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Adiponectin is synthesized and secreted by human and murine cardiomyocytes

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Abstract **Adiponectin is thought to play a decisive role in the relationships among obesity, insulin resistance and cardiovascular risk. This study investigated whether cardiomyocytes synthesize and secrete adiponectin, and the effects of this hormone on cardiac cells. RT-PCR showed that mouse, rat and human cardiomyocytes produced mRNA for adiponectin and adiponectin receptors 1 and 2. Immunohistochemistry confirmed the presence of adiponectin in the cytoplasm of cultured cardiomyocytes, and radioimmunoassay showed that these cells secreted adiponectin into the culture medium. Exogenous adiponectin enhanced glucose and fatty acid uptake and induced AMPK phosphorylation in cultured cardiomyocytes. Our results demonstrate that adiponectin is synthesized and secreted by isolated murine and human cardiomyocytes, and suggest that the local production of this hormone by cardiomyocytes could be involved in the regulation of cardiac metabolism and function.**

Keywords: Cardiomyocyte; Adiponectin; Energy metabolism; AMP-activated protein kinase; Metabolic syndrome

1. Introduction

Recent intensive research on obesity has shown that adipokines mediate many of its cardiovascular and metabolic complications, although the pathogenic relationships among obesity, metabolic syndrome, and cardiovascular complications remain poorly understood. Adiponectin is an approximately 30-kDa protein that is secreted by adipose tissue, circulates in plasma as multimeric complexes at relatively high concentration (~2–10 μg/ml), and plays a crucial role in the association among obesity, type II diabetes, and insulin resistance [1]. In humans, adiponectin is reduced in the serum of type II diabetic and obese patients, and is further decreased in patients with cardiovascular disease, being increasingly recognized as both a potential biomarker for the metabolic syndrome and cardiovascular disease, and a possible therapeutic target [2]. Adiponectin has been reported to have antiatherogenic and antiinflammatory effects [3], to enhance endothelial vasodilation [4], and to play a role in myocardial remodelling after ischaemic injury [5]. On the other hand, plasma adiponectin levels correlate negatively with C-reactive protein levels [6], high plasma adiponectin concentrations are associated with lower risk of myocardial infarct in men [7], and hypoadiponectinaemia is a risk factor for hypertension [8]. Recently, an adiponectin gene mutation has been associated with coronary artery disease as well as with metabolic syndrome [9].

While adiponectin receptors (Adipo-R1 and Adipo-R2) are present in most organs [10], adiponectin itself has generally been written of as it were produced exclusively by adipocytes. However, adiponectin synthesis has recently been observed in other tissues [11–14], where it may have autocrine and/or paracrine functions. In view of this, and given the cardiovascular effects of adiponectin noted above, we wondered whether cardiomyocytes synthesize and secrete this hormone. In this study we found that cardiomyocytes can synthesize and secrete adiponectin and that exogenous adiponectin increases their uptake of glucose and free fatty acids and leads to phosphorylation of AMPK. The present findings demonstrate that adiponectin is not adipocyte specific but local production of adiponectin by cardiomyocytes might have important functions in the regulation of the cardiac function and/or metabolism.

2. Material and methods

All sera and media were from Life Technologies Ltd. (Poole, UK), and all other products from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise stated.

2.1. Cells

HL-1 cells, a line of adult mouse atrial cardiomyocytes, were a generous gift of Dr. W.C. Claycomb of Louisiana State University Medical Center (New Orleans, LA, USA) and primary cultures of human (with cells obtained from fragments of right atrial appendage, previous informed consent obtained from the patients) and neonatal rat cardiomyocytes were cultured as previously described [15].

2.2. Glucose uptake experiments

10 μg/ml full-length recombinant adiponectin (BioCat GmbH, Heidelberg, Germany) [16] was used for the treatments. Glucose uptake was determined as described previously [17]. In some experiments, an

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effective inhibitor of AMPK, 2 mM adenine 9-β-D-arabinofuranoside (araA) (MP Biomedicals, LLC, Eschwege, Germany) was added 20 min before the addition of adiponectin, as previously described [18].

2.3. BODIPY-labeled fatty acid uptake

HL-1 cells (4×10^5) were serum starved for 12 h and then treated for 0.5, 2, 6 and 12 h with 10 μg/ml adiponectin, and incubated 30 s with PBS containing 10 μM 4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoic acid (BODIPY® 500/510 C_1, C_{12}; Molecular Probes, Inc., Eugene, OR, USA), and 20 μM fatty acid-free BSA. Cells were also stained with propidium iodide (1 μM) to determine dead cells and analyzed by flow cytometry in a FACSCALIBUR (Becton & Dickinson, San José, CA, USA) using the program Cell Quest.

2.4. Western blots

HL-1 cells (5×10^5) were starved 12 h and treated for 5, 30, or 120 min with 10 μg/ml adiponectin. Lysates were analyzed by Western blot using either rabbit anti-phospho-AMPK antibody (Cell Signalling Technology, Inc., Beverly, MA, USA) or rabbit anti-AMPK antibody (Cell Signalling Technology, Inc.).

2.5. Immunohistochemistry

Slide-borne cells were fixed in 96% ethanol for 10 min. Antigen retrieval was only done for the adiponectin localization: cells were pretreated for 40 min in 0.1 M sodium citrate buffer (pH 6) in a water bath at 95–99 °C. Immunohistochemical procedure:

1. *Primary antibody*. Goat anti-adiponectin antibody (ACRP-30 N-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA), at a dilution of 1/200, overnight at 4 °C; rabbit anti-Adipo-R1 antibody (41–65) (Phoenix Pharmaceuticals, Belmont, CA, USA) 1/100 for 1 h at room temperature (RT) or rabbit anti-Adipo-R2 antibody (4–39) (Phoenix Pharmaceuticals) 1/50 overnight at RT.
2. 3% Hydrogen peroxide (Merck, Darmstadt, Germany) 10 min.
3. *Detection system.*
   - *LSAB® System HRP* (Dakocytomation, Carpinteria, CA, USA) (multilink biotinylated anti-mouse, rabbit and goat IgG, 30 min, and streptavidin-conjugated horseradish peroxidase, 30 min), for the adiponectin detection, and
   - *Envision® HRP* anti-rabbit and mouse (Dakocytomation) for 30 min, in the case of the adiponectin receptors
4. 3,3′-Diaminobenzidine tetrahydrochloride (Dakocytomation) for 10 min.

Between steps, sections were washed twice for 5 min with TBS (0.05 M Tris buffer of pH 7.6 containing 0.3 M NaCl), and before step 6 with distilled water. Counterstaining was done with Harris' haematoxylin for 1 min.

Double immunofluorescence. Mouse monoclonal antibodies to cardiac myosin heavy chain (MHC, Abcam, Cambridge, UK, clone 3-48) and the same antibodies to adiponectin, Adipo-R1 or Adipo-R2 as above were used. Antigen retrieval using the water bath was only done for double immunofluorescence of adiponectin and MHC. Cells were incubated in:

1. Anti-adiponectin antibody, anti-Adipo-R1 antibody or with anti-Adipo-R2 antibody;
2. 1/200 Alexa Fluor® 488 $F(ab')_2$ fragment of rabbit anti-goat IgG (Molecular Probes, Eugene, OR, USA) for adiponectin detection, 1 h at RT or 1/200 Alexa Fluor® 488 $F(ab')_2$ fragment of goat anti-rabbit IgG (Molecular Probes) for adiponectin receptors detection, 1 h at RT;
3. 1/1000 mouse anti-(cardiac myosin heavy chain) antibody (Abcam; Cambridge, UK); for 1 h at RT and
4. 1/100 Cy3-conjugated $F(ab')_2$ fragment of sheep anti-mouse IgG (Sigma, St. Louis, MO, USA) for 1 h.

Controls. As positive control tissues, mouse and rat adipose tissue was used for adiponectin, mouse and rat striated muscle for Adipo-R1, and mouse and rat liver for Adipo-R2. Negative controls included preadsorption of the anti-adiponectin antibody with 10 nmol/ml human or mouse adiponectin (BioCat), or with blocking peptide (sc-17044, Santa Cruz).

2.6. RT-PCR

1–2 μg of total RNA was back-transcribed and the resulting cDNA was used as a PCR template in a reaction mixture containing the primers for mouse, human and rat adiponectin and adiponectin receptors [10,16,19–21] listed in Table 1.

2.7. Sequence analysis

Human and mouse adiponectin, and mouse Adipo-R1 and Adipo-R2 PCR products were sequenced using a BIGDye™ Terminator kit (Amersham Biosciences) and an ABI Prism automated DNA sequencer (Applied Biosystems, Foster City, CA, USA).

2.8. Adiponectin radioimmunoassay

Adiponectin levels in the media in which cultured HL-1 and human cardiomyocytes had been starved for 24 h were determined using a kit from Linco Research, Inc. (St. Charles, MO, USA).

2.9. Statistical analysis

Results shown are the means ± S.E.M. of at least three independent experiments. The significance of differences was estimated by ANOVA followed by Student–Newmann–Keuls multiple comparison tests; $P < 0.05$ was considered significant.

Table 1
Primers used for RT-PCR

Protein	Primer sequences	Annealing (°C)	Product size (bp)
Mouse Adiponectin [19]	5′-GACGTTACTACAACTGAAGAGC-3′ 5′-CATTCTTTTCCTGATACTGGTC-3′	56	532
Mouse Adipo-R1 [10]	5′-ACGTTGGAGAGTCATCCCGTAT-3′ 5′-CTCTGTGTGGATGCGGAAGAT-3′	60	132
Mouse Adipo-R2 [10]	5′-TCCCAGGAAGATGAAGGGTTTAT-3′ 5′-TTCCATTCGTTCGATAGCATGA-3′	60	72
Human Adiponectin [20]	5′-TGGTGAGAAGGGTGAGAA-3′ 5′-AGATCTTGGTAAAGCGAATG-3′	56	221
Human Adipo-R1 [10]	5′-TTCTTCCTCATGGCTGTGATGT-3′ 5′-AAGAAGCGCTCAGGAATTCG-3′	62	70
Human Adipo-R2 [10]	5′-ATAGGGCAGATAGGCTGGTTGA-3′ 5′-GGATCCGGGCAGCATACA-3′	62	75
Rat Adiponectin [21]	5′-ACCCAGGAGATGCTG-3′ 5′-ACCTGGAGCCAGACTTGGTC-3′	55	405
Rat Adipo-R1 [16]	5′-CTTCTACTGCTCCCCACAGC-3′ 5′-TCCCAGGAACACTCCTGCTC-3′	60	138
Rat Adipo-R2 [16]	5′-CCACACAACACAAGAATCCG-3′ 5′-CCCTTCTTCTTGGGAGAATGG-3′	60	117

3. Results

3.1. Expression of adiponectin and adiponectin receptor genes

RT-PCR for adiponectin in HL-1 cardiomyocytes afforded a cDNA band of the same size as that amplified from mouse white adipose tissue (WAT) (Fig. 1, panel A.1). HL-1 cells also expressed both Adipo-R1 and Adipo-R2 genes at levels similar to those observed in whole mouse myocardium and mouse liver, respectively (Fig. 1, panel B.1). The identities of the adiponectin, Adipo-R1 and Adipo-R2 gene PCR products were confirmed by sequencing (data not shown).

RT-PCR also amplified cDNA for adiponectin from RNAs obtained from primary cultured (PC) human atrial cardiomyocytes, myocardium and fat (Fig. 1, panel A.2), cDNAs for Adipo-R1 from cultured human atrial cardiomyocytes and human skeletal muscle (Fig. 1, panel B.2), and cDNAs for Adipo-R2 from cultured human atrial cardiomyocytes and human liver (Fig. 1, panel B.2).

Similarly, RT-PCR amplified adiponectin cDNA from RNA obtained from adult rat myocardium (data not shown). Adipo-R1 mRNA was present in the hearts of 1-day-old rats, but Adipo-R2 was not (Fig. 1, panel B.3). Adipo-R2 mRNA also appeared to be absent from the hearts of 6-day-old and 9-day-old rats, but was clearly present in those of 25-day-old rats (Fig. 1, panel B.4).

That reverse transcription had proceeded properly was confirmed in all cases by amplification of GAPDH. The number of PCR cycles was in all cases 35.

3.2. Synthesis of adiponectin and adiponectin receptors by cardiomyocytes

The cytoplasm of HL-1 cells showed intense immunoreactivity with anti-adiponectin, anti-Adipo-R1 and anti-Adipo-R2 antibodies (Fig. 2A, F and G, respectively). Preadsorption of the primary anti-adiponectin antibody with 10 nmol/ml of mouse adiponectin did not show any positive reaction (Fig. 2B). Rat and mouse myocardial tissue also stained positive for adiponectin (Fig. 2E shows this for rat). As positive controls, rat striated muscle and liver showed intense immunostaining for adiponectin receptors 1 and 2, respectively (Fig. 2H and I). The cytoplasm of human cardiomyocytes in primary culture also showed intense immunoreactivity with anti-adiponectin antibody (Fig. 2C). Preadsorption of the primary anti-adiponectin antibody with 10 nmol/ml of human

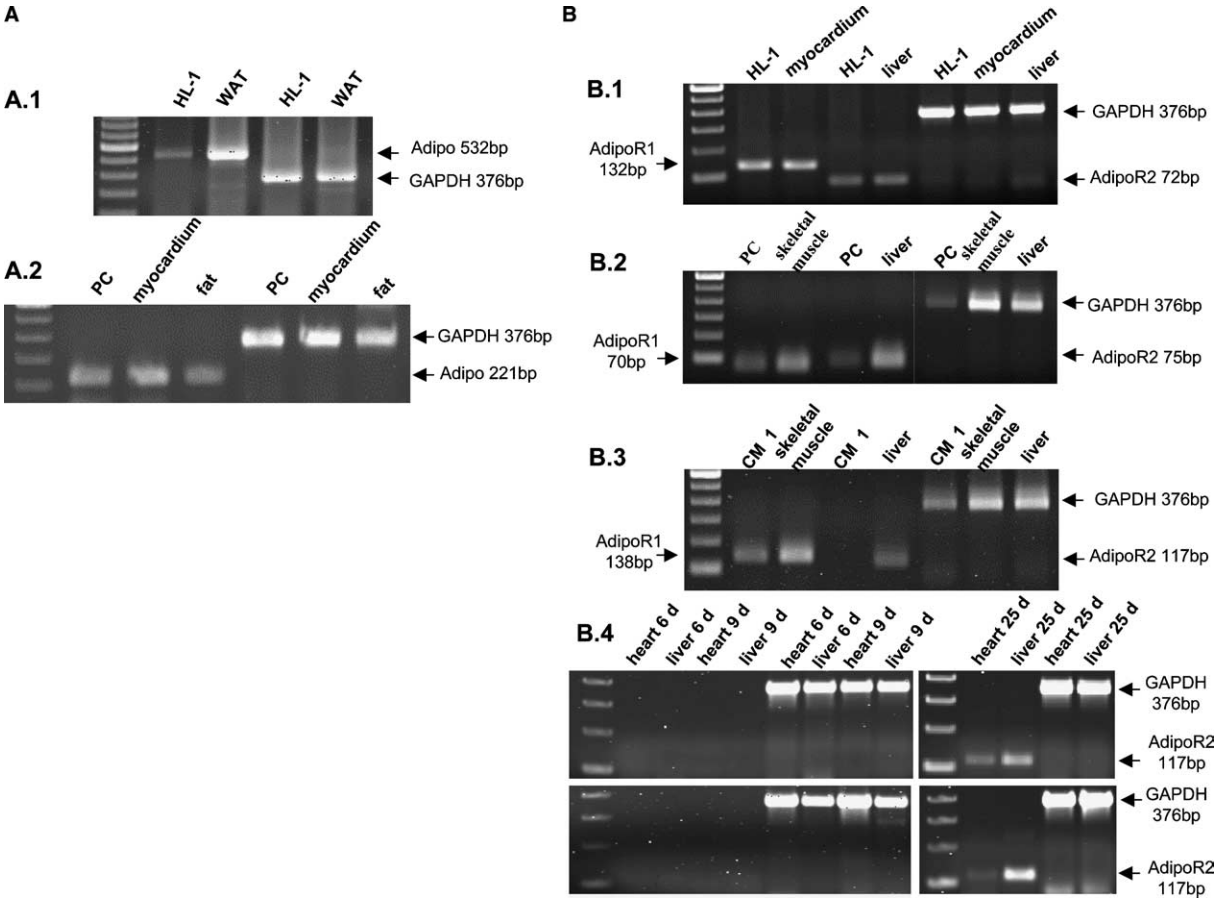

Fig. 1. RT-PCR results showing expression of genes for adiponectin, Adipo-R1 and Adipo-R2. Panel A.1: adiponectin cDNA amplified from mouse HL-1 cardiomyocytes and white adipose tissue. Panel A.2: adiponectin cDNA amplified from human cardiomyocytes in primary culture (PC), human myocardium and human fat. Panel B.1: Adipo-R1 cDNA amplified from HL-1 cardiomyocytes and whole mouse myocardium, and Adipo-R2 cDNA amplified from HL-1 cardiomyocytes and mouse liver. Panel B.2: Adipo-R1 cDNA amplified from human cardiomyocytes in primary culture (PC) and human skeletal muscle, and Adipo-R2 cDNA amplified from human cardiomyocytes in primary culture (PC) and human liver. Panel B.3: Adipo-R1 cDNA amplification from primary cultures of 1-day-old rat cardiomyocytes (CM 1) and rat skeletal muscle, and Adipo-R2 cDNA from primary cultures of 1-day-old rat cardiomyocytes (CM 1) and rat liver. Panel B.4 (upper): Adipo-R2 cDNA amplification from hearts and livers of 6-day-old, 9-day-old, and 25-day-old male rats. Panel B.4 (lower): as for the upper panel, but with female rats.

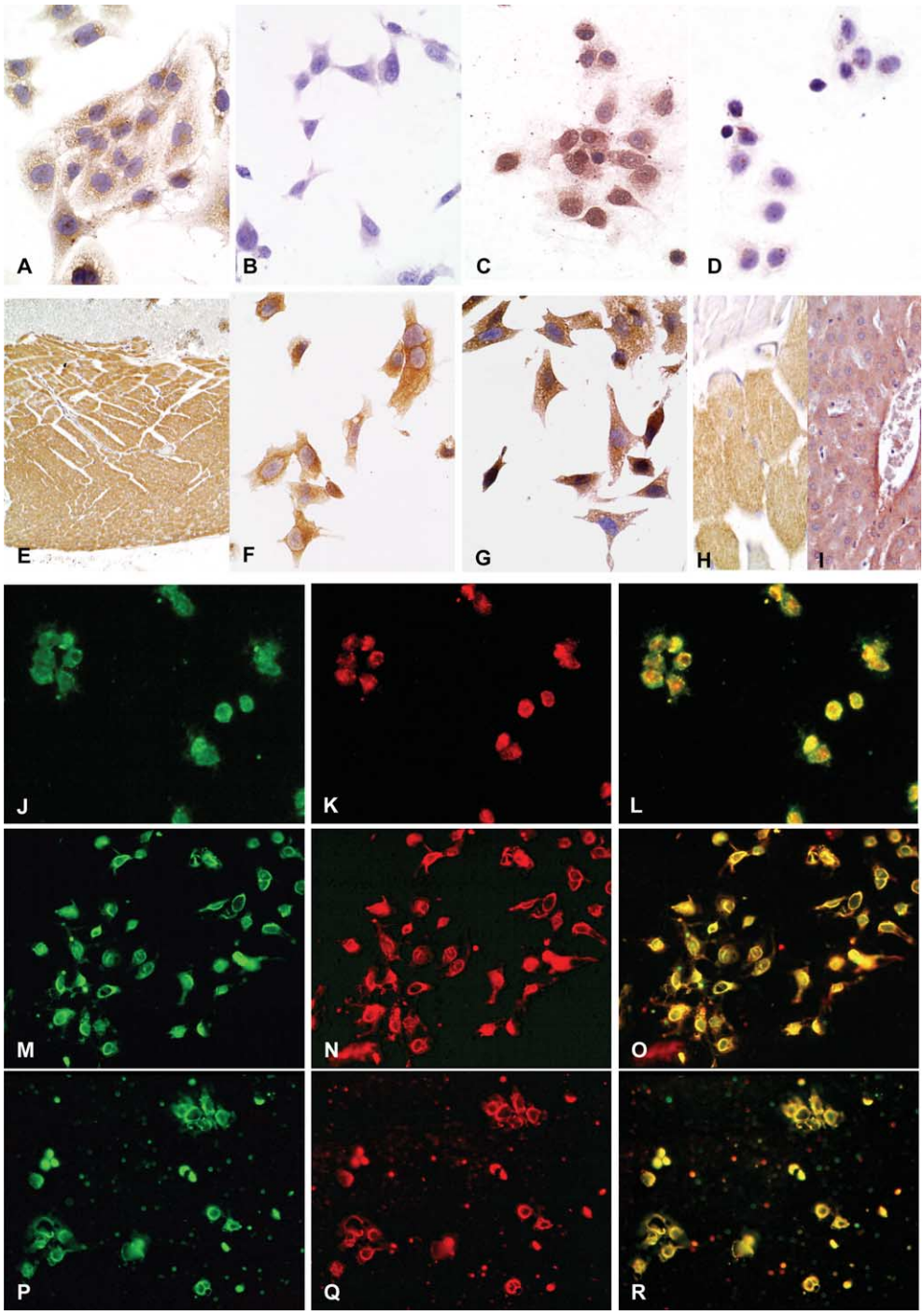

Fig. 2. Immunohistochemical detection of adiponectin, Adipo-R1 and Adipo-R2 in cardiomyocytes and myocardium (objective magnification 40× as otherwise stated). (A) Adiponectin immunostaining was mainly localized in the cytoplasm of HL-1 cells. (B) Preadsorption control with mouse recombinant adiponectin (10 nmol/ml) shows no positive immunostaining. (C) Adiponectin immunostaining was also evident in the cytoplasm of human atrial myocardium cells. Positive reaction was mainly localized in the cytoplasm in the vicinity of the nuclei. (D) Preadsorption control with human recombinant adiponectin (10 nmol/ml) resulted in a negative reaction. (E) Rat left ventricle, showing myocardium positively immunostained for adiponectin (objective magnification 10×). (F–G) HL-1 cells with cytoplasm showing diffuse immunopositivity for Adipo-R1 (F) and Adipo-R2 (G). (H) Rat striated muscle: myocytes, but not connective tissue cells, are positively immunostained for Adipo-R1. (I) Rat liver, positively immunostained for Adipo-R2. (J–L) Photomicrographs of human atrial myocardium cells in primary culture showing immunofluorescence of adiponectin (J), cardiac myosin heavy chain (K), and both these targets (L). (M–O) Photomicrographs of human atrial myocardium cells in primary culture showing immunofluorescence of Adipo-R1 (M), cardiac myosin heavy chain (N), and both these targets (O). (P–R) Photomicrographs of human atrial myocardium cells in primary culture showing immunofluorescence of Adipo-R2 (P), cardiac myosin heavy chain (Q), and both these targets (R).

adiponectin did not show any positive reaction (Fig. 2D). The possibility that the human cells were fibroblasts rather than cardiac muscle fibres was ruled out in colocalization studies showing positivity for adiponectin, Adipo-R1, and Adipo-R2, by their immunoreactivity with antibody against cardiac myosin heavy chain (Fig. 2J–R).

3.3. Secretion of adiponectin by cultured HL-1 cells and primary cultures of human cardiomyocytes

The adiponectin concentrations measured by RIA in the media in which starving HL-1 cells and human cardiomyocytes were cultured were 2.7 ± 0.6 ng/ml ($n = 5$) and 3.1 ± 0.2 ng/ml ($n = 10$), respectively.

3.4. Adiponectin-induced increases in glucose and fatty acid uptake by cardiomyocytes

Treatment with a physiological concentration of adiponectin (10 μg/ml) increased 2-deoxy-D-[^{3}H] glucose uptake by HL-1 cardiomyocytes by 4.5 ± 1.6% at 5 min (not significant, $n = 4$), by 12.9 ± 2.6 at 30 min ($P < 0.01$, $n = 4$), and by 18.5 ± 2.8% at 120 min ($P < 0.01$, $n = 4$), whereas treatment with 300 nM insulin for 20 min increased 2-deoxy-D-[^{3}H] glucose uptake by 47.5 ± 7.6% ($P < 0.001$, $n = 4$) (Fig. 3, panel A).

2-Deoxy-D-[^{3}H] glucose uptake by neonatal rat cardiomyocytes was increased by 49.6 ± 10.4% by treatment for 24 h with 10 μg/ml adiponectin ($P < 0.05$ vs. control, $n = 4$), by 90.1 ± 19.9% by stimulation with 100 nM insulin for 60 min ($P < 0.01$ vs. control, $n = 4$), and by 138.2 ± 20.2% by co-stimulation with insulin and adiponectin ($P < 0.001$ vs. control, $n = 4$) (Fig. 3, panel B.1). In HL-1 cells, 2-deoxy-D-[^{3}H] glucose uptake was enhanced by 27.02 ± 5.5% by 24 h adiponectin ($P < 0.05$ vs. control, $n = 8$), by 48.7 ± 10.4% by insulin ($P < 0.001$ vs. control, $n = 8$), and by 70.5 ± 10.6% by adiponectin plus insulin ($P < 0.001$ vs. control, $n = 8$) (Fig. 3, panel B.2). Thus adiponectin both increased basal glucose uptake and enhanced insulin-stimulated glucose uptake by cardiomyocytes.

Furthermore, adiponectin treatment also increased fatty acid uptake by HL-1 cardiomyocytes in a time dependent manner (Fig. 4), with the highest effect observed at 30 min after treatment (units as BODIPY fluorescence means)(975.2 ± 37.1 vs. 779.8 ± 23.5 of control, $P < 0.01$, $n = 5$). With treatments of 6 h the effect was still evident but it started to decrease (785.2 ± 16.4 vs. 737.6 ± 4.3 of control, $P < 0.05$, $n = 3$), and it disappeared with treatments of 12 h (767.7 ± 39.5 vs. 745.4 ± 11.3 of control, not significant, $n = 3$).

3.5. Adiponectin-induced phosphorylation of AMP-activated protein kinase (AMPK) in HL-1 cardiomyocytes

Phosphorylation of AMPK in HL-1 cells was increased by 72.0 ± 17.3% by 5 min treatment with 10 μg/ml adiponectin ($P < 0.05$, $n = 4$), by 53.0 ± 6.3% by 30 min treatment ($P < 0.05$, $n = 4$), and by 117.0 ± 11.0% by 120 min treatment ($P < 0.001$, $n = 4$) (Fig. 5). Noteworthy, the

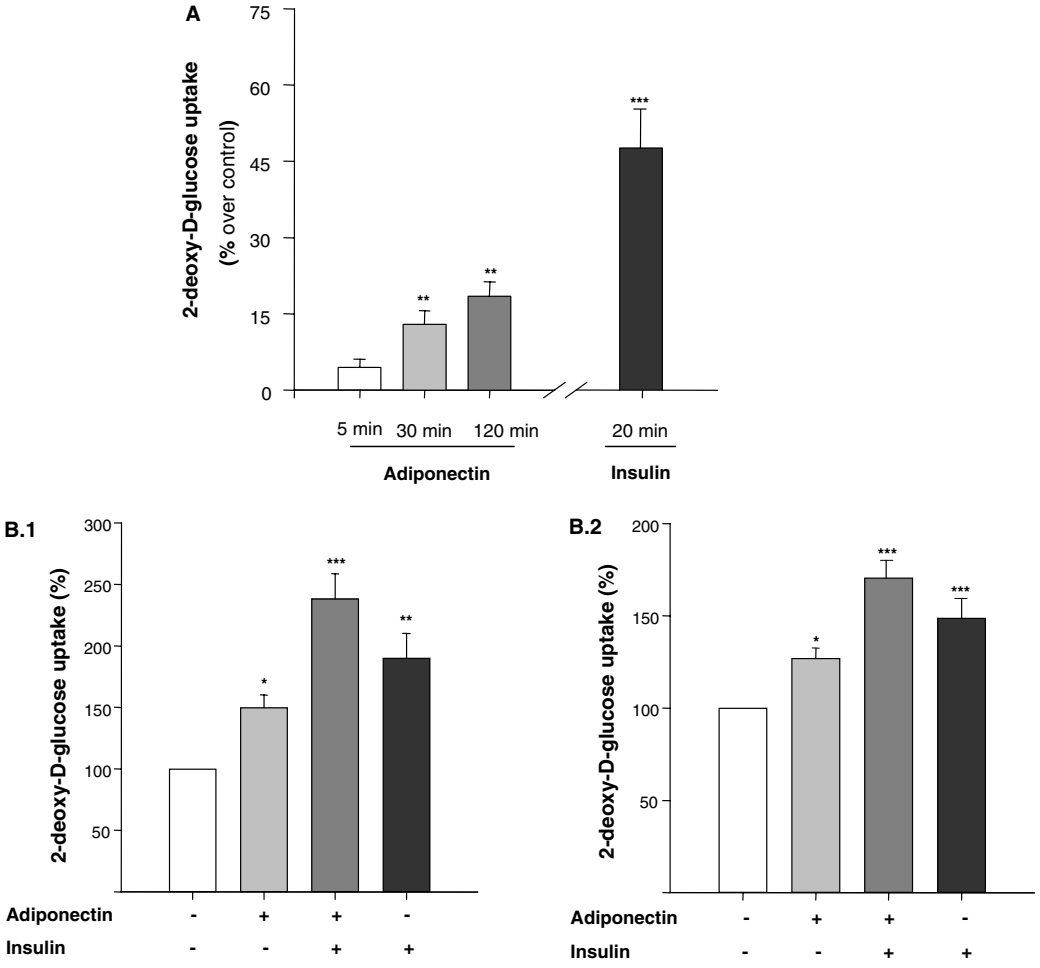

Fig. 3. Uptake of 2-deoxy-D-glucose by cardiomyocytes. Panel A: time course treatment with 10 μg/ml adiponectin or 20 min treatment with 300 nM insulin of HL-1 cardiomyocytes ($n = 4$). Panel B: primary cultures of neonatal rat cardiomyocytes (panel B.1; $n = 4$) and HL-1 cells (panel B.2; $n = 8$) following 24 h treatment with 10 μg/ml adiponectin, 60 min treatment with 100 nM insulin, or both treatments, relative to untreated controls. Statistical significance: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

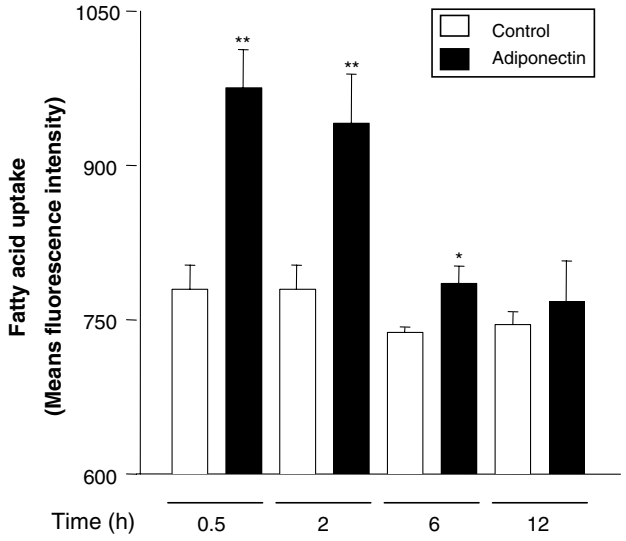

Fig. 4. Statistical analysis (of at least three independent experiments) of free fatty acid uptake by HL-1 cardiomyocytes after 10 μg/ml adiponectin treatment. *$P < 0.05$; **$P < 0.01$.

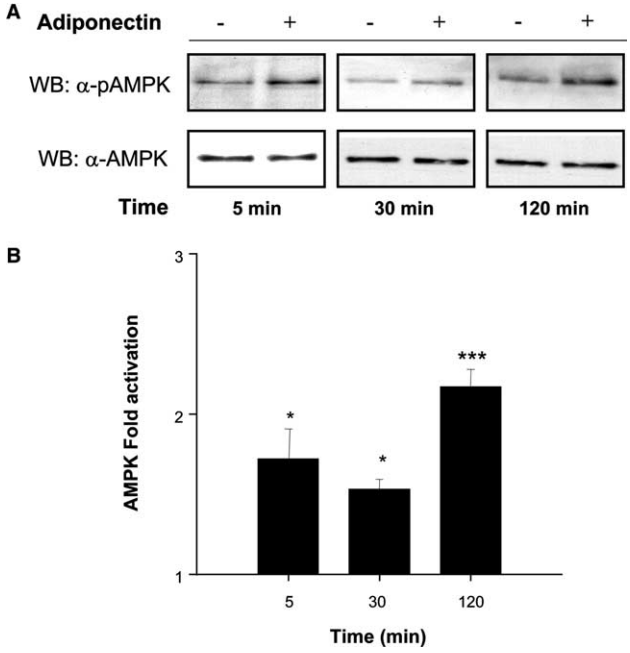

Fig. 5. Panel A: Western blots of HL-1 cell lysates following staining with anti-phospho-AMPK antibody (α-pAMPK) or anti-AMPK antibody (α-AMPK); times indicate the duration of adiponectin treatment. Panel B: Amount of phospho-AMPK, relative to controls, in four independently performed experiments (*$P < 0.05$; ***$P < 0.001$).

adiponectin-induced increase in glucose uptake by HL-1 cardiomyocytes was diminished in a 72.1 ± 5.3% ($P < 0.01$, $n = 3$) by the addition of the AMPK specific inhibitor araA 20 min before the treatment with adiponectin.

4. Discussion

Until recently it appeared to have been generally accepted that adiponectin was only synthesized by adipocytes [3]. However, recent studies have found that mouse liver cells can synthesize adiponectin under appropriate conditions [12]; that adiponectin gene expression can be induced in human myotubes by exposure to an adiponectin-containing HEK293 cell culture supernatant [11]; that adiponectin synthesis can be induced both in myotubes and in muscle in response to pro-inflammatory cytokines [13]; and that adiponectin is expressed in bone-forming cells [14]. Nevertheless, despite these discoveries and the intensive research on the cardiovascular effects of adiponectin, hitherto no studies have been published that have investigated the possibility that cardiac cells synthesize adiponectin.

Our results in this study show in the first place that adiponectin can be synthesized and secreted by cardiomyocytes of both human and murine origin. As far as we know, this is the first report of adiponectin synthesis and secretion by cardiomyocytes. It may be pointed out that although the adiponectin content of cardiomyocytes seems to be less than that of adipose tissue, this difference does not suffice to throw doubts on the efficacy of adiponectin secretion by cardiomyocytes, since cardiomyocyte-secreted adiponectin may well mediate paracrine and/or autocrine signalling pathways rather than long-range mechanisms.

We also found that, as expected, HL-1 cells (a cultured line derived from murine atrial cardiomyocytes that maintains a heart-specific phenotype and is accordingly used as an in vitro model in studies of cardiomyocyte biology [15]) and cultured human cardiomyocytes express genes for the adiponectin receptors Adipo-R1 and Adipo-R2. Interestingly, 1-day-old rat hearts contained Adipo-R1 mRNA but not Adipo-R2 mRNA, which only appeared at some time between the ages of 9 and 25 days. The late appearance of Adipo-R2 may perhaps be related to the sweeping postnatal changes in the intermediate metabolism of the heart, which switches from lactate and glucose to fatty acids as its main sources of energy [22]. Although additional work in this subject is necessary to elucidate the physiological relevance of this finding, this may be a physiological mechanism allowing adaptation of cardiac responsiveness to different physiological and pathological settings.

Although the processes initiated by the binding of adiponectin to its receptors on cardiomyocytes may be manifold, two at least were identified in this study, in which adiponectin significantly enhanced glucose and fatty acid uptake by cardiomyocytes. These findings are in keeping with recent reports of similar adiponectin effects on glucose uptake by C2C12 myocytes [23] and adipocytes themselves [18].

We also found that adiponectin treatment induced AMPK phosphorylation in HL-1 cardiomyocytes. It seems possible that, as in other cell types [23], the adiponectin-promoted enhancement of glucose uptake by cardiomyocytes may be mediated by activation of AMPK, especially since AMPK is known to mediate glucose uptake and GLUT4 translocation in heart muscle [24]. In fact, we have observed that, in HL-1 cardiomyocytes, the AMPK specific inhibitor araA blunted the adiponectin-induced increase on glucose uptake, as it does in adipocytes [18]. Recently, it has been reported that adiponectin inhibits hypertrophic signaling in the myocardium through activation of AMPK [25], further supporting the relevance of adiponectin signaling on cardiomyocyte function.

In conclusion, the main results of this study are that (a) adiponectin can be synthesized and secreted by human and

murine cardiomyocytes in culture, and (b) adiponectin can directly regulate cardiomyocyte metabolism through AMPK activation.

The observed expression and secretion of adiponectin by cardiomyocytes add more complexity to the largely unknown regulation of cardiomyocyte metabolism/function by adipokines. It may be hypothesized that both fat-derived and local cardiac-derived adiponectin could be essential for cardiomyocyte metabolism and functionality, and that the failure of one or both of these mechanisms might be determinant in the development of several cardiac pathologies.

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