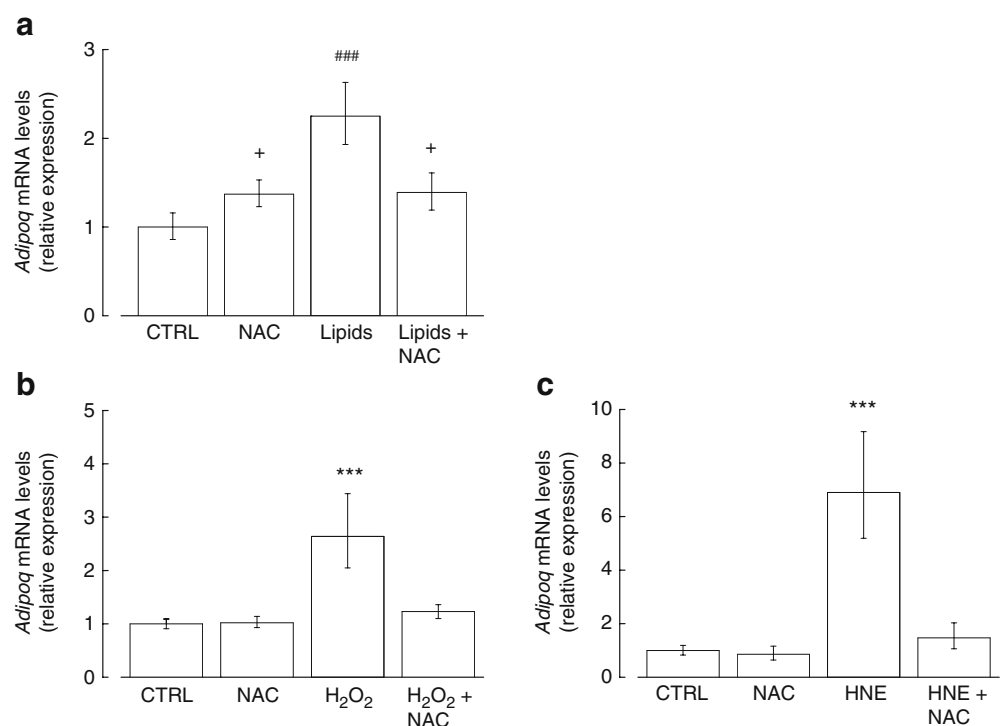
ob/ob mouse characteristics tested	Culture		<i>Adipoq</i> mRNA levels (relative expression)
	Tested agent	Concentration	
Type 2 diabetes			
Hyperglycaemia–glucotoxicity	Glucose	25 mmol/l	1.30 (1.13–1.49)
	Glucosamine	20 mmol/l	0.82 (0.54–1.27)
	AGE	0.25 mg/ml	1.21 (0.63–2.34)
Hyperinsulinaemia	Insulin	100 nmol/l	0.93 (0.70–1.24)
Other endocrine disorders			
Hypercorticozonaemia	Dexamethasone	1 µmol/l	1.30 (0.98–1.73)
Hypoleptinaemia	Leptin	10 nmol/l	0.95 (0.60–1.30)
Hyperlipidaemia			
Hypercholesterolaemia	Cholesterol	50 µg/ml	0.90 (0.86–0.94)

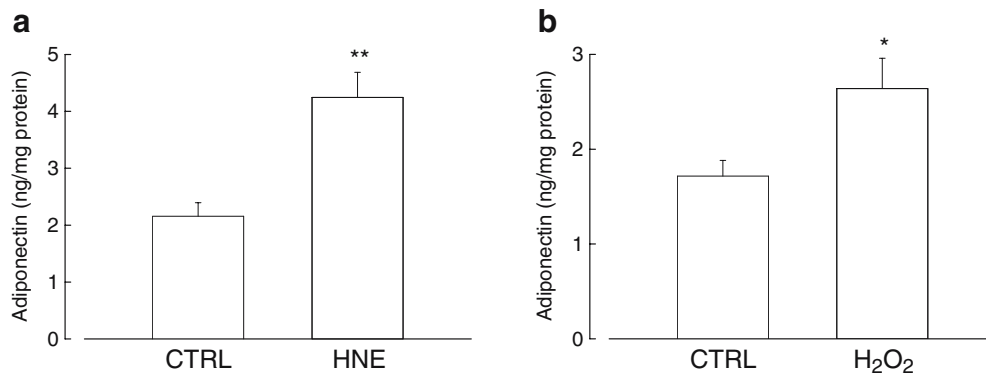
Differentiated C2C12 cells were cultured for up to 48 h (except for glucosamine, 24 h) in the absence (control) or in the presence of the tested agents to mimic the in vivo diabetic or obese situation. *Adipoq* mRNA levels were quantified by RTQ-PCR and are presented as relative expression compared with control values (i.e. basal medium composed of DMEM with 25 mmol/l glucose+2% horse serum except for the study of hyperglycaemia [DMEM with 5.5 mmol/l glucose+2% horse serum]). Results are the means (ranges) for six independent experiments

out, a mixture of triglycerides added to the medium induced *Adipoq* expression. Likewise, inducers of ROS, such as H<sub>2</sub>O<sub>2</sub> and HNE, upregulated adiponectin. Thus, ectopic lipid storage may lead to high amounts of lipoperoxidation end-products, which may in turn produce ROS, thereby inducing adiponectin in muscle. This hypothesis was supported by in vivo data. Obese muscle exhibited both lipid infiltration and signs of oxidative stress. Moreover, muscle *Adipoq* mRNA did positively correlate with plasma TBARS and 8-isoprostanes. Eventually, treatment of the obese mice with the ROS scavenger probucol led to a decrease in plasma and muscle oxidative

stress markers, together with a partial restoration of *Adipoq* mRNA levels and a clear-cut diminution of the adiponectin protein content in muscle. We thus extended the recent concept that lipid accumulation and oxidative stress in adipose tissue deregulate adipokine production to non-adipose tissues [18]. Fourthly, pro-inflammatory cytokines. Diabetes and obesity are now commonly considered as chronic inflammatory states. Plasma concentrations of inflammatory adipokines, such as TNF-α and IL-6, are increased in those pathological situations [35]. We showed that *Tnf* was slightly upregulated in the tibialis anterior of obese mice. Because adiponectin could be induced in

**Fig. 6** Influence of lipids and oxidative stress on *Adipoq* mRNA levels in C2C12 myotubes. **a** C2C12 cells were cultured for 48 h in the absence (control, CTRL) or in the presence of Structolipid (0.2 % v/v; Lipids) and/or *N*-acetylcysteine (NAC; 10 mmol/l). **b**, **c** C2C12 cells were cultured for 18 h (**b**) or 8 h (**c**) in the absence (control, CTRL) or in the presence of 100 µmol/l H<sub>2</sub>O<sub>2</sub> (**b**) or 100 µmol/l 4-hydroxy-2-nonenal (HNE; **c**) and/or NAC (10 mmol/l). The antioxidant was added 30 min before the other products. mRNA levels were quantified by RTQ-PCR and are presented as relative expression compared with control values. Results are normalised to the level of cyclophilin and expressed as means±SEM for six independent experiments. ###*p*<0.001 vs CTRL; +*p*<0.05 vs Lipids; \*\*\**p*<0.001 vs all conditions





**Fig. 7** Influence of oxidative stress on adiponectin levels in C2C12 myotubes. C2C12 cells were cultured for 8 h (a) or 18 h (b) in the absence (control, CTRL) or in the presence of 100  $\mu\text{mol/l}$  4-hydroxy-2-nonenal (HNE; a) or 100  $\mu\text{mol/l}$  H<sub>2</sub>O<sub>2</sub> (b). Adiponectin

levels were measured in cell homogenates by RIA and expressed as nanogram per milligram protein. Results are the means $\pm$ SEM for six independent experiments. \*\* $p$ <0.01 and \* $p$ <0.05 vs CTRL

mouse skeletal muscle and in cultured myotubes in response to LPS or pro-inflammatory cytokines [21], increased TNF- $\alpha$  may further amplify the rise in muscle adiponectin levels in obesity.

The molecular mechanisms underlying adiponectin upregulation in muscle occurred at the pre-translational level. Along with *Adipoq* mRNA, *Ppar $\gamma$*  was upregulated in the skeletal muscle of *ob/ob* mice and its expression was also induced in every condition that stimulated adiponectin in C2C12 myotubes. It is well established that in the adipocyte, PPAR $\gamma$  stimulates the transcriptional activity of the gene encoding adiponectin through a PPAR $\gamma$ -responsive element in the promoter [36]. Whether increased production of PPAR $\gamma$  also stimulates adiponectin production in muscle remains to be established. In contrast to the upregulation of adiponectin and PPAR $\gamma$  found in muscle (this study), oxidative stress downregulated their expression in the adipocyte [18]. Likewise, LPS or pro-inflammatory cytokines upregulated adiponectin production in muscle or myotubes, while inhibiting its production in adipocytes [21, 37]. Thus, in many respects, adiponectin regulation in muscle seems to be fully distinct, if not opposite to that described in adipose tissue.

Although the bulk of adiponectin originates from fat, we estimated, based our own and other data (Table 3, right columns; [38–41]), that as much as 1/10th to 1/15th of systemic adiponectin levels might be accounted for by muscle production in obese mice, while this proportion fell to ~1/175th in lean ones. Yet, the systemic contribution of muscle in obese mice is still insufficient to reverse the hypo adiponectinaemia. Nevertheless, this muscular production of adiponectin in obesity may be of physiological significance because of its local action. Firstly, it is worth noting that *Adipoq* mRNA concentration in obese muscle was rather close to that found in the same amount of fat (Table 3). Secondly, the local efficacy of adiponectin on myocytes might be high since it is directly acting on these cells via autocrine or paracrine mechanisms [42]. Thirdly,

this potency could even be further enhanced by post-translational modifications of the adipokine (e.g. a proteolytic cleavage leading to its globular form) [4]. In consideration of the local action of adiponectin, it should be stressed that, at variance with another study performed in a very small number of *ob/ob* mice [34], the expression of *Adipor1* and *Adipor2* was not modified in the muscle of our obese mice. Our data are in agreement with the unaltered expression of adiponectin receptors in muscle of type 2 diabetic patients [43]. The lack of downregulation of these receptors indicates that the potency of locally produced adiponectin may be preserved.

The physiological relevance of this upregulation of adiponectin in diabetic muscle is still under investigation. On the one hand, adiponectin exerts anti-inflammatory properties on several cell types or tissues and suppresses superoxide generation in endothelial cells [10–12, 14]. As already suggested, induction of adiponectin in muscle could be viewed as a protective mechanism against deleterious inflammatory reactions and oxidative stress. On the other hand, adiponectin upregulation in diabetic muscle could also be a useful means to counteract insulin resistance as well as ectopic lipid deposit [44]. This may be achieved thanks to its ability to stimulate fatty acid oxidation and glucose transport in skeletal muscle [2, 3, 7].

In conclusion, adiponectin is upregulated in muscle of obese and diabetic *ob/ob* mice. This induction may result from lipotoxicity and related oxidative stress. This finding could be viewed as a local protective mechanism to counteract oxidative damage, ectopic lipid storage and insulin resistance.

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