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Possible involvement of AMP-activated protein kinase in obesity resistance induced by respiratory uncoupling in white fat

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Abstract **The AMP-activated protein kinase (AMPK) cascade is a sensor of cellular energy charge that promotes catabolic and inhibits anabolic pathways. However, the role of AMPK in adipocytes is poorly understood. We show that transgenic expression of mitochondrial uncoupling protein 1 in white fat, which induces obesity resistance in mice, is associated with depression of cellular energy charge, activation of AMPK, downregulation of adipogenic genes, and increase in lipid oxidation. Activation of AMPK may explain the complex metabolic changes in adipose tissue of these animals and our results support a role for adipocyte AMPK in the regulation of storage of body fat.**
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Keywords: ATP; Lipid metabolism; Mitochondrion; Adipocyte; Transgenic mouse

1. Introduction

It is becoming evident that metabolism of adipose tissue is important for the control of body fat content [1,2] and that intracellular energy charge is involved in the control of lipid metabolism in adipocytes. In vitro studies demonstrated that a decrease of mitochondrial ATP production resulted in the inhibition of both fatty acid (FA) synthesis [3] and the lipolytic action of catecholamines [4]. In turn, lipolytic hormones decreased ATP levels in adipocytes [5,6]. The intracellular energy charge is also low [7,8] in transgenic mice [9] rendered resistant to obesity by ectopic expression of mitochondrial uncoupling protein 1 (UCP1) in white fat (aP2-*Ucp1* mice). These animals exhibited a reduction of FA synthesis [10] and the lipolytic action of catecholamines [7], as well as increases in mitochondrial biogenesis [11] and endogenous oxygen consumption [12] in white adipose tissue. At that time, a unifying explanation for all of these metabolic changes was not apparent.

The AMP-activated protein kinase (AMPK) is a sensor of cellular energy charge that, once activated by an increase in the cellular AMP/ATP ratio, acts as a metabolic master switch [13,14]. Its known effects include: (a) inhibition of FA synthesis and lipolysis in adipocytes due to phosphorylation of acetyl-CoA carboxylase-1 (ACC-1) and hormone-sensitive lipase [15–17]; (b) stimulation of glucose uptake into adipocytes [18] and myocytes [19]; (c) activation of FA oxidation in muscle due to phosphorylation of ACC-2 [19,20]; (d) induction of mitochondrial biogenesis possibly through activation of nuclear respiratory factor-1 [21]; and (e) downregulation of lipogenic genes in liver [22], as well as in 3T3-L1 adipocytes, in the latter case by downregulating peroxisome proliferator-activated receptor γ (PPARγ; [23]). The role of AMPK in adipose tissue remains relatively unexplored, although recent studies indicate the involvement of AMPK in the effects of physical exercise [24], adiponectin [18], thiazolidinediones [25], and leptin [26,27].

The aim of this study was to verify whether changes in the energy charge of adipocytes in vivo, due to respiratory uncoupling in aP2-*Ucp1* mice, could activate AMPK in adipocytes, and to clarify further the role of AMPK in white adipose tissue.

2. Materials and methods

2.1. Animals

Male C57BL/6J control mice and their transgenic littermates, hemizygous for the aP2-*Ucp1* transgene [9,12,28], were kept in a controlled environment (20 °C; 12 h light/dark cycle) with free access to water and standard chow diet. At 6 months of age, animals were sacrificed by cervical dislocation under diethylether anesthesia. Epididymal and subcutaneous dorsolumbar white fat depots [12] were dissected, flash frozen and stored in liquid nitrogen for biochemical and RNA analysis.

2.2. RNA analysis

Gene expression was analyzed by reverse transcription followed by the real time quantitative PCR (LightCycler Instrument, Roche, Germany) with primers specific for PPARγ, adipocyte lipid binding protein 2 (aP2) and β-actin (Table 1). Levels of β-actin were used to correct for inter-sample variations. Detailed protocol was described before [7].

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Abbreviations: ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; aP2, adipocyte lipid binding protein 2; aP2-*Ucp1* mice, transgenic mice expressing UCP1 gene from aP2 gene promoter; FA, fatty acids; pACC, phosphorylated form of ACC; pAMPK, phosphorylated form of AMPK; PPARγ, peroxisome proliferator-activated receptor γ; TBS, 10 mM Tris–HCl, pH 7.4, 0.5 M NaCl; UCP, uncoupling protein

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Table 1
Sequences of PCR primers

Gene	Sense primer (5′–3′)	Antisense primer (5′–3′)	GenBank Accession No. for cDNA
PPARγ[a]	GGCGAGGGCGATCTTGACAGG	GGGCTTCCGCAGGTTTTTGAGG	U09138
β-Actin	GAACCCTAAGGCCAACCGTGAAAAGAT	ACCGCTCGTTGCCAATAGTGATG	XO3765
aP2	AACACCGAGATTTCCTTCAA	AGTCACGCCTTTCATAACACA	M13385

[a] Both isoforms γ1 and γ2 are amplified.

2.3. Determination of ATP and AMP

Flash-frozen tissues dissected immediately after sacrifice were stored in liquid nitrogen. Tissues were homogenized in 6% (w/v) perchloric acid. After centrifugation, supernatants were neutralized and adenine nucleotides determined using HPLC [7].

2.4. Activity of α1 isoform of AMPK

AMPK was immunoprecipitated from tissue extracts prepared by homogenization in buffer A and the activity was assayed using a peptide substrate [29]. Protein concentration was estimated using bicinchoninic acid procedure and BSA as a standard [7]. AMPK activity is expressed in Units (nmol phosphate/min) per mg of protein in the adipocyte extract used for immunoprecipitation.

2.5. Oxidation of FA

Oxidation of oleic acid was measured using a modified protocol of Wang et al. [30]. Adipose tissue (~35 mg) sliced up into 5–10 fragments was pre-labeled with 40 μCi/mL [9,10(n)-^{3}H]oleic acid in 300 μl of 2% FA-free BSA-KRB buffer containing 5 mM glucose for 75 min at 37 °C and under 5% CO_2 (gentle shaking). Fragments were washed three times and re-suspended in 500 μl of the buffer and incubated for additional 4 h at 37 °C. Oxidation was assessed by measuring the amount of ^3H_2O released into the medium. The content of free FA in adipose tissue fragments was measured in 5% homogenate (w/v in H_2O) using a NEFA C kit from Wako Chemicals (Richmond, VA) and results were calculated according to a published formula [30].

2.6. Quantification of AMPK and ACC

The total content of the α1 catalytic subunit of AMPK (α1 AMPK) and the phosphorylated form of AMPK (pAMPK) was determined in tissue lysates by Western blotting [29,31] using antibodies against total α1 AMPK [31] and phosphospecific antibodies against Thr-172 [32]. For the quantification of total ACC and the phosphorylated form of ACC (pACC), tissue lysates (10 μg protein) were subjected to SDS–PAGE using pre-cast 3–8% Tris-acetate gels (Invitrogen). Protein was transferred to nitrocellulose membranes (BioRad, Hercules, CA) and the membranes incubated in Odyssey Blocking buffer (Li-Cor Biosciences, Lincoln, NE). Phosphospecific sheep antibodies against the Ser-221 site on ACC-2 [29], that also recognize the homologous site (Ser-79) on ACC-1 (1.46 μg/ml in blocking buffer containing 0.2% Tween-20), were used to quantify pACC (incubation for 1 h). The membranes were washed 6 × 5 min with TBS (10 mM Tris–HCl, pH 7.4, 0.5 M NaCl) containing Tween-20 (0.2%) and immersed in blocking buffer containing 0.2% Tween-20 and 1 μg/ml anti-sheep IgG conjugated to IR dye 680 (Molecular Probes, Leiden, The Netherlands) and 1 μg/ml streptavidin conjugated to IR Dye 800 (Rockland Inc., Philadelphia, PA) and left shaking for 1 h, protected from light. The membranes were then washed 6 × 5 min using TBS-Tween (0.2%) and 1 × 5 min in PBS and scanned using the Odyssey IR Imager (Li-Cor Biosciences). The results were quantified using Odyssey software. A linear response was obtained between 0.3 and 20 μg lysate protein analyzed (not shown).

2.7. Statistics

Statistical significance was evaluated using unpaired *t*-tests. Differences were judged to be significant at $P < 0.05$.

3. Results

3.1. Increased cellular AMP/ATP ratio

We observed previously [7] that in white fat of the aP2-*Ucp1* mice ATP content was lower and ADP/ATP ratio was higher than in control mice. These differences were more pronounced in subcutaneous than in epididymal fat, in accordance with a higher content of transgenic UCP1 in the former fat depot [7]. Due to the adenylate kinase reaction [13], we expected that the AMP/ATP ratio should be also affected by ectopic expression of UCP1 in white fat. It was observed that the AMP/ATP ratio in both fat depots of transgenic animals was higher than in control mice (Fig. 1A). No statistically significant differences between the two genotypes could be detected at the level of tissue contents of individual nucleotides (not shown).

3.2. Enhancement of AMPK activity

The increase of the AMP/ATP ratio produced by ectopic expression of UCP1 in white fat suggested that AMPK activity in adipose tissue would be also affected. Indeed, the presence of transgenic UCP1 resulted in a significant, 2-fold increase in the activity of the α1 isoform (α1 AMPK) in subcutaneous fat (Fig. 1B). The activity also tended to be higher in the epididymal fat, although this was not statistically significant (Fig. 1B). The activity of the α2 isoform was negligible in adipose tissue (not shown). Quantification of the expression of α1 AMPK and phosphorylation of Thr-172 using Western blots (Fig. 2A) indicated a significantly higher content of the α1 subunit in transgenic than in control mice in both epididymal and subcutaneous fat (Fig. 2B) and, after correction for α1 content, a significant increase in α1 AMPK phosphoryla-

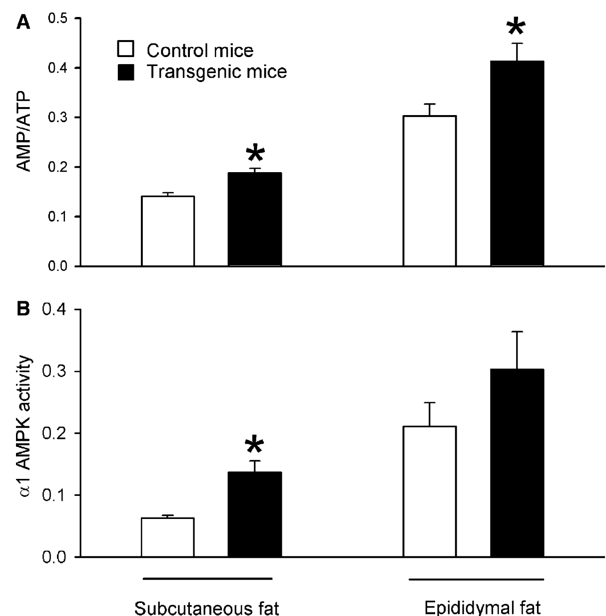

Fig. 1. AMP/ATP ratio and AMPK activity in adipose tissue. (A) Concentrations of ATP and AMP were estimated by HPLC in tissue extracts ($n = 15$). (B) Activity of α1 AMPK (in units; $n = 6$). Values are means ± S.E. Asterisks indicate statistically significant differences between genotypes.

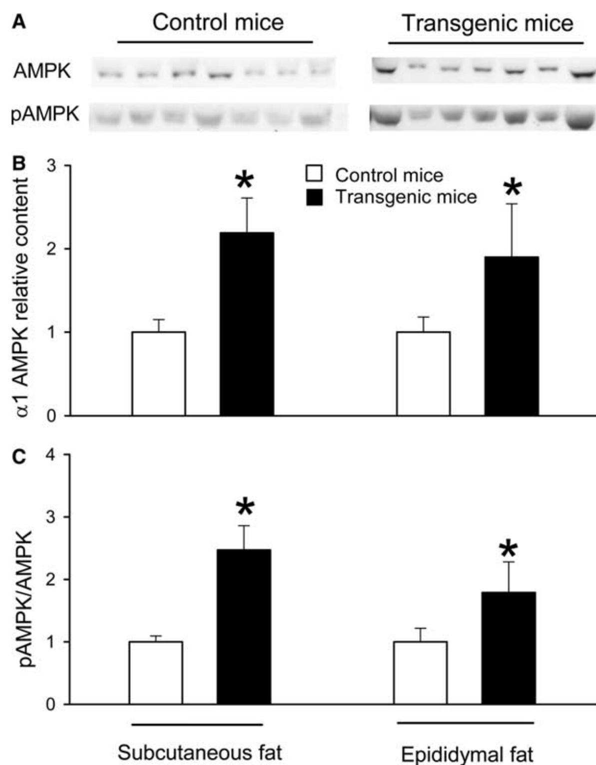

Fig. 2. Content and phosphorylation of AMPK in adipose tissue. (A) Total α1 AMPK and phosphorylated form of α1 AMPK (pAMPK) were quantified using Western blots, as illustrated for the analysis in subcutaneous fat. (B) Total content of α1 AMPK. (C) Phosphorylation of AMPK. Values are means ± S.E. ($n = 6$). Asterisks indicate statistically significant differences between genotypes.

tion in both fat depots due to the transgenic modification (Fig. 2C). Quantification of total ACC and pACC content in adipose tissue lysates (see Section 2) also showed a modest but significant increase of the pACC/ACC ratio (1.3-fold; $P < 0.05$; $n = 6$) in subcutaneous fat due to the transgenic modification. Increased phosphorylation of both α1 AMPK and ACC in the transgenic mice further supports the activation of AMPK by the ectopic UCP1 in white fat.

3.3. Decreased expression of PPARγ and aP2 genes

In order to further characterize the complex changes in lipid metabolism in the transgenic mice, expression of the PPARγ gene was analyzed. PPARγ plays a crucial regulatory role in adipogenesis and its expression is inhibited by AMPK activation during differentiation of 3T3-L1 adipocytes in culture [23]. A significant diminution of PPARγ mRNA level was found in subcutaneous but not in epididymal fat of aP2-*Ucp1* mice (Table 2). Moreover, the expression of a PPARγ target gene, i.e., aP2, was also downregulated in subcutaneous fat (Table 2).

3.4. Enhancement of FA oxidation

Previous experiments showed a higher rate of oxidation of endogenous substrates in white fat of transgenic compared with control mice [12]. Measurement of oleate oxidation in adipose tissue fragments (Fig. 3) revealed a similar rate of oxidation in the subcutaneous and epididymal fat of control mice. However, the presence of transgenic UCP1 resulted in a significant, 2.7-fold increase in the oleate oxidation in subcutaneous fat, but not epididymal fat.

Table 2
Downregulation of PPARγ and aP2 gene expression in adipose tissue of transgenic mice

Sample	mRNA level (relative units %)	
	PPARγ	aP2
Subcutaneous fat		
+/+	100.0 ± 15.6	100 ± 17.6
tg/+	22.9 ± 6.2*	32.8 ± 6.4*
Epididymal fat		
+/+	100.0 ± 11.6	100 ± 12.5
tg/+	68.1 ± 26.1	80.7 ± 12.6

Transcripts were quantified in adipose tissue of control (+/+) and transgenic mice (tg/+). Data are means ± S.E. ($n = 5$ to 6). Asterisks indicate statistically significant differences between genotypes. Results were verified using Northern blot analysis (not shown).

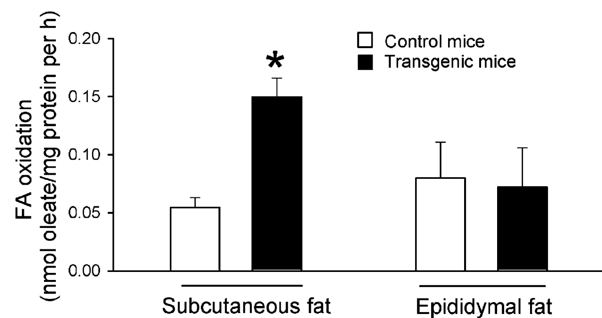

Fig. 3. Oxidation of oleate in adipose tissue. Values are means ± S.E. ($n = 6$). Asterisk indicates statistically significant differences between genotypes.

4. Discussion

Our results demonstrate that persistent depression of energy charge in adipocytes of the transgenic mice results in both an increase of α1 AMPK expression, and an increase in its phosphorylation and activity. Increased expression of α1 has been observed in other tissues in response to treatments that would be expected to persistently activate AMPK, e.g., during pressure overload hypertrophy in rat heart [33], or endurance training in human skeletal muscle [34]. The differential effect of the transgene on AMPK activity in the subcutaneous versus the epididymal fat depots was in accordance with the 2- to 3-fold higher expression of the transgene in the former, detected at both the mRNA and protein levels [10,11], and also with the preferential effect of the transgene on lipogenesis [10], lipolysis [7], and FA oxidation (this report, Fig. 3) in the subcutaneous fat. The strong induction of FA oxidation, the increase in mitochondrial content [11], and the depression of lipogenesis [10] in subcutaneous fat of the transgenic mice are consistent with the known effects of AMPK in white fat [15,16] and other tissues [13,14,20,21] and explain the preferential reduction of subcutaneous fat in the transgenic mice [9,28]. Moreover, the strong downregulation of PPARγ and aP2 genes in the subcutaneous fat of the transgenic mice, most probably due to the activation of AMPK [23], may suppress the adipogenic potential of the tissue. Thus, activation of AMPK in white fat of aP2-*Ucp1* mice, by depression of intracellular energy charge, helps to explain the complexity of the changes in adipose tissue metabolism and obesity resistance in this transgenic model.

Besides the effect of transgenic UCP1 on adipose tissue, several other studies support a link between energy charge and metabolism in adipocytes, and the involvement of AMPK: (a) hyperleptinemia [26] and bezafibrate treatment [35] deplete body fat in rats, whereas expression of UCP1 and UCP2 in white adipose tissue is up-regulated, FA oxidation is increased, and expression of lipogenic genes is profoundly suppressed; (b) in isolated adipocytes leptin inhibits lipid synthesis while up-regulating UCP2 [36]; (c) antidiabetic drugs such as thiazolidinediones stimulate AMPK in adipose tissue [25], while inducing glycerol kinases [37] and phosphoenolpyruvate carboxykinase [38], leading presumably to futile cycling of FA re-esterification in adipocytes. Thiazolidinediones also stimulate AMPK in muscle cells while increasing the intracellular AMP/ATP ratio [39]. In fact, the induction of UCPs by leptin in adipose tissue, leading to a drop in the intracellular energy charge, may partly explain the activation of AMPK in adipose tissue under these conditions [27].

AMPK appears to represent a switch that converts adipocytes to lipid burning cells while suppressing in situ lipogenesis. This mechanism, which reduces the fat content of adipocytes, exists not only in aP2-*Ucp1* mice, but also under other circumstances such as hyperleptinemia [27]. Interestingly, FA oxidation and synthesis in adipose tissue are also reciprocally regulated during fasting [30]. Finally, the control of adipose tissue metabolism by intracellular energy charge and AMPK may represent a basic biological mechanism that contributes to the regional differences in the metabolic properties of adipose tissue depots. This mechanism could be affected by physiological stimuli, as well as by pharmacological agents, and it represents a promising target for the development of strategies for the treatment of both obesity and insulin resistance.

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