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Journal of Cellular Biochemistry 116:754–766 (2015)

Journal of Cellular Biochemistry

Autocrine/Paracrine Function of Globular Adiponectin: Inhibition of Lipid Metabolism and Inflammatory Response in 3T3-L1 Adipocytes

Yulia Lazra,[1,2] Alona Falach,[1,2] Lital Frenkel,[1,2] Konstantin Rozenberg,[1] Sanford Sampson,[2,3] and Tovit Rosenzweig[1*]

[1]*Departments of Molecular Biology and Nutrition, Ariel University, Ariel 40700, Israel*
[2]*Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan, Israel*
[3]*Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot 76101, Israel*

ABSTRACT

Adiponectin is an adipose-derived hormone, with beneficial effects on insulin sensitivity and inflammation. The aim of this study was to clarify the autocrine/paracrine effects of globular adiponectin (gAd) administrated during differentiation on the function of the mature adipocytes. Experiments were performed on 3T3-L1 preadipocytes treated with gAd (10 nM), starting at an early stage of differentiation. gAd treatment during differentiation was without effect on mRNA expression of adiponectin and AdipoR2, but increased AdipoR1 expression. PPARgamma, perillipin and FABP4 mRNA expressions were lower in gAd-treated adipocytes, accompanied by a reduction in lipid accumulation. While mRNA expression of HSL was not affected by gAd compared to untreated adipocytes, both ATGL and FAS were reduced, indicating that gAd regulates both lipolysis and lipogenesis. PPARα, ACOX2 and UCPs mRNA expressions were not affected by gAd, indicating that the reduction in lipid content is not attributed to an increase in fatty-acid oxidation. In accord with the lower PPARγ expression, there was reduced Glut4 mRNA expression; however, insulin-induced PKB phosphorylation was enhanced by gAd, and glucose uptake was not altered. To investigate the effect of gAd on LPS-induced inflammatory response, cells were treated with gAd either during differentiation or 24 h before induction of inflammation in differentiated adipocytes. LPS-induced inflammatory response, as indicated by increase in IL6 and MCP-1 mRNA expression. gAd given through differentiation was much more effective in abrogating LPS-dependent cytokines production than gAd given to the mature adipocyte. In conclusion, elevated gAd at differentiation of 3T3-L1 cells leads to reduced lipid content, reduced lipid metabolism and high resistance to inflammation. J. Cell. Biochem. 116: 754–766, 2015.

KEY WORDS: ADIPOCYTE; DIFFERENTIATION; GLOBULAR ADIPONECTIN; INFLAMMATION

The role of adipose tissue in regulating diverse physiological functions in health and disease has been investigated in depth in the last two decades. By secreting wide range of adipokines, adipose tissue affects a wide range of functions, including whole body energy homeostasis, insulin sensitivity, inflammatory state, blood pressure, hemostasis, fertility, bone mass, to name a few [Bluher, 2012]. Among the enormous battery of adipokines, adiponectin is uniquely known as an important and beneficial factor, which is positively correlated with insulin sensitivity, and negatively correlated with the risk to develop metabolic syndrome and systemic inflammation [Berg et al., 2002; Turer and Scherer, 2012].

Adiponectin is a 30 kDa protein, constituting approximately 0.01–0.05% of plasma proteins. Paradoxically, although adiponectin is an adipocyte-derived hormone, its plasma concentrations are inversely correlated to adipose mass [Arita et al., 1999]. Several additional factors affect adiponectin secretion, including the location of adipose depots, gender, ethnicity, and age [Ryan et al., 2003; Pereira et al., 2011]. The beneficial metabolic activities of adiponectin are well recognized, based on studies showing its positive effect on various tissues involved in metabolic regulation [Berg et al., 2002; Turer et al., 2012]. In pancreatic β-cells, adiponectin increases both glucose-induced insulin secretion, and the viability and survival of the cells

Conflicts of Interest: The authors have no conflict of interest to declare.
*Corresponding to: Tovit Rosenzweig, Departments of Molecular Biology and Nutrition, Ariel University, Ariel, 40700, Israel. E-mail: tovitro@ariel.ac.il
Manuscript Received: 2 December 2014; Manuscript Accepted: 4 December 2014
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 10 December 2014
DOI 10.1002/jcb.25031 • © 2014 Wiley Periodicals, Inc.

[Chetboun et al., 2012]. In skeletal muscle and the liver, adiponectin improves insulin sensitivity, as reflected by increased blood glucose disposal and reduced glucose hepatic production [Berg et al., 2001; Yamauchi et al., 2002; Miller et al., 2011]. Adiponectin also exerts a cardioprotective function, by improving plasma lipid profile, increasing the resistance of cardiomyocytes to ischemia/reperfusion, and improving endothelial function [Yamauchi et al., 2003b; Shibata et al., 2005; Yamauchi and Kadowaki, 2008]. Unquestionably, the main favorable activity of adiponectin is related to its anti-inflammatory effects. Adiponectin reduces inflammatory responses both locally in adipose tissue, and systemically. Related to this, macrophages seem to be the main target for adiponectin function. Macrophage inflammatory activity is reduced under the influence of adiponectin, as manifested by reduced phagocytic activity and a shift in the profile of secreted cytokines from pro-inflammatory (IL6, TNFα, and MCP) toward anti-inflammatory (IL-10). Circulating adiponectin exists in several forms; full-length adiponectin, which is multimerized to the generation of trimer, hexamer and high molecular weight adiponectin, and the 19 kDa cleaved form of the protein, globular adiponectin. While some of the biological activities of the various forms are similar, there seem to be some distinct functions [Kadowaki and Yamauchi, 2005].

Another interesting target tissue of adiponectin is the adipose tissue itself. The two forms of adiponectin receptors, AdipoR1 and AdipoR2 are expressed in adipocytes [Rasmussen et al., 2006], indicating the existence of some local autocrine/paracrine effects of adiponectin. Several studies have been conducted in order to clarify the local effect of adiponectin on mature adipocytes, investigating its effect on glucose uptake [Wu et al., 2003], lipolysis [Wedellova et al., 2013] and inflammatory response. In relation to its anti-inflammatory response, administration of adiponectin to fully differentiated 3T3-L1 adipocytes, reduces LPS-induced IL6 and TNFα secretion via inhibition of the NFkappaB signaling pathway [Zoico et al., 2009]. Adiponectin-overexpressing mice showed improved metabolism, including reduction in blood glucose, higher insulin sensitivity and enhanced lipid flux [Shetty et al., 2012]. Several tissues are involved in lipid turnover, mainly white adipose tissue, brown adipose tissue and the liver. Investigating the effect of adiponectin on the isolated adipocyte is important in order to clarify the specific effect of the hormone on these cells.

During adipocyte differentiation there is an activation of several key transcription factors responsible for the coordinated induction and silencing of vast amount of genes regulating both morphology and physiology of the mature adipocyte [Cristancho and Lazar, 2011]. The environment of the differentiating cells has a major effect on the process of adipogenesis, with stable and long term consequences on the adult, as was demonstrated for the effect of endocrine disruption chemicals on the function of the mature adipose tissue [Janesick and Blumberg, 2011]. Despite the important characteristics adipocytes acquire through their differentiation, the effect of adiponectin on the differentiation of adipocytes has not yet been elucidated. In this study, we investigated the notion that globular adiponectin might program differentiating adipocytes to a more favorable and healthy mode. To clarify this, we investigated the effect of gAd given at an early stage of differentiation on the dynamic expression of several key genes in adipocyte function, and on the function of the fully differentiated adipocytes in relation to lipid metabolism, insulin sensitivity and inflammatory response.

MATERIALS AND METHODS

CHEMICALS, KITS AND REAGENTS

Mouse globular adiponectin (gAd) was purchased from Peprotech (Israel). Isobutylmethylxanthine (IBMX), dexamethasone, insulin, lipopolysaccharide (LPS), 2-deoxy-d-glucose (2-DG) and inhibitors of proteases and phosphatases were purchased from Sigma. Glycerol assay was obtained from Cayman (Ann Arbor). [^{3}H]2-DG (1 mCi) was purchased from Perkin-Elmer, Metformin from CalBiochem and XTT cell proliferation kit was obtained from Biological Industries (Beit Haemek, Israel). BSA, reagents and media for cell cultures were also obtained from Biological Industries (Beit Haemek, Israel). Anti-phospho PKB (S473), PPARγ and AMPK (T172) were purchased from Cell-signaling Technology, anti-actin was purchased from MP, and secondary antibodies were purchased from Jackson ImmunoResearch Laboratories.

CELL CULTURE

3T3-L1 pre-adipocytes were cultured and differentiated as described by Frost and Lane [1985]. Briefly, cells were grown to confluence in DMEM containing 10% calf serum (CS), 2 mM glutamine and 1% ampicillin. Two days after full confluence (day 0), the cells were induced to differentiate by 3 days incubation in DMEM containing 10% FBS, 0.5 mM IBMX, 1 μM dexamethasone and 100 nM insulin. This was followed by 2 days of incubation in DMEM containing 10% FBS and 100 nM insulin. The cells were grown for an additional 5–7 days in DMEM containing 10% FBS. 3T3-L1 adipocytes were used for the experiment 10–14 days after the initiation of differentiation, when 80–90% of cells exhibited adipocyte morphology.

gAd ADMINISTRATION PROTOCOLS

In order to study the effect of gAd given continually from early stage of differentiation, globular adiponectin (10 nM) was administered at day 0. Fresh gAd was added whenever culture media was replaced (long term, LT). For short term treatment (ST) gAd was given to fully differentiated adipocytes for 24 h.

INDUCTION OF INFLAMMATORY RESPONSE

Differentiated adipocytes were transferred to a starvation medium (serum free, 0.1% fatty acid–free BSA) for 24 h. Inflammatory response was induced by LPS (1 ng/ml) given for 6 h.

ANALYSIS OF mRNA EXPRESSION BY PCR REACTION

Total RNA was extracted from cells using TRI reagent (Molecular Research Center, Inc. Cincinnati, OH), according to manufacturers' instruction. 2.5 ng of total RNA were reverse transcribed by oligo dT priming (Stratascript 5.0 multi-temperature reverse transcriptase, Stratagene) according to the manufacturers' instructions. Real-time PCR amplification reactions were performed using SYBRGreen Master mix (ROVALAB), by the MxPro QPCR instrument (Stratagene). Primers for real time PCR reactions were synthesized by Integrated DNA Technologies (IDT, Israel).

Primer sequences were as follows:

ACOX2: forward 5′- aagtggccaggtttctgatg-3′, reverse 5′- tcttggtgtggcgagataca-3′.
Adiponectin: forward 5′- gacaccaaaagggctcaggat-3′, reverse 5′- aagaggaacaggagagcttgca-3′.
AdipoR1: forward 5′- tcgtgtataaggtctgggag-3′, reverse 5′- gtgaaatagcacaaaaccaa-3′.
AdipoR2: forward 5′- cacaaccttgcttca-3′, reverse 5′- atact-gaggggtggc-3′.
ATGL/PNPLA2: forward 5′- ggaaccaaaggacctgatga-3′, reverse 5′- gctctttcatccaccggata-3′.
FABP4: forward 5′- agcctttctcacctggaaga-3′, reverse 5′- aagcc-cactcccacttcttt-3′.
FASn: forward 5′- ttgctggcactacagaatgc-3′, reverse 5′- aacagcct-cagagcgacaat-3′.
Glut4: forward 5′- accctgggctctgtatccc-3′, reverse 5′- ggttcgtcatt-gagcgaggag-3′.
HSL: forward 5′- tgctcttcttcgagggtgat-3′, reverse 5′- tctcgttgcgtttgtagtgc-3′.
IL1β: forward 5′- gcccatcctctgtgactcat-3′, reverse 5′- aggcca-caggtattttgtcg-3′.
IL6: forward: 5′- cctaccccaatttcc-3′, reverse 5′- ttggtccttacc-cactccttc-3′.
IL10: forward 5′- tgcactaccaaagccacaag-3′, reverse 5′- tgggaagtgggtgcagttat-3′.
MCP-1: forward 5′- cactcacctgctgctactcatcatt-3′, reverse 5′- tctggacccattccttcttg-3′.
PPARα: forward 5′- atgccagtactgccgttttc-3′, reverse 5′- ccgaatctttcaggtcgtgt-3′.
PPARgamma: forward 5′- gccagtttcgatccgtagaa-3′, reverse 5′- aatccttggccctctgagat-3′.
Perilipin: forward 5′- ccagttcacagctgccaatg-3′, reverse 5′- ttcgaaggcgggtagagatg-3′.
TNFα: forward: 5′- ccctcacactcagatcatcttct-3′, reverse 5′- gctac-gacgtgggctacag-3′.
UCP1: forward 5′- gccttcagatccaaggtgaa-3′, reverse 5′- taagccggctgagatcttgt-3′.
UCP2: forward: gcgttctgggtaccatccta-3′, reverse 5′- gctctgagcccttggtgtag-3′.
UCP3: forward 5′- gtctgcctcatcagggtgtt-3′, reverse 5′- cctggtccttaccatgcagt-3′.
Mouse HPRT was used as housekeeping gene: forward 5′- gttgttggatatgcccttg-3′, reverse 5′- aaagcctaagatgagcgca-3′.

OIL RED STAINING

Differentiated 3T3-L1 cells were fixed in 20% PFA for 20 min. After washing thoroughly with distilled water, cells were incubated with a working solution of Oil Red O (36% triethyl phosphate) for 30 min, and washed with water. To quantify the staining, the dye was extracted using isopropanol, and absorbance was measured using Tecan Infinite F200 microplate reader (Tecan, Salzburg, Austria), wavelength of 510 nm.

TRIGLYCERIDE (TG) DETERMINATION

Cells were lysed using RIPA buffer containing anti-proteases and anti-phosphatases. The samples were homogenized and centrifuged at 14,000 rpm for 20 min. The supernatant was collected and protein concentration was measured using the Bradford method. TG contents of 3T3-L1 adipocytes was measured immediately using Triglycerides liquid reagent set (Pointe Scientific), and results were normalized according to protein concentration of each sample.

GLYCEROL RELEASE ASSAY

3T3-L1 cells were treated with gAd during differentiation as described (gAd Administration Protocols Section). Differentiated adipocytes were preincubated in DMEM containing 2% fatty acid-free bovine serum albumin, in the absence of FCS for 12 h. Glycerol level in the medium was measured immediately using Glycerol release assay kit (Cayman Chemical).

WESTERN IMMUNOBLOT ANALYSIS

Differentiated 3T3-L1 cells were treated with gAd according to the appropriate protocol as described. Protein lysates were prepared using RIPA buffer supplemented with protease and phosphatase inhibitors. The samples were homogenized and centrifuged at 14,000 rpm for 20 min. The supernatant was collected and protein concentration was measured using the Bradford method. 20 μg protein per lane was separated by SDS-polyacrylamide gel electrophoresis. Proteins were electrophoretically transferred onto nitrocellulose membranes. The membranes were blocked in 10% dry milk, incubated with the appropriate antibodies and proteins immunodetected using the enhanced chemiluminescence method.

CELL VIABILITY

Viability of preadipocytes and differentiating adipocytes was assessed either by direct measurement of cell number, using trypan blue and automated cell counter (Luna, Logos Biosystems) or by using XTT assay, which is a cell proliferation kit, based on the ability of viable cells to reduce tetrazolium salt (XTT) into colored compounds of formazans. The dye intensity is measured according to manufacturer's instruction, by an ELISA reader (Tecan, Salzburg, Austria). The effect of gAd on viability was measured in preadipocytes and on differentiating adipocytes. In order to measure preadipocyte viability, cells were subcultured on a96-well plate at a concentration of 5×10^4 cells/ml in growth medium containing gAd. The medium was discarded and replaced daily with medium containing fresh gAd. Viability was measured at day 0 (8 h following seeding), and 24 and 48 h later. In order to measure the effect of gAd on the viability of differentiating adipocytes, cells were cultured as described in Cell Culture Section, gAd was administrated as described in gAd Administration Protocols Section starting at day 0 and viability was measured at days 3, 5, 7, 10, and 12.

GLUCOSE UPTAKE

Differentiated adipocytes, pretreated with gAd beginning at day 0 of differentiation (LT) or 24 h before measuring glucose uptake (ST) were preincubated for 2 h in serum-free DMEM. Starvation media was replaced with PBS with or without the presence of 100 nM insulin for 30 min. cells were washed twice with warm (37°C) PBS, the final wash being replaced immediately with PBS containing 0.1 mM 2DG and 0.5 μCi [^{3}H]-2DG. Non-specific glucose uptake was measured by the addition of 20 μM of cytochalasin B. Cells were then incubated for 10 min in 37°C, washed three times with cold PBS, and lysed with 1% SDS. The contents of each well were transferred to a

different plate containing scintillation liquid (Optiphase, Perkin-Elmer), and counted using microbeta (Perkin-Elmer).

STATISTICAL ANALYSIS

Values are presented as means ± SEM. Statistical differences between the treatments and controls were tested by unpaired two-tailed Student's *t*-test or one-way analysis of variance (ANOVA), followed by Bonferroni's posthoc testing, when appropriate. Analysis was performed using the GraphPad Prism 5.0 software. A difference of $P<0.05$ or less in the mean values was considered statistically significant.

RESULTS

EFFECT OF gAd ON THE EXPRESSION OF ADIPONECTIN AND ITS RECEPTORS

In this study we attempted to clarify the possible autocrine/paracrine effect of gAd given continuously during differentiation on 3T3-L1 adipocyte differentiation and function. We first investigated whether treatment with gAd might affect the endogenic production of adiponectin by the cells. The effect of gAd on mRNA expression of adiponectin receptors was followed as well. As can be seen in Figure 1A, treatment with gAd did not affect mRNA production of adiponectin by the cells, suggesting that there is no downregulation of adiponectin production. There are two different receptors for adiponectin, AdipoR1 and AdipoR2. The expression of AdipoR1 is higher than AdipoR2 in both preadipocytes and differentiated adipocytes (Fig. 1B). Figure 1C shows the expression of AdipoR1 as a function of age. During differentiation, mRNA level of AdipoR1 is only temporarily increased at day 6; thus the expression of AdipoR1 mRNA in fully-differentiated 3T3-L1 is similar to the expression of this receptor in 3T3-L1 preadipocytes (−2 day). In gAd-treated cells, the increased expression on the 5th day was not detected; however, gAd induced an increase in mRNA expression of this receptor in fully-differentiated adipocytes. AdipoR2 mRNA expression is increased in differentiated 3T3-L1 adipocyte, and was not affected by administration of gAd (Fig. 1D).

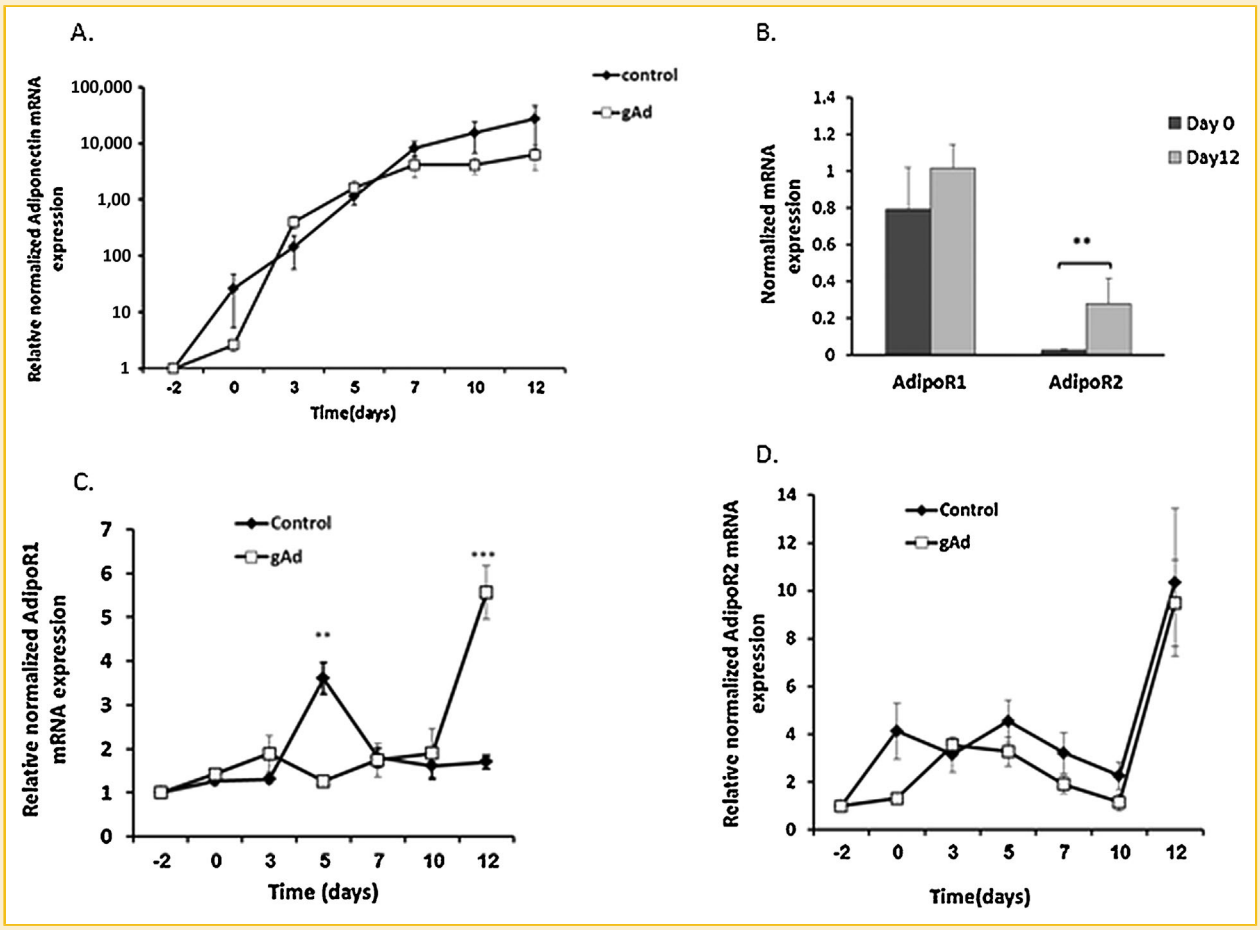

Fig. 1. Effect of gAd administration on mRNA expression of adiponectin and its receptors. gAd (10 nM) was administrated to differentiating preadipocytes as described in Materials and methods section. mRNA of adiponectin (A), AdipoR1 (C) and adipoR2 (D) was measured by real-time PCR. Results were normalized to the expression of housekeeping gene, HPRT, and are presented as fold change compared to the expression in preadipocytes. Results are mean ± SEM of three independent experiments. *$P<0.05$, **$P<0.005$, ***$P<0.0005$ compared to untreated cells at the matched-day of differentiation, by Student's *t*-test. B: Relative expression of AdipoR1 and adipoR2 in untreated preadipocytes and adipocytes. Results are mean ± SEM of three independent experiments. **$P<0.005$ by Student's *t*-test.

LIPID ACCUMULATION IS DECREASED BY gAd

The main physiological role of adipocytes is to store excess energy in the form of lipids, mainly Triglycerides (TG). TGs are stored in lipid droplets, surrounded by perilipin which separates the TG from the cytoplasmic soluble lipolytic enzymes. Although the adipocyte is capable of enlarging its volume with increased lipid storage, some functions of the adipocyte are disturbed by excess hypertrophy, as detected by altered profile of secreted cytokines and adipokines, macrophage infiltration toward the hypertrophic adipose tissue etc.

We next investigated the effect of gAd on lipid accumulation in adipocytes. Figure 2A shows that although lipid content was markedly increased in gAd-treated and untreated cells, gAd caused a 20% reduction in lipid accumulation in cells treated throughout the differentiation process, as determined by ORO staining. This result was confirmed by specifically measuring intracellular TG (Fig. 2B), showing that gAd-treated cells contained 1.41 mg TG/mg protein, compared to 1.71 mg TG/mg protein in untreated adipocytes. In accord with these findings, gAd-treated differentiated cells displayed a lower expression of perilipin mRNA than untreated adipocytes, as illustrated in Figure 2C. The reduction in lipid accumulation is in accordance with lower PPARγ mRNA and protein expression in gAd-treated adipocytes compared to untreated differentiated cells (Fig. 3A and B). As PPARγ is a key regulator of adipocyte differentiation, expressed at early stages of differentiation [Cristancho and Lazar, 2011], we followed the effect of gAd on PPARγ expression through the differentiation process (Fig. 3C). It can be seen that although gAd reduced PPARγ expression at the end of differentiation compared to untreated adipocytes, the adipokine increased the expression of this transcription factor at day 8 of differentiation.

Reduced lipid accumulation in adipocytes may result from different mechanisms such as reduced lipid synthesis, increased lipolysis or increased fatty acid oxidation and energy expenditure. In order to distinguish among the different pathways, we measured the expression of several genes involved in regulating the metabolic pathways in adipocytes (Fig. 4A). mRNA expression of hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL), enzymes known to play a key role in lipolysis, was measured. While mRNA expression of HSL was not affected by gAd, the expression of ATGL was reduced in cells treated by the hormone, compared to untreated differentiated adipocytes. Glycerol was measured in medium of differentiated adipocytes, and found to be lower in gAd treated cells, indicating reduced rate of lipolysis (Fig. 4B), in accordance with mRNA level of ATGL. In addition, as is shown in Figure 4A, mRNA expression of FAS, an enzyme catalyzing the elongation of malonyl-coA to fatty acids during lipogenesis, was also reduced in gAd treated cells compared to its expression level in preadipocytes, suggesting a reduction in the overall turnover of lipids in the adipocyte. Expression levels of FABP4 (fatty acid binding protein 4, also known as ap2) mRNA were measured as well, FABP4 is a fatty acid binding protein expressed in adipose tissue, having a role in fatty-acid import, storage, and export. Expression of FABP proteins seems to be proportional to the rates of fatty-acid metabolism in most cells [Furuhashi and Hotamisligil, 2008]. mRNA expression of FABP4 was increased during adipocytes differentiation, but its expression level was lower in gAd-treated adipocytes, compared to untreated adipocytes, in accord with the reduced lipid content in gAd-treated cells (Fig. 2A and B), reduced expression of PPARγ (Fig. 3) and

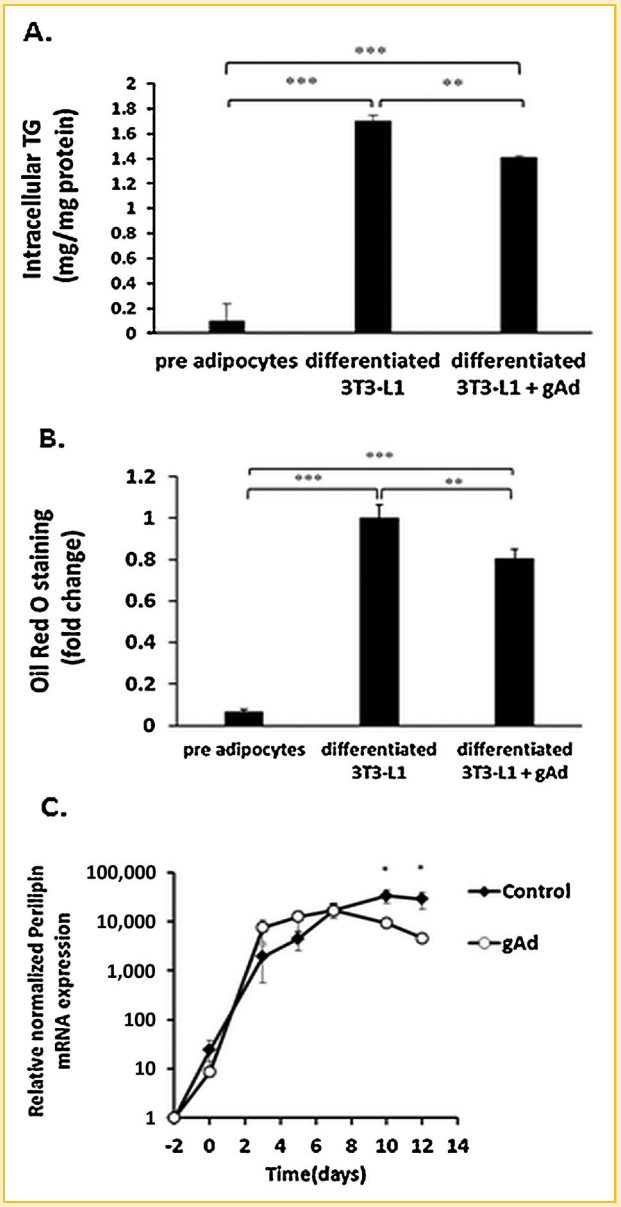

Fig. 2. gAd reduces lipid accumulation in differentiated 3T3-L1 adipocytes. gAd (10 nM) was administrated to differentiating preadipocytes as described in Materials and methods section. A: Preadipocytes and gAd-treated and untreated differentiated adipocytes were stained with ORO, stains were extracted by isopropanol and optical density was measured. Relative results are presented. B: TG content was measured in extract of adipocytes using Triglycerides liquid reagent set. Results were normalized to protein concentration. $^{**}P<0.005$, $^{***}P<0.0005$ by one way ANOVA followed by Bonferroni's multiple comparison posthoc test. Dynamic mRNA expression of perilipin (C) was measured by real-time PCR. Results were normalized to the expression of housekeeping gene, HPRT, and are presented as fold change compared to the expression in preadipocytes. Results are mean ± SEM of three independent experiments. $^{*}P<0.05$ compared to untreated cells at the matched-day of differentiation, by Student's *t*-test.

lower expression of genes involved in lipid metabolism; ATGL and FAS. Expression of PPARα, a nuclear receptor playing a role in fatty acid oxidation [Vega et al., 2000], was found to be very low in both preadipocytes and differentiated cells, with no difference between untreated and gAd-treated differentiated adipocytes. mRNA expression of ACOX2 (Acyl Co-A Oxidase 2), a gene catalyzing the key step in peroxisomal β-oxidation was increased during differentiation, but not affected by gAd. AMPK is known to be phosphorylated and activated by adiponectin and is recognized as a major player in the transduction of adiponectin signaling in several cells [Yamauchi et al., 2002; Huypens et al., 2005], mediating the effect of adiponectin on lipid metabolism. As can be shown in Figure 4C, AMPK was phosphorylated by metformin, used as positive control, but was not affected by gAd.

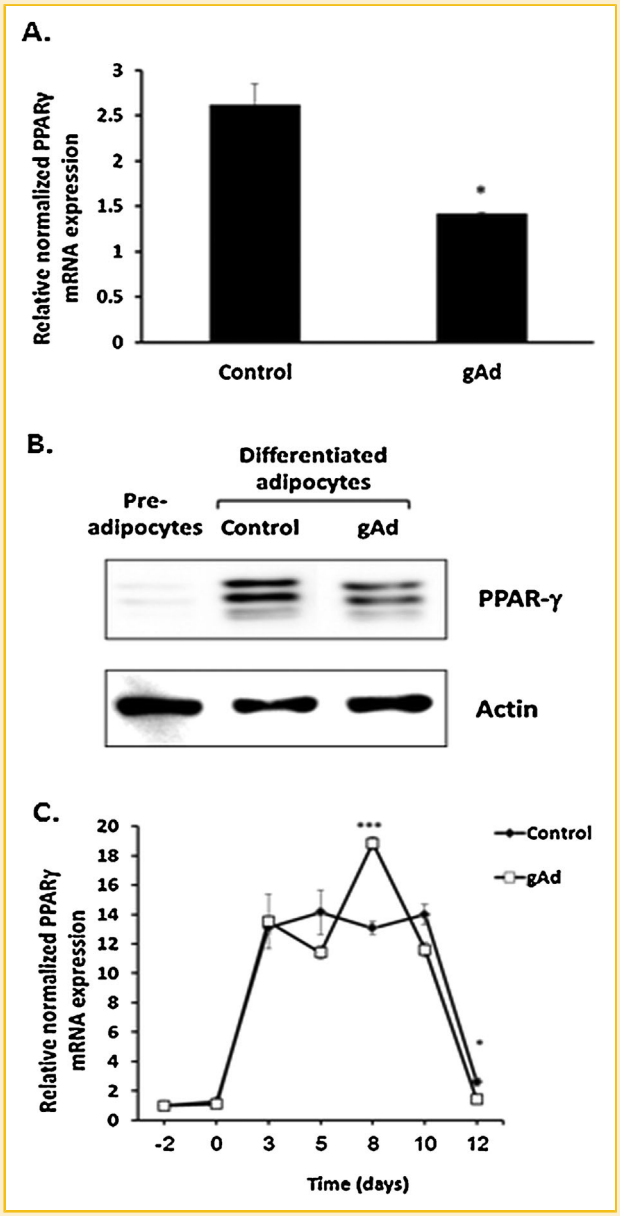

Fig. 3. gAd reduces PPARγ expression in differentiated 3T3-L1 adipocytes. gAd (10 nM) was administrated to differentiating preadipocytes as described in Materials and methods section. A: mRNA expression of PPARγ in differentiated 3T3-L1 adipocytes with and without the administration of gAd. Results were normalized to the expression of housekeeping gene, HPRT, and are presented as fold change compared to the expression in preadipocytes. B: Western blot analysis was performed on cell lysates using anti PPARγ or anti actin, this is a representative blot of three independent experiments. C: Dynamic mRNA expression of PPARγ during differentiation by real-time PCR. Results were normalized to the expression of housekeeping gene, HPRT, and are presented as fold change compared to the expression in preadipocytes. *$P<0.05$, ***$P<0.0005$ compared to untreated cells at the matched-day of differentiation, by Student's *t*-test.

Uncoupling proteins (UCPs) are located at the inner membrane of the mitochondrion, enabling proton flux toward the mitochondrial matrix, leading to reduced proton gradient and reduced efficiency of ATP production by ATP synthase. While brown adipose tissue was the first tissue described to express UCPs, mainly UCP1, other members of the UCP family were also found to be expressed in other tissues. In order to clarify whether gAd reduced lipid content in adipocytes via enhancing energy expenditure mechanisms, we measured the expression of UCPs in these cells. Both preadipocytes and differentiated adipocytes express negligible levels of UCP1 and UCP3 (data not shown). Expression of UCP2 is temporarily increased through the differentiation process (3rd day). However, no difference in expression of UCP2 could be detected between gAd-treated and untreated differentiated adipocytes (Fig. 5A), suggesting that inefficient mitochondrial energy production is not the main mechanism responsible for reduced lipid content in gAd-treated adipocytes. Measuring the reduction of tetrazolium salts (XTT) supports the conclusion that metabolic activity of adipocytes is not affected by gAd. XTT is reduced to a colorimetric formazan product in the presence of metabolically active cells. The formation of XTT-formazan is in proportion to both the quantity and activity of cells [Sieuwerts et al., 1995]. Confluent, differentiating 3T3-L1 cells, are unable to proliferate, thus, measuring XTT in differentiated adipocytes represents their metabolic activity. gAd did not affect metabolic activity of adipocytes, as is shown in Figure 5B. This is in difference from its effect on pre-adipocytes, showing that gAd increased the production of XTT-formazan in pre-adipocytes by 115% and 125% (24 and 48 h treatment, respectively) compared to control (Fig. 5C), but did not affect cell number, as measured by an automated cell counter (data not shown) indicating that the increase in XTT is attributed to increased metabolic rate in pre-adipocytes.

EFFECT OF gAd ON INSULIN SENSITIVITY

Adipose tissue is considered to be one of the main target tissues for regulation of blood glucose by insulin. Adipocytes participate in glucose disposal following glucose challenge, by increasing glucose uptake via glucose transporter-4 (Glut4). Adiponectin is known to improve insulin sensitivity. The possibility that adiponectin improves adipocyte response to insulin by an autocrine/paracrine mechanism was investigated by measuring Glut4 mRNA expression (Fig. 6A), glucose uptake (6B) and PKB phosphorylation (6C and D). gAd reduced mRNA expression of Glut4. Neither short (ST, 24 h) nor long (LT, during differentiation) exposure to gAd was accompanied by reduced basal or insulin-dependent glucose uptake. Similarly, gAd did not affect insulin-induced glucose uptake when using lower concentrations of insulin (1 and 10 nM, data not shown).

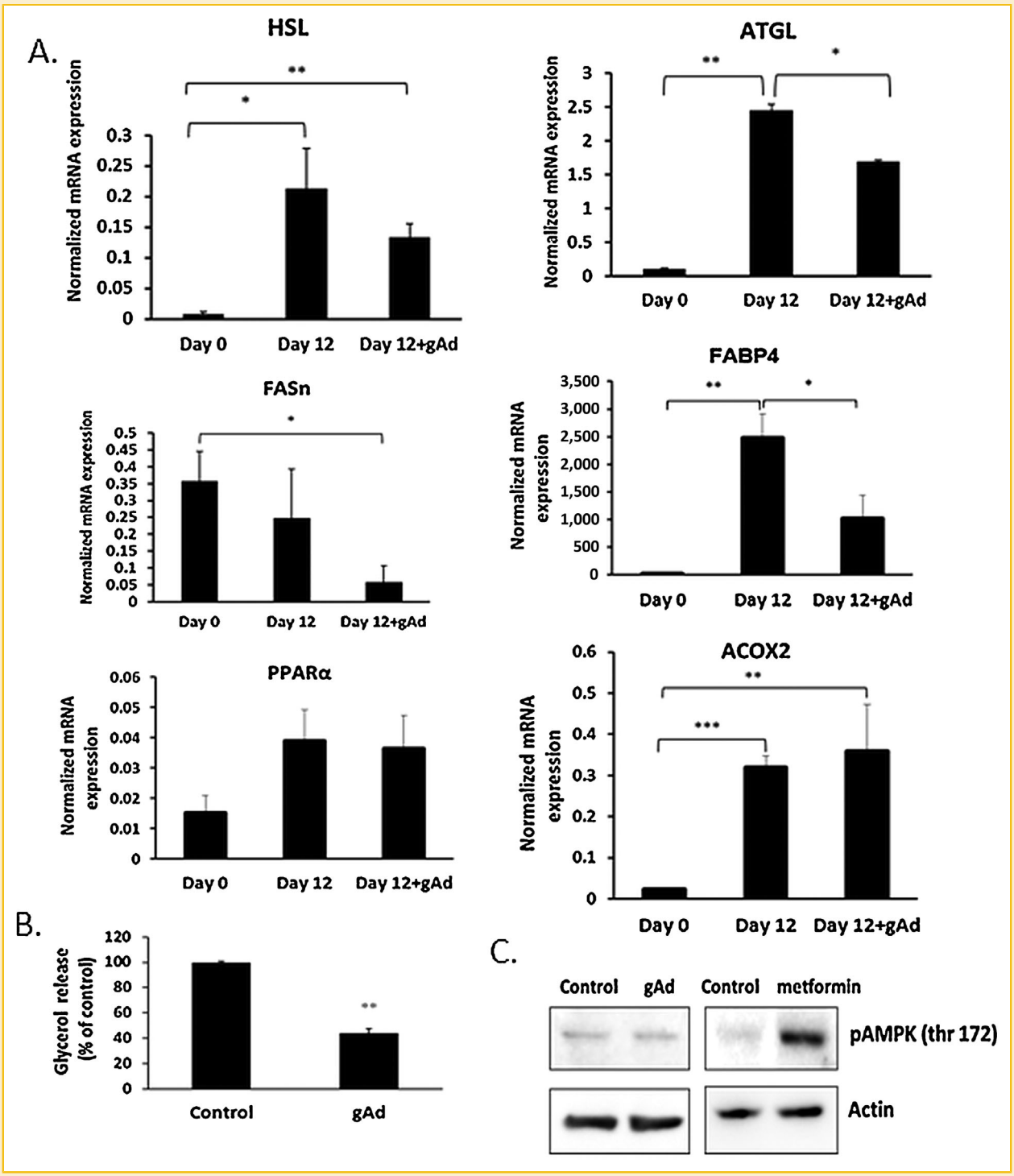

Fig. 4. gAd inhibits lipid turnover in 3T3-L1 adipocytes independently of AMPK phosphorylation. gAd (10 nM) was administrated to differentiating preadipocytes as described in Materials and methods. A: mRNA expression of HSL, ATGL, FAS, FABP4, PPARα, and ACOX2 was measured by real-time PCR. Results were normalized to the expression of housekeeping gene, HPRT. *$P<0.05$, **$P<0.005$, ***$P<0.0005$ by Student's *t*-test in HSL, FASn and ACOX2. One way ANOVA followed by Bonferroni's multiple comparison posthoc test was conducted on ATGL and FABP4 results. B: glycerol was measured in media of gAd-treated and untreated adipocytes as described in Materials and methods section. **$P<0.005$, by Student's *t*-test. C: Metformin (5 mM) was used as positive control for AMPK phosphorylation, given to differentiated adipocytes, 6 h before protein extraction. Western blot analysis was performed on cell lysates using anti AMPK (T172) or anti actin, this is a representative blot of three independent experiments.

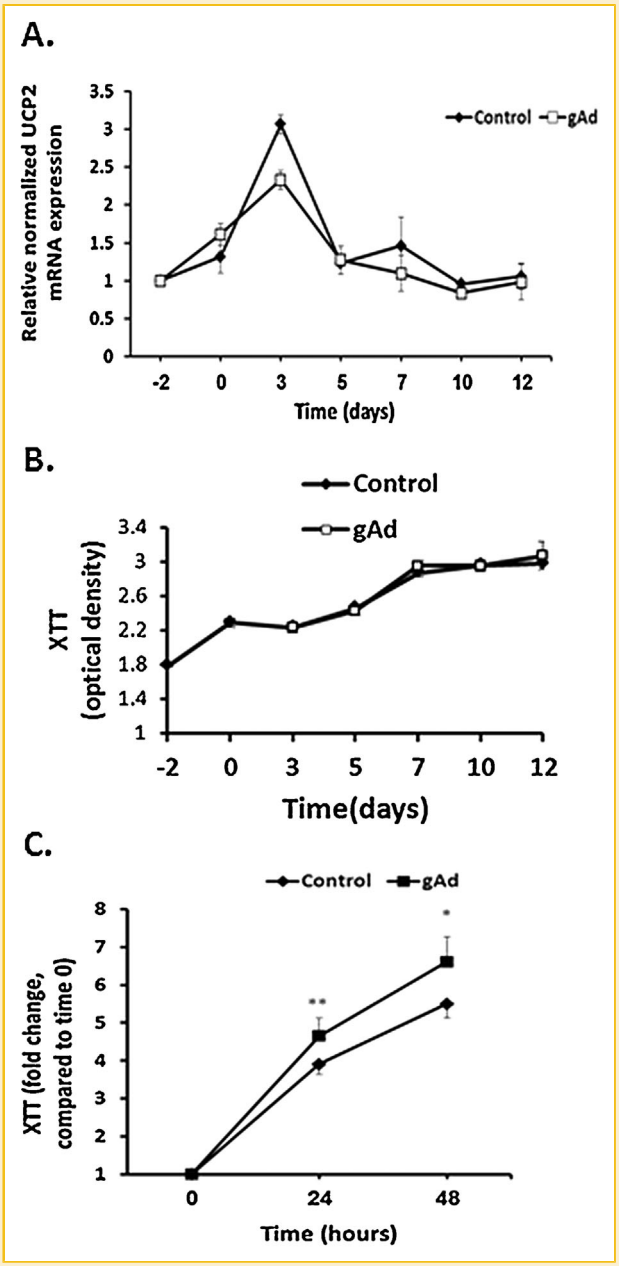

Fig. 5. Metabolic rate is not affected by gAd 3T3-L1 adipocytes. A and B. gAd (10 nM) was administrated to differentiating preadipocytes as described in Materials and methods section. A: mRNA expression of UCP2 was measured by real-time PCR. Results were normalized to the expression of housekeeping gene, HPRT, and are presented as fold change compared to the expression in preadipocytes. B: XTT was measured during differentiation in gAd-treated and untreated adipocytes. C: Preadipocytes were subcultured on a 96-well plate at a concentration of 5×104 cells/ml in growth medium with or without the addition of gAd (10 nM). Viability was measured 24 and 48 h later using XTT method. Results are mean $\pm$ SEM of three independent experiments. *$P<0.05$, **$P<0.005$ compared to untreated cells by Student's t-test.

Protein kinase B (PKB) is a key enzyme required for the transmission of insulin signaling, and phosphorylation of PKB on ser473 is necessary for its activation by insulin. The effect of gAd on PKB phosphorylation is shown in Figure 6C and D. It can be seen that while short term treatment with adiponectin only slightly increased insulin-induced PKB phosphorylation, chronic treatment with the adipokine had a significant insulin sensitizing effect.

EFFECT OF gAd ON LPS-INDUCED INFLAMMATORY RESPONSE

Obesity is accompanied by the presence of sub-clinical, low-grade inflammation, which is abetted by the hypertrophic, inflamed adipose tissue. The inflammatory state, characterized by elevated pro-inflammatory cytokines, is a common denominator of some of the complications related to obesity, such as atherosclerosis and insulin resistance. Adipocytes differentiated in the presence of gAd expressed higher levels of IL6 mRNA than untreated adipocytes (1.37 and 6.58 fold increase relative to undifferentiated cells in control and gAd treated cells, respectively, Fig. 7A), however this induction of IL6 expression is much lower than that obtained by LPS, as shown in Figure 7B. Stimulating cells by LPS models the inflammatory stimuli. The effect of gAd on LPS-induced inflammatory response was investigated, by measuring the mRNA expression of the pro-inflammatory cytokines IL6, IL1β, TNFα and MCP-1, and IL10, an anti-inflammatory cytokine. As can be seen in Figure 7B, LPS stimulated the expression of IL6 by 82 fold compared to untreated adipocytes. gAd given through the differentiation process almost completely protected cells from the inflammatory stimuli, leading to a reduction of 94% in IL6 expression compared to LPS-treated cells. Differentiated adipocytes treated with gAd for 24 h before the administration of LPS, showed lower resistance to LPS (50% compared to the effect of LPS on untreated cells). Similar results were found related to MCP-1 expression (Fig. 7C). IL1β, TNFα, and IL10 mRNA were barely detected in differentiated 3T3-L1 either with or without LPS stimulation (not shown).

DISCUSSION

The beneficial properties of adiponectin on the metabolic state are well established, and are associated with increased insulin sensitivity, reduced risk to atherosclerosis and inflammation [Berg et al., 2002]. These effects are mediated via its actions on several target tissues, including the most extensively studied skeletal muscle [Tomas et al., 2002; Yamauchi et al., 2002], liver [Berg et al., 2001; Miller et al., 2011] and pancreatic β-cells [Huypens et al., 2005; Wijesekara et al., 2010; Chetboun et al., 2012] as well as renal podocyte [Sharma et al., 2008] and cardio-myocytes [Shibata et al., 2005]. In addition, both AdipoR1 and AdipoR2 are expressed by adipocytes, indicating the existence of paracrine/autocrine pathways. However the effect of adiponectin on adipocyte function has not been fully clarified.

In this study, globular adiponectin was supplemented to the media of cells at early stage of differentiation. Although the dominant isoform in serum is the full length adiponectin, the globular form is highly effective in improving hyperglycemia and hyperinsulinemia [Yamauchi et al., 2003a]. Some differences in the biological activities of the isoforms had been reported, such as higher activity of the full length adiponectin in hepatocytes, while the globular form found to be more effective in skeletal muscle [Yamauchi et al., 2002]. However, the metabolic effect of the globular and full-length

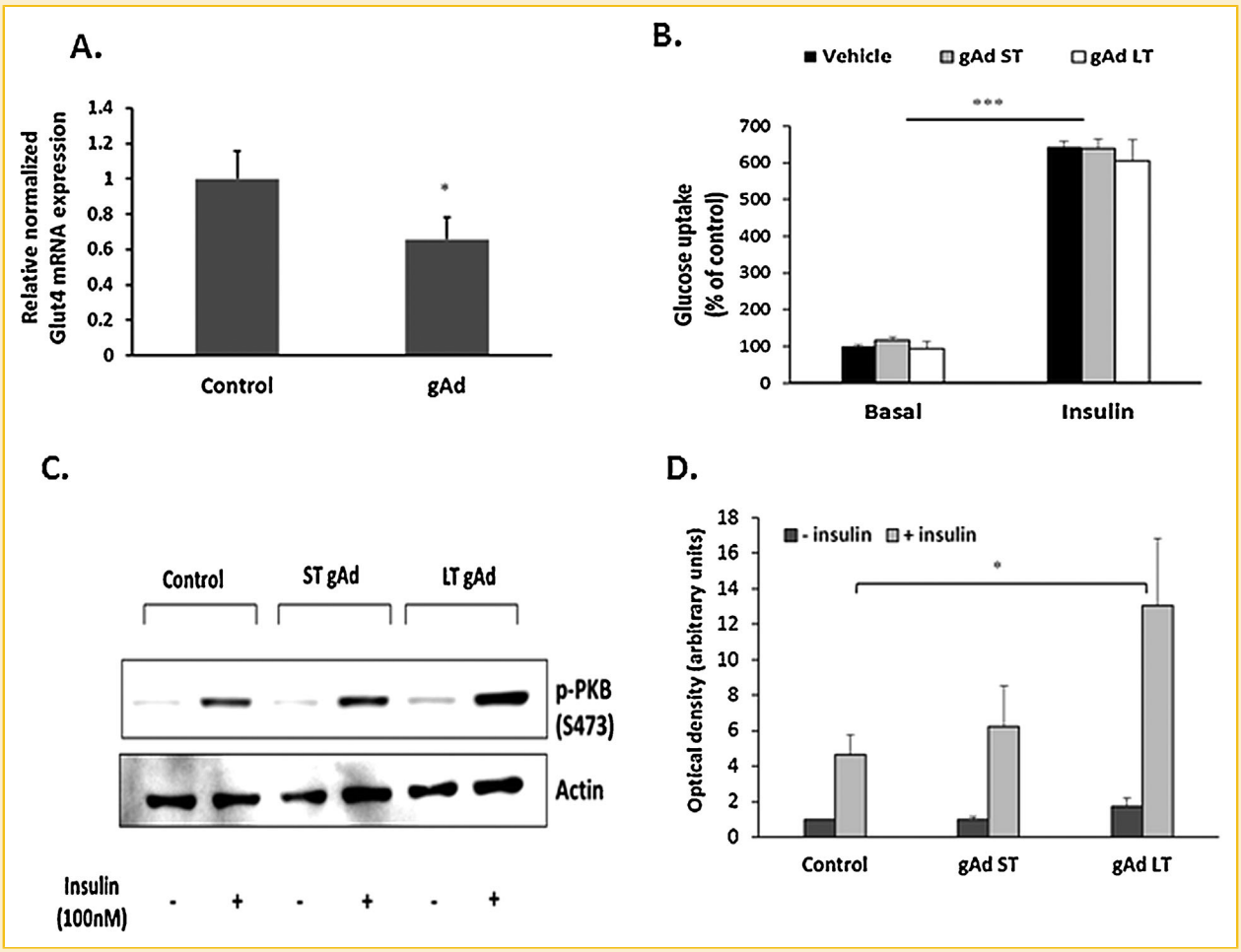

Fig. 6. Effect of gAd on insulin sensitivity of 3T3-L1 adipocytes. A: gAd was administrated at day 0 of differentiation as described. Glut4 mRNA expression was measured by real-time PCR. Results were normalized to the expression of housekeeping gene, HPRT, and are presented as fold change compared to untreated cells. *$P<0.05$, by Student's *t*-test. B and C: gAd was administrated at the beginning (LT) or at the end of differentiation (ST) as described in materials and methods section. B: Adipocytes were transferred to serum-free medium. Basal and insulin-induced uptake of [3H]2-deoxy-d-glucose into cells were determined as described. Each bar represents the mean ± SEM of a measurement made on three replicates in each of three independent experiments. Data are expressed as percent of basal uptake in control cells. C: Western blot analysis was performed on cell lysates using anti PKB (S473) or anti actin, this is a representative blot of three independent experiments. D: Optical density of PKB phosphorylation. Results were normalized to actin.

isoforms were found to be similar in most models, and both isoforms induce the activation of signaling molecules such as AMPK [Yamauchi et al., 2002] and PPARα and the propagation of downstream pathways [Yamauchi et al., 2003a; Palanivel et al., 2007]. The possible variation in activity of full-length and gAd had not been elucidated yet in adipocytes, and should be clarified further in future studies.

It is important to note that administration of gAd did not alter endogenic mRNA production of the hormone by the cells, suggesting that no downregulation of endogenous hormonal production machinery occurred, and the treatment actually increased gAd level in the environment of differentiating adipocytes. In addition, mRNA expression of adiponectin receptors was not downregulated. Similar to previous studies [Rasmussen et al., 2006], we found that differentiated adipocytes express higher mRNA levels of AdipoR1 than AdipoR2. However, while the expression of AdipoR1 is more or less stable during differentiation, AdipoR2 expression is markedly induced in differentiated adipocytes, indicating a specific role of AdipoR2 in the functional adipocyte. gAd was found to increase the expression of AdipoR1 in differentiated adipocytes. The selective increase of AdipoR1 expression in gAd-treated cells may be due to its higher binding affinity for gAd that was used in this study, and weak affinity for fAd [Yamauchi et al., 2003a], while AdipoR2 which has a higher affinity for binding to fAd, was not affected by the administration of gAd in this study. Specific inhibition of these receptors might be used in future studies in order to clarify the specific role of each receptor in the differentiation and function of the adipocyte.

We found that although gAd-treated cells differentiated into mature adipocytes, gAd reduced the lipid content of differentiated adipocytes, as indicated by direct measurement of triglycerides, and also supported by reduced mRNA expression of perilipin, a protein that coats the lipid droplets in the adipocyte. It is known that adipose cell size, independently of body mass index, is inversely correlated

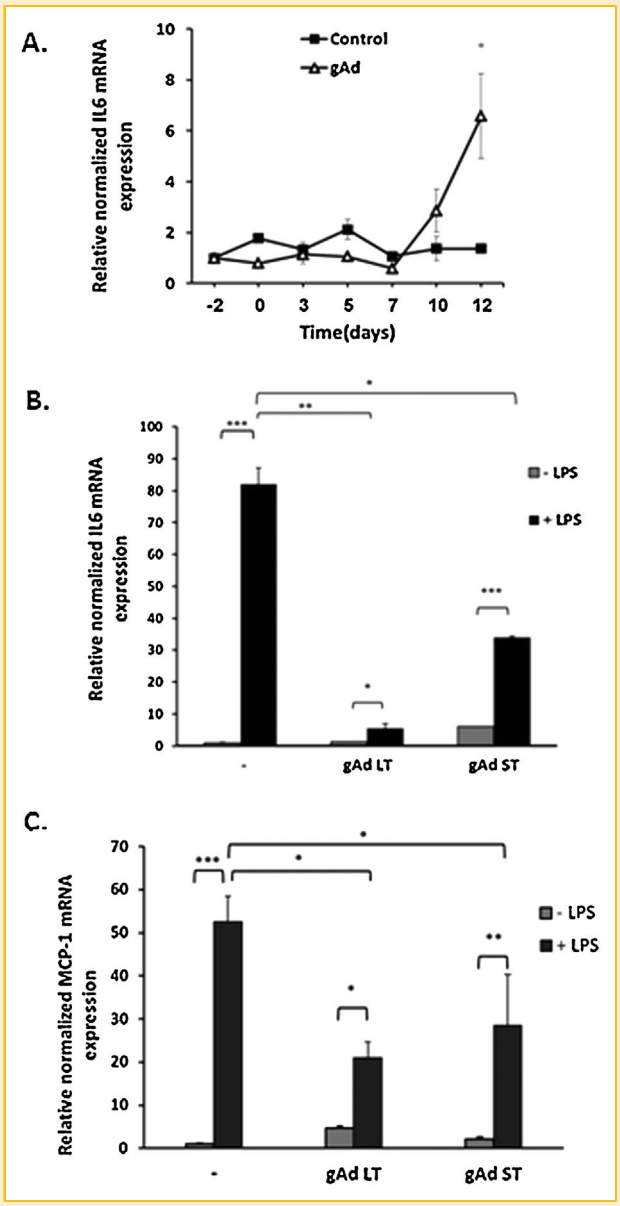

Fig. 7. gAd reduced the sensitivity of 3T3-L1 to LPS. A: gAd stimulated IL6 expression in differentiated adipocytes. gAd (10 nM) was administrated to differentiating preadipocytes as described in Materials and methods section. mRNA of IL6 was measured by real-time PCR. B and C: Cells were treated with gAd at the beginning (LT) or at the end of differentiation (ST) as described in Materials and Methods section. Differentiated adipocytes were stimulated with LPS (100 ng/ml) for 6 h. IL6 (A) and MCP-1 (B) mRNA expression was measured by real-time PCR. Results were normalized to the expression of housekeeping gene, HPRT. *$P<0.05$, **$P<0.005$, ***$P<0.0005$ by one-way Anova, followed by Bonferroni's posthoc test.

with metabolic perturbations, mainly insulin resistance and disturbed lipid profile, as well as induction of inflammatory pathways and fatty liver [O'Connell et al., 2010]. Our study suggests that gAd directs the adipocytes to a preferred function and eliminates excess lipid storage by adipocytes. We hypothesize that by autocrine/paracrine mechanisms gAd maintains homeostasis of lipid storage in the adipocyte, thus eliminating surplus lipid storage, that insult the functionality of the cell (Mlinar and Marc, 2011), leading to alteration in the profile of adipokines secreted by the cells, including alteration in the secretion of adiponectin. Studies on effects of expression of fAd in 3T3 preadipocyte by stable transfection show increased lipid storage in the mature adipocyte, in contrast to our results [Fu et al., 2005]. However, in addition to the difference between studies in the isoform used, stable transfection of adiponectin showed increased adiponectin level only 7 days following the induction of differentiation [Fu et al., 2005], while we supplemented the media with constant concentration of gAd at day 0 of differentiation. In addition, in the study of Fu et al., downregulation of the expression of the two receptors was demonstrated, which might explain the differences between the two studies.

PPARγ mRNA expression is increased at day 3 of differentiation in both treated and untreated cells, but is higher at the 8th day in gAd-treated cells. On the other hand, gAd reduced the expression of PPARγ in fully differentiated adipocytes. PPARγ is a major and critical transcription factor that regulates the transcription of various genes encoding proteins involved in creating the adipocyte phenotype [Ntambi and Young-Cheul, 2000]. In addition, although its expression is normally reduced at late differentiation stages in relation to earlier stages, PPARγ is also crucial for maintaining the appropriate function of the mature adipocyte, and is associated with both local and systemic effects, including increased glucose uptake, and whole body insulin sensitivity. PPARγ also increased lipogenesis, attributed to recruitment of preadipocytes and hyperplasia of adipocytes, mainly in subcutaneous depots [Kajita et al., 2013]. In addition, PPARγ improved secretory profile of the adipocyte, increasing secretion of insulin sensitizing factors, such as adiponectin and reducing pro-inflammatory cytokines [MacKellar et al., 2009]. Thus, while PPARγ is a known positive regulator of adiponectin expression [Iwaki et al., 2003], our data show that gAd also affects PPARγ expression, in a complicated biphasic pattern; gAd increased the expression of PPARγ through differentiation, but reduced its expression in mature adipocytes. These observations are in accordance with the lower lipid accumulation of the gAd-treated mature adipocyte, and lower expression of perilipin and FABP4, which are known to be highly regulated during differentiation and their mRNA is transcriptionally controlled by fatty acids and PPARγ [Furuhashi and Hotamisligil, 2008].

Reduced PPARγ expression in gAd-treated differentiated adipocytes was also accompanied by lower expression of ATGL and FAS. Low expression of these genes suggests reduced turnover of lipids in gAd-treated cells. In adiponectin-overexpressing mice, a reduction in FAS mRNA was also found, along with increased expression of genes involved in fatty acid oxidation [Shetty et al., 2012], however, our results indicate that gAd did not affect AMPK phosphorylation, PPARα expression, fatty acid oxidation and metabolic rate in differentiated adipocytes, and the reduced lipid content should be attributed to low rate of lipogenesis. AMPK phosphorylation and activation is a known event involved in the propagation of adiponectin signaling [Yamauchi et al., 2002; Huypens et al., 2005; Shibata et al., 2005], and was found to mediate its effects on glucose metabolism and fatty-acid oxidation in several target tissues

of the hormone, such as skeletal muscle and hepatocytes. However, our results show that gAd did not induce AMPK phosphorylation, and support the existence of an AMPK-independent pathway as was demonstrated by several studies [Holland et al., 2011; Miller et al., 2011; Gu and Li, 2012], suggesting a role of ceramidase activation and sphingolipids metabolism in adiponectin signaling cascade. The involvement of ceramidase in gAd effects in adipocytes should be investigated in depth.

Reduced GLUT4 mRNA found in gAd adipocytes may also result from reduced PPARγ expression; however insulin sensitivity was not disturbed, as was demonstrated by measuring basal and insulin-induced glucose uptake. Moreover, insulin-induced PKB phosphorylation was higher in gAd-treated cells, indicating that gAd sensitizes the adipocytes to insulin signaling. Induction of PKB phosphorylation by adiponectin, independent of AMPK phosphorylation was also demonstrated in pancreatic β-cells [Wijesekara et al., 2010]. We show that gAd sensitizes insulin signaling pathway in adipocytes as well, and suggest that this event compensates for the reduction in PPARγ and Glut4 expression, enabling the preservation of intact glucose uptake in gAd treated adipocytes.

Adiponectin is known to be negatively correlated with inflammation [Yamauchi and Kadowaki, 2008]. Paradoxically, we found that chronic exposure to gAd lead to increased expression of IL6, a pro-inflammatory cytokine. It was suspected that recombinant gAd used in this study is contaminated with very low amounts of LPS which may exert some inflammatory response which are actually unrelated to adiponectin [Turner et al., 2009]. However, in the current study cells were treated with gAd at the beginning of the differentiation (day 0), while the increase in IL6 mRNA was detected 12 days later. Thus, it seems that the induction of IL6 expression is not related to residual LPS contamination, if it exists, and is specific for the effect of gAd on the mature adipocytes. These results are in accord with those of Awazawa et al., who used IL6 KO mice and neutralizing antibodies to show that adiponectin upregulates IRS2 protein expression and insulin sensitivity via the induction of moderate production of IL6 by macrophages [Awazawa et al., 2011]. In addition, high lipid levels were found in IL6-deficient mice [Wallenius et al., 2002], while increased IL6 was accompanied by low plasma cholesterol and TG [Hashizume et al., 2010], indicating that IL6 at physiological concentrations play a role in maintaining lipid metabolism. Our finding, showing increased IL6 expression in adipocytes by gAd, a hormone correlated with an improved metabolic state, supports the idea that IL6 has some physiologic functions. The effects of low IL6 concentrations on adipocytes function should be further clarified.

In contrast to the induction of moderate levels of IL6 mRNA expression, gAd abrogated the pathological LPS-dependent IL6 expression, In this study we showed that in addition to the known mechanism, in which adiponectin ameliorates inflammation through mediating local and systemic activity of macrophages leading to reduced secretion of pro-inflammatory cytokines by the immune cells [Yokota et al., 2000; Wulster-Radcliffe et al., 2004], gAd also enhances the resistance of the adipocytes to inflammatory stimuli. Adipocytes treated with gAd before the induction of inflammation by LPS, acquire resistance to the inflammatory stimuli, as indicated by abrogation of LPS-induced IL6 and MCP-1 mRNA expression. TNFα and IL10 expression by 3T3-L1 adipocytes were almost undetectable in both LPS-treated and untreated cells, as was also reported previously [Hoch et al., 2008]. These results are in accord with previous studies showing reduced expression of pro-inflammatory cytokines in adipocytes treated with adiponectin. This effect was suggested to be mediated by reduced activation of NFκB [Zoico et al., 2009]. However, our results demonstrated that while gAd given to differentiated adipocytes, as administrated in the previous studies, only partially reduced inflammatory response, treating the adipocytes continuously during differentiation almost completely blocked the elevation of pro-inflammatory cytokines, mainly IL6. This suggests that early exposure to gAd may provide higher resistance to inflammatory stimuli. As expanded adipose tissue is characterized by pro-inflammatory cell infiltration, causing chronic, low-grade inflammation, which has significant systemic adverse consequences, such as insulin resistance and the progression of atherogenic processes [Sell and Habich, 2012], identification of additional strategies to reduce adipose inflammation is important.

Adiponectin-overexpressing mice showed improved metabolic function under high fat diet. In their study, Asterholm et al. [2010] suggest that adiponectin increases metabolic flexibility of the organism by balancing the ability to enhance expansion of adipose tissue while also enhancing the ability to respond to lipolytic stimuli, and suggest that under the effect of adiponectin, adipose tissue acts as an efficient "metabolic sink." They hypothesize that adiponectin increases clonal expansion of preadipocytes as well as adipocyte differentiation. Our study supports this idea, demonstrating the benefits of early exposure of the developing adipose tissue to gAd.

In conclusion, this study demonstrates the potential beneficial effect of increased adiponectin level in the environment of the developing adipose tissue in order to program the cells to differentiate toward a healthy tissue. The molecular mechanisms mediating this programing, and the possible involvement of epigenetic events involved in the effect of gAd on the differentiating adipocytes are currently under study.

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