

Short-Term Regulation of Adiponectin Secretion in Rat Adipocytes

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Summary

Adiponectin belongs to the group of biologically active substances secreted by adipocytes and referred to as adipokines. Disturbances in its secretion and/or action are thought to be involved in the pathogenesis of some metabolic diseases. However, regulation of adiponectin secretion is poorly elucidated. In the present study, short-term regulation of adiponectin secretion in primary rat adipocytes was investigated. Isolated rat adipocytes were incubated in Krebs-Ringer buffer containing 5 mM glucose and insulin alone or in the combination with epinephrine, dibutyryl-cAMP, adenosine A₁ receptor antagonist (DPCPX), palmitate, 2-bromopalmitate or inhibitor of mitochondrial electron transport (rotenone). Adipocyte exposure for 2 h to insulin (1-100 nM) significantly increased secretion of adiponectin compared with secretion observed without insulin. Furthermore, secretion of adiponectin from adipocytes incubated with glucose and insulin was reduced by 1 and 2 μM epinephrine, but not by 0.25 and 0.5 μM epinephrine. Under similar conditions, 1 and 2 mM dibutyryl-cAMP substantially diminished secretion of adiponectin, whereas 0.5 mM dibutyryl-cAMP was ineffective. Secretion of adiponectin was found to be effectively decreased by DPCPX. Moreover, adipocyte exposure to rotenone also resulted in a substantial diminution of secretory response of adipocytes incubated for 2 h with glucose and insulin. It was also demonstrated that palmitate and 2-bromopalmitate (0.06-0.5 mM) failed to affect secretion of leptin. The obtained results indicated that in short-term regulation of adiponectin secretion, insulin and epinephrine exert the opposite effects. These effects appeared as early as after 2 h of exposure. Moreover, deprivation of energy or blockade of adenosine action substantially decreased secretion of adiponectin.

Key words

Adipocytes • Adiponectin • Secretion • Regulation

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Introduction

Diabetes mellitus is a serious disease affecting about 5 % people worldwide. Type 2 diabetes is the most frequent and accounts for almost 90 % of all types of diabetes. This type of diabetes is mainly characterized by impaired insulin secretion and action. Environmental factors, such as high calorie diet and low physical activity, are thought to play very important role in the pathogenesis of type 2 diabetes. It is well established that the incidence of type 2 diabetes is highly related to obesity and that increased adipocyte lipid storage contributes to reduced insulin action (Leahy 2005). However, adipose tissue is not only a reservoir of energy, but is also recognized as an endocrine organ since it secretes biologically active factors referred to as adipokines. Disturbances in secretion and/or action of some adipokines are also known to contribute to the onset of type 2 diabetes (Rabe *et al.* 2008).

Adiponectin is one of the adipocyte-derived factors, the role of which in the pathogenesis of type 2 diabetes is quite well elucidated not only in rodents but also in humans (reviewed by Haluzik *et al.* 2004, Nedvídková *et al.* 2005, Ziemke and Mantzoros 2010). It is known that diminished levels of blood adiponectin contribute to obesity-related insulin resistance and diabetes, whereas increased blood adiponectin exerts the opposite effect. It was also demonstrated that exogenous adiponectin effectively ameliorates insulin resistance

(Yamauchi *et al.* 2001). On the other hand, adiponectin resistance, induced by a high-fat diet, causes dysregulation of lipid metabolism in skeletal muscles and contributes to the development of insulin resistance (Mullen *et al.* 2009). An inverse correlation between adiposity and blood adiponectin is well documented (Arita *et al.* 1999, Matsubara *et al.* 2002, Yamamoto *et al.* 2002). Importantly, weight reduction significantly increases blood adiponectin levels in both diabetic and nondiabetic subjects (Hotta *et al.* 2000).

The mechanism of adiponectin action at the cellular level is only partially known. This action is preceded by adiponectin binding to its membrane receptors (AdipoR1 and AdipoR2) found in different tissues (Yamauchi *et al.* 2003) and involves activation of adenosine-5'-monophosphate-activated protein kinase (AMPK) (Yamauchi *et al.* 2002, Huypens *et al.* 2005, Wang *et al.* 2007) and reduction of serine phosphorylation of insulin receptor substrate mediated by p70 S6 kinase and mammalian target of rapamycin (Wang *et al.* 2007). As a result, adiponectin sensitizes insulin signaling (Wang *et al.* 2007) and improves insulin action (Yamauchi *et al.* 2001, Berg *et al.* 2001).

Despite a large body of evidence pointing to adiponectin as an adipocyte-derived factor which plays an important role in preventing some metabolic diseases, little is known about regulation of its secretion. Moreover, the majority of results arises from long-term experiments in which cells were incubated for many hours or even days. In the present study, short-term regulation of adiponectin secretion in isolated rat adipocytes was investigated under different experimental conditions.

Methods

Animals

In each experiment, male Wistar rats that weighed 260-280 g and obtained from Brwinow (Poland) were used. Rats were maintained in cages in an air-conditioned room at a constant temperature of 21 ± 1 °C with a 12:12-h dark-light cycle. Animals were fed a standard laboratory chow (Labofeed, Poland) and had free access to drinking water. The experiments were performed according to rules and protocols accepted by Local Ethical Commission for Investigations on Animals.

Isolation of adipocytes

Fat cells were isolated as described previously

(Rodbell 1964) with modifications (Szkudelska *et al.* 2000). In brief, the animals were slaughtered by decapitation and the epididymal fat tissue was collected and rinsed with 0.9 % NaCl. Large blood vessels were removed, fat tissue was cut into small pieces and incubated with gentle shaking for 90 min at 37 °C in Krebs-Ringer buffer containing 3 % bovine serum albumin, 3 mM glucose, 10 mM HEPES and 2 mg/ml collagenase. Before use, the buffer was gassed for 20 min with a mixture of O₂/CO₂ (95 % / 5 %) and pH was adjusted to 7.4. Afterwards, the adipocytes were filtered through a nylon mesh, rinsed with Krebs-Ringer buffer without collagenase and counted under the microscope with a Bürker-Türk counting chamber.

Incubations of adipocytes

In order to investigate the mechanisms involved in the short-term regulation of adiponectin secretion, adipocytes were exposed to different agents which potentially could affect secretory activity of these cells. In each experiment, cells (10^5 /ml) were incubated in plastic tubes containing Krebs-Ringer buffer with 3 % bovine serum albumin (except for experiments with palmitate and 2-bromopalmitate) and 10 mM HEPES. Incubations were made at 37 °C with gentle shaking.

In the first part of the study, the effects of insulin on adiponectin secretion were studied. In these experiments, fat cells were incubated without insulin or with 0.1, 1, 10 and 100 nM insulin. All incubations were performed for 2 h in Krebs-Ringer buffer containing 5 mM glucose. Additionally, adipocytes were incubated for 2 or 4 h in the buffer without glucose and insulin or in the presence of 5 mM glucose and 10 nM insulin.

To determine the effects of epinephrine on adiponectin secretion, isolated cells were incubated for 2 h in the medium containing 5 mM glucose and 10 nM insulin or 5 mM glucose, 10 nM insulin and 0.25, 0.5, 1 or 2 µM epinephrine. Furthermore, the effects of dibutyryl-cAMP, a non-hydrolysable cAMP analogue, on adiponectin secretion were investigated. In these experiments, fat cells were incubated for 2 h with 5 mM glucose and 10 nM insulin alone or in the combination with 0.5, 1 or 2 mM dibutyryl-cAMP.

The role of adipocyte-derived adenosine in the short-term regulation of adiponectin secretion was also determined. For this purpose, adipocytes were incubated for 2 h with 5 mM glucose and 10 nM insulin alone or in the presence of 0.5, 1 or 2 µM DPCPX, an adenosine A₁ receptor antagonist.

Additionally, the effects of palmitate and 2-bromopalmitate on adiponectin secretion from isolated rat adipocytes were studied. In these experiments, fat cells were incubated for 2 h in the medium containing 5 mM glucose with 10 nM insulin alone or in the presence of palmitate or 2-bromopalmitate. Both fatty acids were tested at concentrations 0.06, 0.125, 0.25 or 0.5 mM. In the experiments with palmitate and 2-bromopalmitate, the concentration of albumin in the buffer was diminished to 0.1 %.

The effects of diminished ATP concentration on adiponectin secretion were also tested. In this part of the study, adipose cells were incubated for 2 h with 5 mM glucose and 10 nM insulin or were exposed to glucose and insulin in the presence of 1.25, 2.5, 5 and 10 μ M rotenone, a potent and specific inhibitor of mitochondrial electron transport.

Determination of adiponectin, glycerol and ATP

At the end of each incubation, adipocytes were removed and adiponectin concentrations in the medium were measured by radioimmunoassay using kits provided by Linco Research, Inc. (USA).

Epinephrine, dibutyryl-cAMP and DPCPX are known lipolytic agents. To ensure that they are effective, in each experiment employing these agents, glycerol release from adipocytes to the incubation medium was determined. At the end of incubations, adipocytes were aspirated and aliquots of the incubation buffer were taken and frozen until analysis. The concentration of glycerol was measured colorimetrically according to the method described by Foster and Dunn (1973).

In the experiments with rotenone, ATP concentrations were determined. At the end of each incubation with rotenone, the lysis reagent was added, the tubes were vortexed, left in room temperature for a few min and the upper phase was removed. Afterwards, ATP was measured by a luminometric method using a kit containing firefly luciferase and luciferine.

Viability of adipocytes

Cell viability was assessed *via* Trypan blue exclusion. Adipocytes were incubated for 2 h at 37 °C in Krebs-Ringer buffer containing 5 mM glucose, 10 nM insulin, 3 % BSA and 10 mM HEPES. At the end of the incubation, cells were suspended in Trypan blue solution and were observed under the microscope. Viability of adipocytes was no less than 95 %.

Reagents

D-glucose, bovine serum albumin (fraction V), collagenase (EC 3.4.24.3, from *Clostridium histolyticum*, type II), insulin (from bovine pancreas), dibutyryl-cAMP, DPCPX, epinephrine, rotenone, palmitate, 2-bromopalmitate, lysis reagent (somatic cell ATP releasing reagent; Sigma catalogue symbol FLSAR), DMSO, kits used to determine ATP, trypan blue (0.4 %) and all reagents used to prepare Krebs-Ringer buffer were from Sigma (St. Louis, MO, USA). Stock solutions of rotenone, DPCPX, palmitate and 2-bromopalmitate were prepared in dimethyl sulfoxide and 5 μ l of the solution per 950 μ l of Krebs-Ringer buffer with adipocytes was added. The composition of Krebs-Ringer buffer was the following (in mM): 118 NaCl, 4.8 KCl, 1.3 CaCl₂, 1.2 KH₂PO₄, 1.2 MgSO₄, 24.8 NaHCO₃.

Statistical analysis

The means \pm S.E.M. were obtained from three independent experiments in quadruplicate and were evaluated statistically using analysis of variance and Duncan's multiple range test. Differences were considered significant at $p < 0.05$.

Results

Effects of insulin on adiponectin secretion

It was demonstrated that insulin, present in the incubation medium at concentrations 0.1, 1, 10 and 100 nM, increased secretion of adiponectin by 7 (non significant), 15, 21 and 23 %, respectively, compared with results noticed without insulin. These observations were made in the presence of 5 mM glucose (Fig. 1). Further studies revealed that the combination of 5 mM glucose and 10 nM insulin enhanced secretion of adiponectin, after 2 and 4 h of incubation, by 32 and 56 %, respectively, compared with secretion found in adipocytes incubated without glucose and insulin (Fig. 1).

Effects of epinephrine, dibutyryl-cAMP and adenosine A₁ receptor antagonist on adiponectin secretion

Adipocyte exposure to 0.25 μ M epinephrine failed to affect adiponectin secretion in the presence of 5 mM glucose and 10 nM insulin. Similar lack of effect was noticed when cells were incubated with 0.5 μ M epinephrine. Interestingly, 0.5 μ M epinephrine did not affect adiponectin secretion despite a significant rise in lipolysis. However, incubation of fat cells with 1 and 2 μ M epinephrine significantly affected both adiponectin

secretion and lipolysis. Under these conditions, secretion of adiponectin was diminished by 22 % and 25 %, respectively, compared with results obtained without epinephrine (Fig. 2).

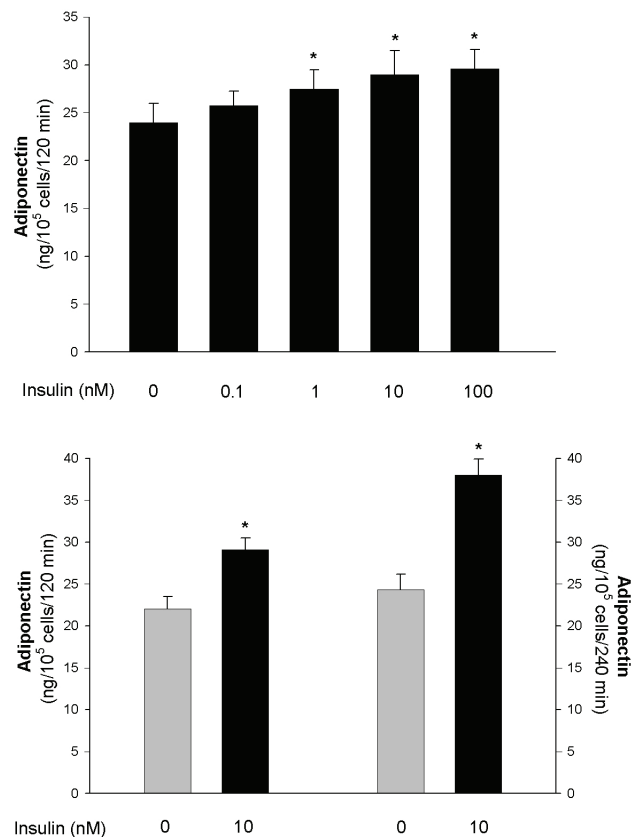


Fig. 1. The effect of glucose and insulin on adiponectin secretion from isolated rat adipocytes. Adipocytes were incubated without glucose and insulin (gray bars) or with 5 mM glucose in the presence of different concentrations of insulin (black bars). Bars represent mean \pm SEM of 12 determinations from 3 separate experiments. * Differences statistically significant vs. incubations without insulin (upper plot) or vs. incubations without insulin and glucose (lower plot), $P < 0.05$.

Secretion of adiponectin tested in the presence of 5 mM glucose and 10 nM insulin tended to be diminished by 0.5 mM dibutyryl-cAMP. This effect was not statistically significant despite increased lipolysis. However, adiponectin secretion was reduced by 24 and 37 %, respectively, when 1 and 2 mM dibutyryl-cAMP was present in the incubation buffer. This effect was accompanied by a substantial increase in lipolysis (Fig. 3).

Adenosine A₁ receptor blockade significantly affected secretion of adiponectin from isolated rat adipocytes. Fat cells incubated with 5 mM glucose and 10 nM insulin and exposed to 0.5, 1 or 2 μ M DPCPX released less adiponectin, by 23, 25 and 26 %, respectively, compared with adipocytes incubated without DPCPX. Simultaneously, 0.5, 1 and 2 μ M DPCPX significantly increased glycerol release from adipocytes to the incubation medium (Fig. 4).

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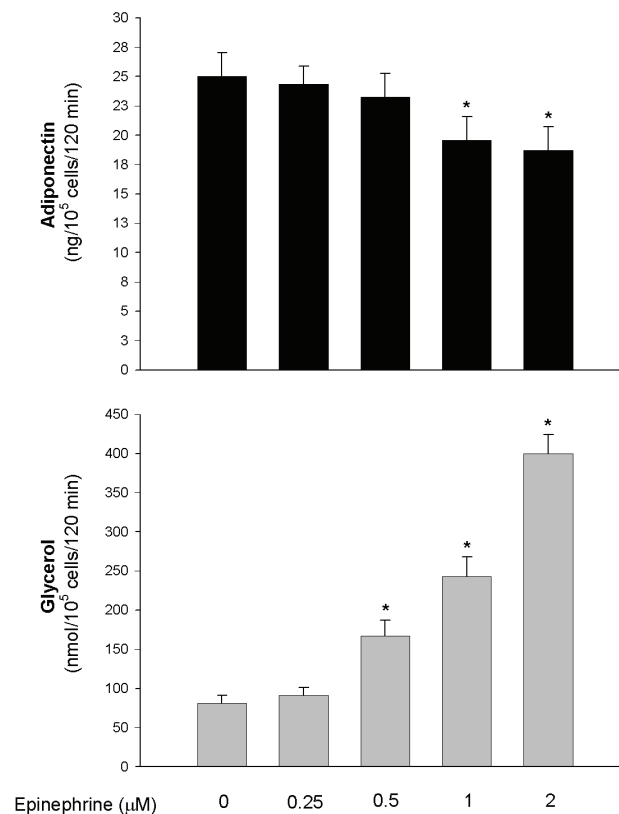


Fig. 2. The effect of epinephrine on adiponectin secretion (upper plot) or glycerol release (lower plot) from isolated rat adipocytes. Adipocytes were exposed to 5 mM glucose and 10 nM insulin without epinephrine or in the presence of different concentrations of this hormone. Bars represent mean \pm SEM of 12 determinations from 3 separate experiments. * Differences statistically significant vs. incubations without epinephrine, $P < 0.05$.

Effects of palmitate and 2-bromopalmitate on adiponectin secretion

Adipocyte exposure to 0.06, 0.125, 0.25 and 0.5 mM palmitate failed to affect secretion of adiponectin from adipocytes incubated for 2 h in the presence of 5 mM glucose and 10 nM insulin. Similarly to palmitate, its non-metabolisable analogue, 2-bromopalmitate, was also ineffective and did not change secretion of adiponectin (Fig. 5).

Effects of energy deprivation on adiponectin secretion

In the present study, adipocytes incubated with 5 mM glucose and 10 nM insulin and exposed to rotenone released less adiponectin compared with cells

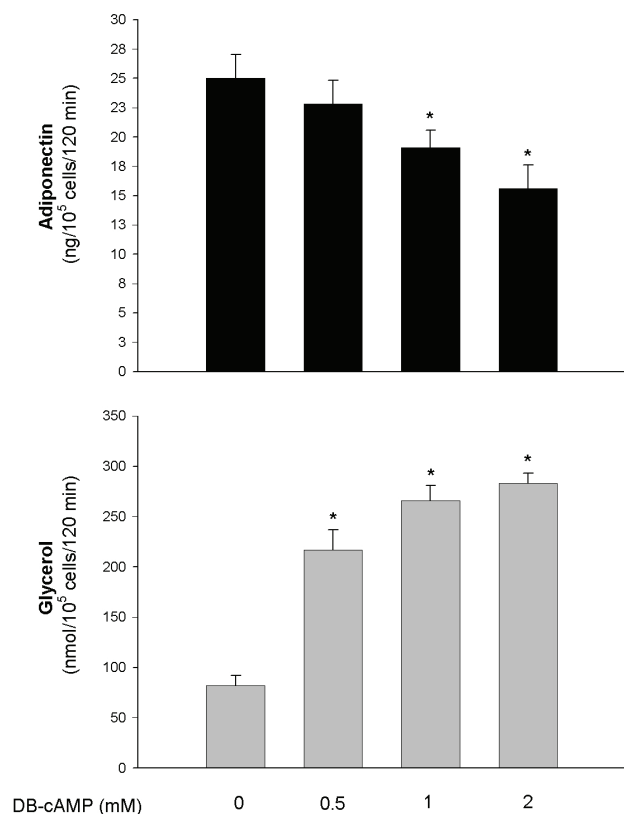


Fig. 3. The effect of dibutyl-cAMP (DB-cAMP) on adiponectin secretion (upper plot) or glycerol release (lower plot) from isolated rat adipocytes. Adipocytes were exposed to 5 mM glucose and 10 nM insulin without DB-cAMP or in the presence of different concentrations of this compound. Bars represent mean \pm SEM of 12 determinations from 3 separate experiments. * Differences statistically significant vs. incubations without DB-cAMP, $P < 0.05$.

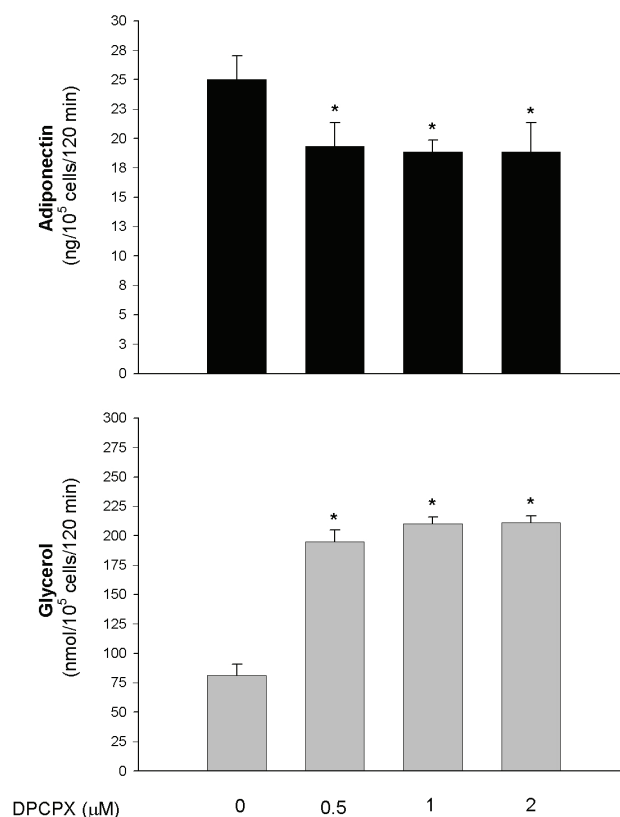


Fig. 4. The effect of adenosine A₁ receptor antagonist (DPCPX) on adiponectin secretion (upper plot) or glycerol release (lower plot) from isolated rat adipocytes. Adipocytes were exposed to 5 mM glucose and 10 nM insulin without DPCPX or in the presence of different concentrations of this compound. Bars represent mean \pm SEM of 12 determinations from 3 separate experiments. * Differences statistically significant vs. incubations without DPCPX, $P < 0.05$.

incubated with glucose and insulin without rotenone. It was found that 1.25, 2.5, 5 and 10 μ M rotenone diminished secretion of adiponectin by 45, 47, 51 and 49 %, respectively. As expected, adipocyte exposure to rotenone resulted in a profound reduction of intracellular ATP (Fig. 6).

Discussion

Insulin is one of the pivotal physiological factors regulating different adipocyte functions, including secretion of leptin. Insulin increases secretion of leptin *via* pleiotropic action involving changes in leptin gene expression, activation of mTOR (mammalian target of rapamycin), stimulation of glucose transport and metabolism, increased release of adenosine, diminution of cAMP and inhibition of lipolysis (reviewed by Szkudelski 2007). However, the regulatory role of insulin in adiponectin secretion is still elusive. Our present study revealed the stimulatory effect of insulin in the presence

of 5 mM glucose on adiponectin secretion after 2 and 4 h of incubation. The maximal secretory response to insulin, used at concentration as high as 100 nM, was 23 %. Cong *et al.* (2007) have previously shown the stimulatory effect of 20 nM insulin on adiponectin secretion from rat adipocytes incubated for 4 h in the presence of 25 mM glucose, however, after 8-24 h of exposure, insulin completely failed to enhance secretion of adiponectin. It should be also mentioned that the secretory response of adipocytes to insulin differs in various adipose tissue compartments. Motoshima *et al.* (2002) demonstrated no effect of insulin on adiponectin secretion from subcutaneous adipocytes, whereas cells obtained from omental fat tissue secreted more adiponectin upon insulin exposure. Results of our present study revealed the ability of insulin to increase secretion of adiponectin from epididymal rat adipocytes.

Taking into account data from the literature and results obtained in this study, it can be ascertained that the effects of insulin on adiponectin secretion are rather

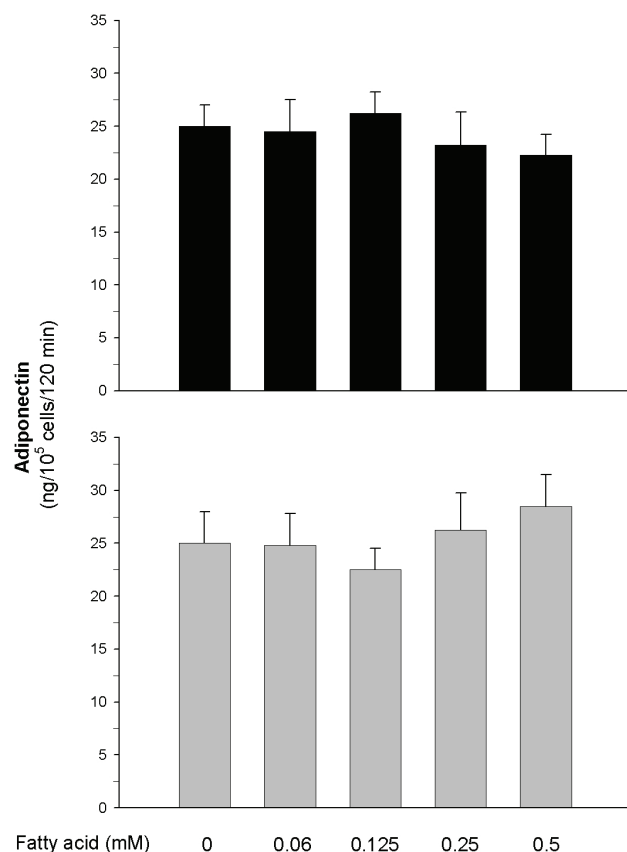


Fig. 5. The effect of palmitate (upper plot) and 2-bromopalmitate (lower plot) on adiponectin secretion from isolated rat adipocytes. Adipocytes were incubated in the presence of 5 mM glucose and 10 nM insulin without fatty acids or in the presence of different concentrations of those compounds. Bars represent mean \pm SEM of 12 determinations from 3 separate experiments.

small. This may have physiological relevance since in different conditions, such as obesity, diabetes and fasting, blood adiponectin seems not to be correlated with changes in insulinemia. It is known that plasma adiponectin is low in obesity and type 2 diabetes despite concomitant hyperinsulinemia (Weyer *et al.* 2001). In patients with type 1 diabetes, plasma adiponectin is reported to be usually increased (Lindström *et al.* 2006, Peczyńska *et al.* 2008). Moreover, fasting significantly affects adiponectinemia neither in rats (Zhang *et al.* 2002) nor in humans (Gavrila *et al.* 2003) despite reduced blood insulin.

Apart from insulin, under physiological conditions numerous factors, including epinephrine and adipocyte-derived adenosine, change intracellular cAMP and thereby affect metabolism of adipocytes. Adrenergic stimulation increases cAMP levels and enhances lipid release. This is accompanied by reduced secretion of leptin (Gettys *et al.* 1996, Cammisotto and Bukowiecki

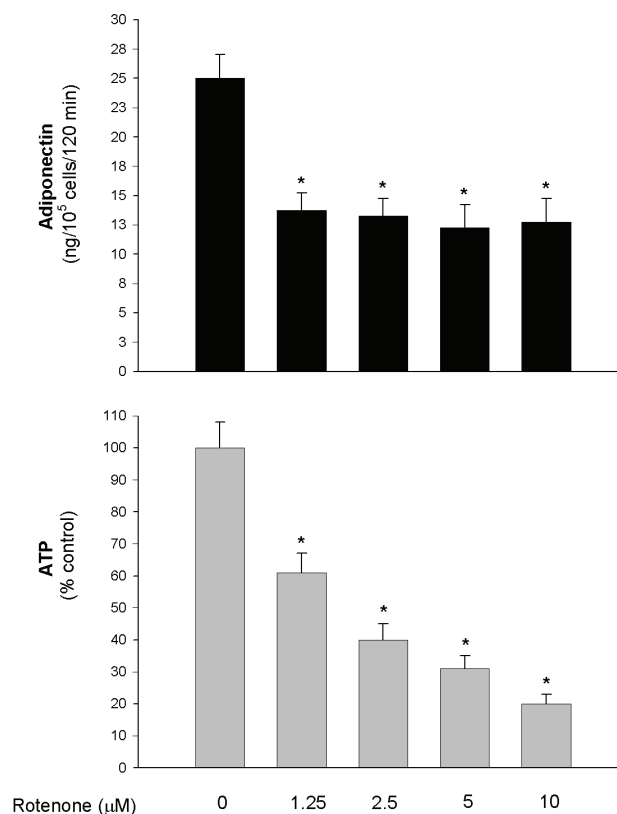


Fig. 6. The effect of rotenone on adiponectin secretion from isolated rat adipocytes (upper plot) or ATP concentrations in adipocytes (lower plot). Adipocytes were exposed to 5 mM glucose and 10 nM insulin without rotenone or in the presence of different concentrations of this compound. Bars represent mean \pm SEM of 12 determinations from 3 separate experiments. * Differences statistically significant vs. incubations without rotenone, $P < 0.05$.

2002, Szkudelski *et al.* 2005). Long-term exposure of adipocytes to different β -adrenergic agonists and cAMP analogues has been previously reported to down-regulate adiponectin mRNA (Cong *et al.* 2007). In the present study, secretion of adiponectin from fat cells incubated with glucose and insulin appeared to be reduced upon exposure for 2 h to 1 and 2 μ M epinephrine. However, under similar experimental conditions, 0.25 and 0.5 μ M epinephrine was ineffective. The lack of effects noticed at lowest concentrations of epinephrine may be explained by the presence of insulin in the incubation medium. Insulin activates cAMP phosphodiesterase 3B (PDE3B), diminishes cAMP in adipocytes and thereby attenuates effects of epinephrine (Smith *et al.* 1991, Eriksson *et al.* 1995).

This assumption is supported by results demonstrating that dibutyryl-cAMP, a cAMP analogue which is not decomposed by PDE3B, was more effective as an inhibitor of adiponectin secretion compared with

epinephrine. Moreover, it was previously shown that in adipocytes exposed for 24 h to isoprenaline, the inhibitory effect of β -adrenergic agonist on adiponectin expression and secretion was suppressed by insulin (Cong *et al.* 2007).

Adipocyte-derived adenosine is another physiological candidate, acting *via* changes in cAMP in adipocytes, that could be implicated in the regulation of adiponectin secretion. Adenosine generated in adipocytes is known to regulate pivotal functions of these cells. The nucleoside is released from fat cells, binds to adenosine A₁ receptor, decreases cAMP content causing tonic inhibition of lipolysis and enhances the lipogenic and antilipolytic action of insulin (Londos *et al.* 1978, Liang *et al.* 2002, Szkudelski *et al.* 2009). It is well established that adenosine is one of the important factors regulating secretion of leptin (Rice *et al.* 2000, Cheng *et al.* 2000, Szkudelski 2007). Results of the present study revealed for the first time that adipocyte-derived adenosine is also involved in the short-term regulation of adiponectin secretion. This conclusion arises from the observation that adipocyte exposure to DPCPX, an antagonist of adenosine A₁ receptor, resulted in a substantial inhibition of adiponectin secretion. This effect was accompanied by increased lipolysis indicating that the inhibitory effect of DPCPX on adiponectin secretion results from increased cAMP in adipocytes. Our results point that signaling *via* adenosine pathway in adipocytes is necessary for the proper secretion of adiponectin, whereas reduced action of adenosine decreases secretion of this adipokine. This may have pathophysiological relevance since the proper action of adenosine is known to be important in preventing obesity and insulin resistance (LaNoue and Martin 1994, Dong *et al.* 2001, Dhalla *et al.* 2007).

Data from the literature and results obtained in this study imply that the increase in cAMP plays an inhibitory role in both short- and long-term regulation of adiponectin secretion. The rise in cAMP concentration in adipocytes results, among others, in increased triglyceride breakdown and increased release of glycerol and fatty acids. It is possible that free fatty acids generated during lipolysis may be involved in the inhibitory effect of epinephrine and other lipolytic agents on adiponectin secretion. This effect was previously found in the case of leptin (Cammisotto *et al.* 2003). However, results of the present study demonstrated that palmitate failed to affect secretion of adiponectin. Similar lack of effects was demonstrated for 2-bromopalmitate, a nonmetabolisable palmitate analogue which is the inhibitor of mitochondrial

palmitate carnitine transferase. These results indicate that the increase in concentration of fatty acids (at least to 0.5 mM) or inhibition of their mitochondrial oxidation do not affect secretion of adiponectin from isolated rat adipocytes.

Previous studies demonstrated an important role of energy for the proper secretion of leptin from rat adipocytes (Levy *et al.* 2000). In the experiments studying the secretion of adiponectin from isolated adipocytes, a strong inverse relationship between the increase in anaerobic utilization of glucose and the decrease in adiponectin secretion after 96 h of incubation was shown (Pérez-Matute *et al.* 2007). In our present study, inhibition of ATP formation by rotenone, a potent and specific inhibitor of mitochondrial electron transport, resulted in a substantial deterioration of adiponectin secretion from fat cells incubated in the presence of glucose and insulin already after 2 h of incubation. Interestingly, the inhibitory effect resulting from ATP depletion was greater than the effect induced by epinephrine, dibutyryl-cAMP or DPCPX. These results indicate that different factors reducing ATP content in adipocytes may also decrease secretion of adiponectin. However, in our short-term studies, the inhibitory effect of rotenone on adiponectin secretion appeared not to be proportional to the depletion of ATP. This observation is in accord with our previous experiments in which secretion of leptin was investigated. In these studies, a dramatic reduction of ATP content in isolated adipocytes only slightly decreased secretion of leptin after 2 h of incubation. However, after 6 h, the effect of ATP depletion on leptin secretion was significantly greater (Szkudelska *et al.* 2009). These results allow to conclude that in short-term regulation of adiponectin and leptin secretion, even a profound depletion of ATP in rat adipocytes causes significant, but not proportional to the decrease in intracellular energy, diminution in secretion of these adipokines.

In conclusion, our results demonstrated that secretion of adiponectin from epididymal rat adipocytes is susceptible to the short-term regulation by insulin, epinephrine, adenosine and energy deprivation. It was found that insulin potentiated adiponectin secretion after 2 h of incubation, whereas epinephrine exerted the opposite effect. Moreover, blockade of adenosine action or deprivation of energy caused a clear-cut inhibition of adiponectin secretion from isolated rat adipocytes. These findings provide new information on the short-term regulation of secretion of this adipokine in rat adipocytes.

Conflict of Interest

There is no conflict of interest.

Abbreviations

DB-cAMP, dibutyryl-cAMP (N⁶, 2'-O-dibutyryl-

adenosine 3', 5'-cyclic monophosphate sodium salt); DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; DMSO, dimethyl sulfoxide; mTOR, mammalian target of rapamycin; HEPES, (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])

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