Physiological Research Pre-Press Article

Adiponectin inhibits spontaneous and catecholamine-induced lipolysis in human adipocytes of non-obese subjects through AMPK-dependent mechanisms

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Short title: Adiponectin inhibits lipolysis in humans

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Summary:

Adiponectin is an adipokine increasing glucose and fatty acid metabolism and improving insulin sensitivity. The aim of this study was to investigate the role of adiponectin in the regulation of adipocyte lipolysis. Human adipocytes isolated from biopsies obtained during surgical operations from 16 non-obese and 17 obese subjects were incubated with 1) human adiponectin (20 µg/ml) or 2) 0.5 mM AICAR - activator of AMPK (adenosine monophosphate activated protein kinase). Following these incubations, isoprenaline was added (10^{-6} M) to investigate the influence of adiponectin and AICAR on catecholamine-induced lipolysis. Glycerol concentration was measured as lipolysis marker. We observed that adiponectin suppressed spontaneous lipolysis by 21 % and isoprenaline-induced lipolysis by 14% in non-obese subjects. These effects were not detectable in obese individuals, however statistically significant differences in the effect of adiponectin between obese and non-obese were not reached with two way ANOVA test. The inhibitory effect on lipolysis of AICAR and adiponectin was reversed by Compound C. Our results suggest, that adiponectin in physiological concentrations inhibits spontaneous as well as catecholamine-induced lipolysis. This effect might be lower in obese individuals; however our data cannot unmistakably confirm this. This regulation seems to involve AMPK.

Key words: obesity, adiponectin, lipolysis, adipose tissue, AMPK

Introduction:

Obesity characterized by excessive lipid accumulation in adipose tissue as well as in ectopic localizations (especially muscle and liver) was shown to increase the risk of developing insulin resistance, type 2 diabetes mellitus, hypertension, cardiovascular diseases, several types of cancer and other chronic diseases (Hayes *et al.* 2008). Among the major mediators possibly contributing to a development of insulin resistance, pancreatic endocrine dysfunction, atherosclerosis and whole body pro-inflammatory state belong the excessive flux of non-esterified fatty acids (NEFA) from adipose tissue and a family of proteins produced in adipose tissue, collectively named adipokines (Boden *et al.* 2008).

Non-esterified fatty acids represent primary energy source for metabolically active tissues, including skeletal muscle (Bickerton *et al.* 2007), however increasing amount of evidence suggests, that the excessive flux of NEFA from adipose tissue of obese individuals (Baldeweg *et al.* 2000) is an important factor in the etiopathogenesis of obesity-related metabolic impairments, mainly insulin resistance (Frayn *et al.* 2001). Insulin resistance-inducing effect of NEFA in muscle has been firstly conceptualized by Randle (Randle *et al.* 1998) who noticed the substrate competition between fatty acids and glucose in myocytes. This concept was further investigated and mechanisms of NEFA-induced impairments of insulin signaling were described at the molecular level in muscle as well as liver. Furthermore, NEFA play an important role also in this process of endocrine pancreatic dysfunction, which represents the second step in the development of type 2 diabetes, and contribute thus to diminished insulin output from pancreas in response to glucose stimulus (Yaney *et al.* 2000).

Adipose tissue lipolysis is regulated by a complex interaction between lipolytic and anti-lipolytic hormones. Under physiological circumstances, catecholamines and atrial natriuretic peptide (ANP) are the most important lipolysis stimulating factors (Lafontan *et al.* 2008), while insulin is a potent anti-lipolytic agent. During fasting, stress and exercise is lipolysis stimulated by growth hormone to spare glucose and proteins at the expense of lipids (Moller *et al.* 2009). Obese individuals have been shown to suffer from several dysregulations in lipolysis regulation, including increased rate of spontaneous lipolysis in subcutaneous adipose tissue *in vitro* as well as *in vivo* (Arner *et al.* 2005) and decreased stimulation of lipolysis during exercise or following local *in-situ* catecholamine administration, compared to healthy subjects, partially due to enhanced anti-lipolytic effect mediated through α_2-adrenergic receptors (Stich *et al.* 2003). Obesity is associated with suppressed levels of circulating growth hormone compared with normal-weight subjects (Veldhuis *et al.* 1991, Laessle *et al.* 1996), what may contribute to maintenance of obesity, because fat deposits are maintained (Laessle *et al.* 1996). Recently, the role of adipokines in lipolysis regulation has been investigated and the strong lipolytic effect of tumor necrosis factor-α (TNF- α) and interleukin-6 (IL-6) was established together with their physiological role in pro-inflammatory state induction (TNF- α) and substrate flux regulation during exercise (IL-6) (Cawthorn *et al.* 2008). Adiponectin is a unique adipokine produced in mature adipocytes with profound insulin-sensitising, anti-inflammatory and anti-atherosclerotic effects (Yamauchi *et al.* 2002, Haluzik *et al.* 2004, Nedvidkova *et al.* 2005). Adiponectin increases glucose uptake and fatty acid oxidation in muscle and reduces hepatic gluconeogenesis (Yamauchi *et al.* 2002). Its cellular effects in muscle and liver are at least partially mediated by the activation of 5'-AMP-activated protein kinase (AMPK) (Yamauchi *et al.* 2002). Adiponectin receptors are ubiquitously expressed on several human tissues including muscle, liver, pancreas and adipocytes (Yamauchi *et al.* 2003). However, the paracrine/endocrine role of adiponectin on adipocytes has never been thoroughly investigated. Available literature identified AMPK as a key regulator of cellular energy metabolism including regulation of lipogenesis, β-oxidation and lipolysis (Daval *et al.* 2006). We hypothesized that adiponectin

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stimulates AMPK also in human adipocytes and participates in the regulation of lipolysis at the tissue level by paracrine and (via systemic circulation) also endocrine mechanisms.

The main aim of this study was to investigate the *in-vitro* effect of adiponectin at physiological concentrations on the spontaneous and catecholamine-stimulated lipolysis using isolated adipocytes from subcutaneous adipose tissue. Subsequently we investigated whether lipolytic response of adipocytes from obese and non-obese individuals is different following adiponectin administration.

Methods:

Subjects

The samples of adipose tissue were obtained during planned laparoscopic operations (cholecystectomy, hernioplastics) or plastic surgery (liposuction). The samples of tissue were taken by the surgeons during laparoscopy surgery under direct vision from the abdominal adipose tissue located immediately under the skin, at the beginning of the surgery. During liposuction the tissue had been taken at the beginning of the surgery as well, before the tissue was infiltrated by adrenaline, to avoid the contamination of the tissue by any pharmaceuticals. All samples were taken from the abdominal area. No sample of adipose tissue in this study had been taken by transcutaneous biopsy. The samples of adipose tissue were given to a saline solution and immediately transported to the laboratory for experiments. The adipose tissue was obtained from 17 obese women (BMI = 37.6 ± 5.6 kg/m^2, age = 41.9 ± 10.9 years) and 16 non-obese subjects (4 men, 12 women, BMI = 23.6 ± 2.2 kg/m^2, age = 45.4 ± 15.9 years). Before including the patients to the study we were informed about their concomitant diseases and only patients without any acute or chronic disease except of obesity were included to the study and the sample of adipose tissue had been taken during the surgery. However, in the group of non-obese patients two of them were on thyroid substitution. The hypothyreosis was in both cases stationary, so we do not suppose any effect on the metabolism of adipocytes.

For the additional experiment (Indirect evidence of AMPK activation using Compound C, see below) we used the adipose tissue obtained from 3 human non-obese subjects (females, BMI 23.4 ± 2.0 kg/m2). All participants gave written informed consent before starting the study. All aspects of the study were performed in accordance with the Declaration of Helsinki and were approved by the Ethical committee of the Third Faculty of Medicine, Charles University (Prague, Czech Republic).

Adipocyte isolation

Immediately after the biopsy was taken, the tissue was transported to the laboratory for experiments. Isolated adipocytes were obtained as previously described by Rodbell (Rodbell M 1964). Briefly, the whole tissue was cut into small pieces and subsequently digested by 1.25 mg/ml collagenase (Sigma Collagenase from Clostridium histolyticum, Prod. No. C6885) in Krebs Ringer Bicarbonate buffer containing 10 mmol/l Hepes, 2 % fatty acid free bovine serum albumin (KRBHA) and 6 mmol/l glucose at pH 7.4, under shaking at 100 cycles/min at 37°C during 30 min. Adipocytes were filtered through a silk screen (250 µm) and washed 3 times with KRBHA buffer to eliminate collagenase.

In-vitro incubation and lipolysis determination

The freshly isolated fat cells were divided in 4 batches, each containing 95 ul of cell suspension and 80 ul of KRBHA buffer. Cells in each batch were left resting for one hour prior to following experiments to enable cells recover after collagenase digestion and manipulation. Four different incubations were performed in parallel with cells obtained from each subject: the first batch (control) was than incubated for 4 hours without any pharmacological intervention, second batch was incubated without pharmacological substances for first 2 hours and subsequently isoprenaline was added at the final concentration of 10^{-6} mol/l for last 2 hours, third batch was incubated with the final concentration of 20 µg/ml of adiponectin for 2 hours and subsequently with isoprenaline (10^{-6} M), fourth batch was incubated for 2 hours in the final concentration of 0.5 mmol/l AICAR (aminoimidazole carboxamide ribonucleotide, activator of AMPK) and subsequently with isoprenaline (10^{-6} M) for last 2 hours. Glycerol was determined by colorimetric assay (Glycerol kit, Randox laboratories, Crumin, United Kingdom) in cultivation media after one hour of rest and subsequently following 2 hours of incubation with adiponectin or AICAR and finally, after 2-hour incubation with isoprenaline in all the batches. Following the incubations, total lipid content in each tube was extracted with heptane and isopropylalcohol and subsequently quantified using the Dole method. Values of glycerol concentration in media were expressed as per 100mg of lipids.

Determinantion of AMPK involvement

Indirect evidence of AMPK activation was evaluated using a specific cell-permeable agent named Compound C (6-[4-(2-Piperidin-1-yl-ethoxy)-phenyl)]-3-pyridin-4-yl-pyrazolo[1,5-a] pyrimidine), which was shown to effectively block AMPK activation in several tissues including adipocytes (Zhou *et al.*, 2001, Gao *et al.*, 2008, Koh *et al.*, 2007). For these experiments, adipose tissue of non-obese subjects was processed as described above and isolated cells were firstly pre-incubated for 2 hours with Compound C (0.5 mmol/l). After this period of pre-incubation, subsequent 2-hour incubations with adiponectin 20mg/L or 0.5mM AICAR were performed.

Statistical analysis

Statistical analysis was performed using SPSS 13.0 for Windows SPSS Inc., Chicago, IL). The effect of pharmacological substances was evaluated using paired Student's t-test. Differential effects of treatments between groups (obese versus non-obese) were tested using Two-way ANOVA analysis where treatment and obesity status were considered as independent variables in the model. Data are presented as mean values ± SD. A level of $p \leq$ 0.05 was considered statistically significant in all tests.

Results:

Spontaneous lipolysis

Data were expressed in absolute values of glycerol concentration in media normalized to 100mg of lipids. The cumulative 2-hour glycerol concentration representing spontaneous lipolytic rate was 458.4 ± 365.7 µmol/l/100mg lipids in the control tube, while incubation with adiponectin led to a suppression of lipolysis by 21% (p = 0.06) and co-incubation with AICAR suppressed lipolysis by 64 % (p = 0.005), in non-obese individuals. Spontaneous lipolysis was 2.6 times higher in obese individuals (p = 0.02). Incubation with AICAR inhibited spontaneous lipolysis by 35 % (p < 0.001) in obese subjects, while the effect of adiponectin was diminished (inhibition by 2%, p = 0.43). However no statistically significant differences between obese and non-obese individuals were observed for AICAR as well as adiponectin treatments, according to two-way ANOVA testing. These data are displayed in Figure 1.

Catecholamine-induced lipolysis

2-hour incubation with isoprenaline stimulated lipolysis 4.4 times in non-obese and 2.4 times obese individuals (both p < 0.05). Adiponectin inhibited isoprenaline-induced lipolysis in non-obese by 14 % (p = 0.03), while AICAR inhibited isoprenaline-induced stimulation of lipolysis by 71 % and 35 % in non-obese and obese subjects (p = 0.006 and p = 0.04, resp.). Despite lower inhibitory effect of adiponectin in obese individuals, no statistical differences were observed between obese and non-obese individuals for AICAR as well as adiponectin treatments, according to two-way ANOVA test. Data are summarized in Figure 2.

AMPK inactivation by Compound C

In additional experiment cells were incubated for 2 hours separately with AICAR or adiponectin and in parallel with addition of AMPK inactivator (Compound C). Anti-lipolytic effect of both, AICAR as well as adiponectin was reversed by Compound C. Addition of Compound C increased lipolysis by 37.8 % (p < 0.001) when co-incubated with adiponectin and by 49.5 % (p < 0.001) following the co-incubation with AICAR. Data are summarized in Figure 3.

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Discussion

This study investigates for the first time the role of adiponectin in the regulation of lipolysis in human adipocytes. The major finding is that adiponectin moderately inhibits spontaneous as well as catecholamine-induced lipolysis in non-obese individuals, while this effect seems to be diminished in obese subjects. Furthermore, we suggest, that AMPK is involved in the effect of adiponectin on lipolysis in human adipocytes.

Adiponectin was shown to activate AMP-kinase in human liver and muscle by specific phosphorylation of Thr-172 residue through yet not fully clarified mechanisms. After binding of adiponectin to membrane receptors (AdipoR1, AdipoR2) that were also identified in human adipose tissue (Tan *et al.* 2006), activation of APPL1 (Adaptor protein with plecthrin homology) was described in liver, muscle and adipocytes (rat) (Saito *et al.* 2007; Deepa *et al.* 2008; Mao *et al.* 2006) with subsequent involvement of several signaling cascades, including PPARα, MAPK and AMP-kinase pathway (Kadowaki *et al.* 2006). However, it remains unclear, whether APPL1 is mediating adiponectin-induced effects in human adipose tissue as well. Activated AMP-kinase subsequently phosphorylates hormone sensitive lipase (HSL), a key regulator of adipocyte lipolysis, at the Ser-565 position, also referred as "Site-2" (Garton *et al.* 1990; Garton *et al.* 1989). Phosphorylation at this position was shown to prevent HSL phosphorylation at Ser-563 position, also referred as "Site-1" (Carmen *et al.* 2006; Holm *et al.* 2003), which is a common substrate for cAMP-dependent lipolysis-activating pathways (e.g. β-adrenergic stimulation) and leads to HSL activation and lipid hydrolysis (Langin *et al.* 2005; Arner *et al.* 1990). AMPK activation thus inhibits/prevents HSL activation. Additional possible mechanism explaining adiponectin anti-lipolytic effect might represent interaction between adiponectin signaling and insulin signaling pathways. It has been shown, that APPL1 interacts with key parts of the insulin-signaling pathway in adipocytes (as well as myocytes and endothelial cells) (Deepa *et al.* 2008), especially PI3K and Akt/PKB (Yang *et al.* 2003), and is essential for insulin-mediated metabolic effects in adipocytes (including Akt phosphorylation, glucose uptake, GLUT 4 translocation) (Saito et al. 2007; Mitsuuchi *et al.* 1999). It can be hypothesized, that adiponectin-induced activation of APPL1 in human adipocytes leads to subsequent activation of Akt and to anti-lipolytic effect through well described mechanisms identical to insulin-mediated anti-lipolytic cascade.

Our finding that stimulation of AMPK by AICAR and to a smaller extent by adiponectin in our study inhibits spontaneous as well as catecholamine-induced lipolysis in lean subjects is congruent with the evidence obtained mainly in animal models and suggests that this regulation is present also in human adipocytes. Furthermore, we showed that the anti-lipolytic effect of adiponectin can be reversed by Compound C, a potent AMPK inactivator, suggesting that AMPK activation plays a role in adiponectin signaling in adipose tissue, which is in line with previously published study showing that Compound C blocks AMPK in hepatocytes (Zhou *et al.* 2001).

The present study supports the concept of adiponectin paracrine effects in adipose tissue. Subcutaneous abdominal, femoral and visceral adipose tissues have different expression of adiponectin gene and possibly also protein production. As plasma levels of adiponectin are rather high, a true endocrine effect can be suggested as well. Adiponectin might serve as a messenger regulating lipolysis between different adipose tissue depots. Additionally, other tissues, especially skeletal muscle and liver, were shown to posses the ability of lipolysis, mediated through similar mechanisms as in adipose tissue (including the activation of HSL) (Qvisth *et al.* 2006; Jocken *et al.* 2008). It can be thus speculated, that adiponectin might serve as inter-organ regulator of lipolysis, influencing also lipid

degradation localized in muscle and liver. Whether this regulation has some physiological relevance needs to be investigated in future studies.

Based on recent literature showing that effects of adiponectin in muscle are diminished in obesity (Bruce *et al.* 2005; Mullen *et al.* 2009), we hypothesized that adiponectin might inhibit lipolysis in adipose tissue of lean subjects, while adipose tissue of obese individuals might be *"adiponecin resistant"* and thus the effect of adiponectin decreased or absent. Adiponectin resistance in adipose tissue, together with decreased levels of adiponectin in obese individuals and insulin resistance of adipocytes (Arner *et al.* 2005; Large *et al.* 1998), might subsequently contribute to elevated plasma levels NEFA (non-esterified fatty acids) in obese individuals. Higher NEFA participate in the induction of the insulin resistance, pancreatic dysfunction and related metabolic disturbances (Yaney *et al.* 2000; Paolisso *et al.* 1995; Chen *et al.* 1999; Diaz-Guerra *et al.* 1991). Unfortunately, based on our data, we cannot unequivocally confirm this hypothesis. We observed suppression of spontaneous and catecholamine-induced lipolysis in lean subjects and absent effect of adiponectin in obese individuals when this effect was tested in each group separately; however, two-way ANOVA test failed to show significant differences in response to adiponectin treatment between both groups. This lack of significance is due to large inter-individual variability of both spontaneous as well as catecholamine-induced lipolysis in human isolated adipocytes.

Interestingly, we observed that the effect of AICAR on lipolysis is preserved in obese as well as non-obese individuals. This observation is in agreement with previously published findings in skeletal muscle (Steinberg *et al.* 2004; Hojlund *et al.* 2004), and suggests that potentially insufficient suppression of lipolysis by adiponectin in obese individuals might be due to defect localized upstream of AMPK. Both, receptor and post-receptor processes can be involved. Indeed, the expression of AdipoR1 and AdipoR2 in myocytes was found to be negatively influenced by obesity and elevated insulin levels in humans as well as animal models (Tsuchida *et al.* 2004; Civitarese *et al.* 2004), however these findings were not uniformly replicated and other authors claim that triglycerides, growth hormone or first-phase insulin secretion are more important determinants of adiponectin receptors expression (Fasshauer *et al.* 2004; Staiger *et al.* 2004). Additionally, impaired ability of adiponectin to simulate AMPK was found in muscle of obese and type 2 diabetic subjects by mechanisms independent of adiponectin receptors, suggesting a post-receptor defect (Chen *et al.* 2005).

Our study has several limitations that need to be taken into account. First, we have not explicitly (using two-way ANOVA) shown that the effect of adiponectin is different between obese and lean subjects. Our hypothesis, that adipocytes from obese subjects are resistant to the effect of adiponectin is based on simple paired comparisons in each group separately and thus need to be interpreted as preliminary and with caution. Future studies are needed to definitely clear this issue. Second, there exists an inherent limitation to the interpretation of this study resulting from the *in-vitro* nature of experiments and the use of AICAR for AMPK activation. Despite the use of AICAR for *in-vivo* as well as *in-vitro* experiments, it needs to be noted, that AICAR does not have exclusive specificity to AMPK and subsequently, activation of other enzymes dependent on cAMP appears in parallel. However, specific activators of AMPK are not available so far and the above mentioned limitation could be probably solved by transgenic animal models and/or gene expression manipulations in other studies. Third, we used samples of subcutaneous abdominal adipose tissue from both men and women in the group of non-obese subjects. At the present time it is not fully understood if there are any differences in metabolism of adipose tissue between men and women so we do not know if using of both gender can influence our results. In the literature can be found studies considering an *in-vitro* lipolysis where the study groups were either homogenous: only men (Hoffstedt *et al.* 1997, Polak *et al.* 2007) or women, or men and women together (Kolehmainen *et al.* 2000, Harmelen *et al.* 1997). Few studies describe the gender variations

in adipose tissue lipolysis. It had been described no difference in basal and catecholamine stimulated lipolysis in vitro between men and women (Mauriege *et al.* 1999, Lundgren *et al.*, 2008), and no difference in gender in maximal lipolytic responses to isoprenaline (Mauriege *et al.* 1999), in another study is described higher basal lipolysis of isolated adipocytes in men than in women (Aguado *et al.* 2008). Another author presents data that catecholamine regulation of lipolysis in subcutaneous adipocytes from men tends to be higher than in women (Lundgren *et al.*, 2008). In the future, it would be necessary to study the effect of adiponectin on lipolysis in men in comparison to women.

In conclusion, our study showed that adiponectin interacts with lipolytic regulation in human adipocytes under basal conditions as well as after catecholamine stimulation. These effects seem to be at least partially mediated by the activation of AMPK and subsequent interaction with intracellular signaling cascades. Furthermore, we provide some indirect indices suggesting, that adiponectin-mediated anti-lipolytic effect might be diminished in obese individuals. Further studies are necessary to definitely confirm presumed lower effect of adiponectin in obesity as well as to elucidate the role of adiponectin in lipolysis regulation in other tissues, especially muscle as well as the role of physical training and diet intervention on the suggested adiponectin resistance.

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Figure comments

Figure 1.
Adipocytes of obese and non-obese subjects were incubated for 2 hours without any treatment (spontaneous lipolysis) or with the presence of 20 mg/L adiponectin or 0.5 mM AICAR. Data are expressed as the mean glycerol concentration in media following the incubation period.

* $p < 0.05$ for comparison between spontaneous and pharmacological incubation (AICAR)

$p = 0.06$ for comparison between spontaneous and pharmacological incubation (Adiponectin)

Figure 2.
Adipocytes of obese and non-obese subjects were incubated for 2 hours with 10^{-6} M isoprenaline without any other treatment (Isoprenaline) and with the presence of 20 mg/L adiponectin or 0.5 mM AICAR. Data are expressed as the relative change of mean glycerol concentration in media during the incubation period.

* $p < 0.05$ for comparison between spontaneous and pharmacological incubation (Adiponectin or AICAR)

Figure 3.
Adipocytes of non-obese subjects were incubated with 20 mg/L adiponectin or 0.5 mM AICAR in
the presence or absence of 0.5 mM Compound C (inhibitor of AMPK). Data are expressed as the mean glycerol concentration in media following the incubation period.

* $p < 0.01$ for comparison between spontaneous and pharmacological incubation (Adiponectin or AICAR)

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Fig. 1

Glycerol concentration in media following 2-hour incubation

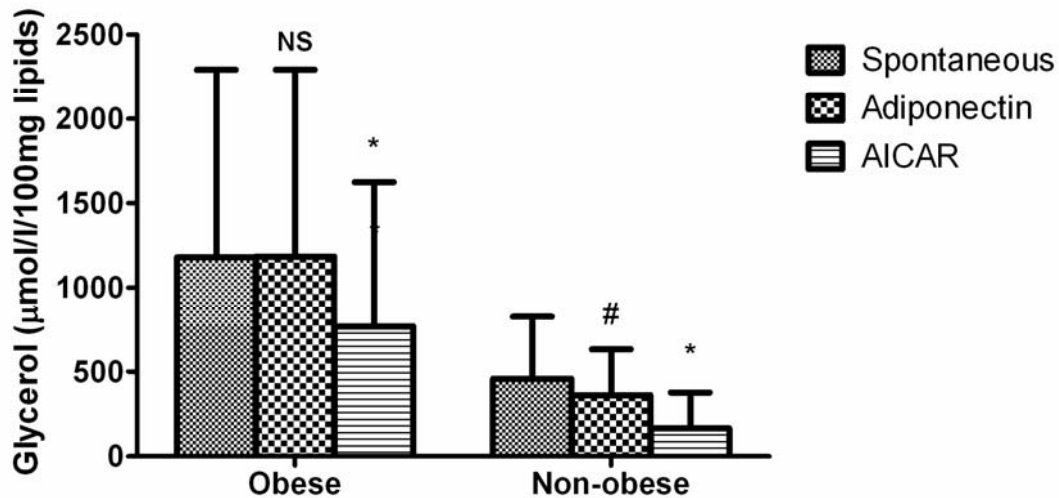**Fig. 2**

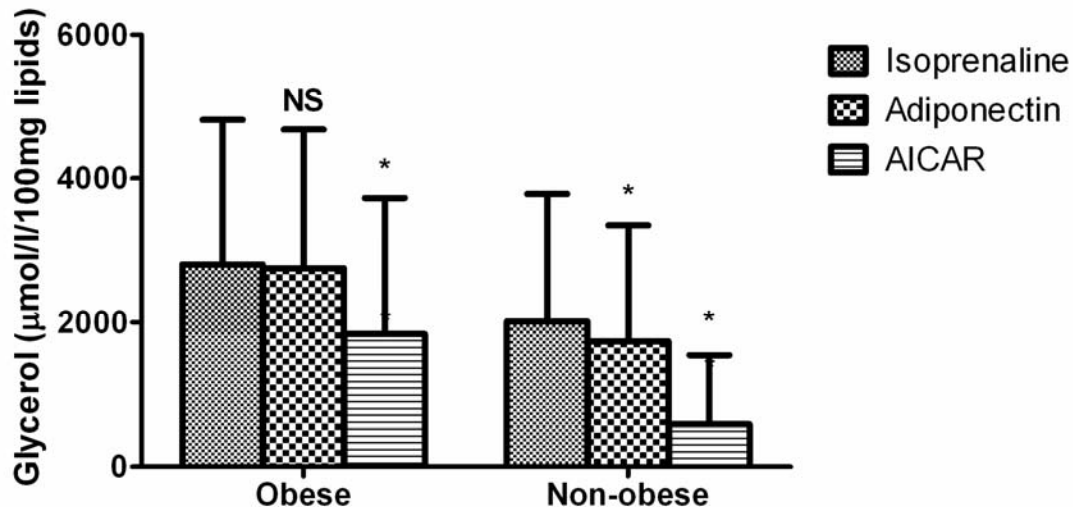Isoprenaline-induced lipolysis following 2-hour incubation

Fig. 3

Glycerol concentration after 4-hour co-incubation of adiponectin or AICAR with Compoud C