BIOCHEMICAL PHARMACOLOGY 74 (2007) 1628–1635

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

A_1 receptor deficiency causes increased insulin and glucagon secretion in mice

Stina M. Johansson [a,1,*], *Albert Salehi* [b,1], *Marie E. Sandström* [a], *Håkan Westerblad* [a], *Ingmar Lundquist* [b], *Per-Ola Carlsson* [c], *Bertil B. Fredholm* [a], *Abram Katz* [a]

[a] *Department of Physiology and Pharmacology, Karolinska Institutet, S-171 77 Stockholm, Sweden*
[b] *Department of Clinical Science, CRC, University of Lund, Malmö, Sweden*
[c] *Department of Medical Cell Biology/Department of Medical Sciences, Uppsala University, Uppsala, Sweden*

ARTICLE INFO

Article history:
Received 30 May 2007
Accepted 6 August 2007

Keywords:
Adenosine
Metabolism
Islet hormone secretion
Insulin sensitivity
Glucose uptake
A_1 receptor

ABSTRACT

Adenosine influences metabolism and the adenosine receptor antagonist caffeine decreases the risk of type 2 diabetes. In this study the metabolic role of one adenosine receptor subtype, the adenosine A_1R, was evaluated in mice lacking this receptor [A_1R (−/−)]. The HbA1c levels and body weight were not significantly different between wild type [A_1R (+/+)] and A_1R (−/−) mice (3–4 months) fed normal lab chow. At rest, plasma levels of glucose, insulin and glucagon were similar in both genotypes. Following glucose injection, glucose tolerance was not appreciably altered in A_1R (−/−) mice. Glucose injection induced sustained increases in plasma insulin and glucagon levels in A_1R (−/−) mice, whereas A_1R (+/+) control mice reacted with the expected transient increase in insulin and decrease in glucagon levels. Pancreas perfusion experiments showed that A_1R (−/−) mice had a slightly higher basal insulin secretion than A_1R (+/+) mice. The first phase insulin secretion (initiated with 16.7 mM glucose) was of the same magnitude in both genotypes, but the second phase was significantly enhanced in the A_1R (−/−) pancreata compared with A_1R (+/+). Insulin- and contraction-mediated glucose uptake in skeletal muscle were not significantly different between in A_1R (−/−) and A_1R (+/+) mice. All adenosine receptors were expressed at mRNA level in skeletal muscle in A_1R (+/+) mice and the mRNA $A_{2A}R$, $A_{2B}R$ and A_3R levels were similar in A_1R (−/−) and A_1R (+/+) mice. In conclusion, the A_1R minimally affects muscle glucose uptake, but is important in regulating pancreatic islet function.

1. Introduction

Adenosine is the endogenous ligand for four pharmacologically well defined G protein-coupled adenosine receptors, the A_1, A_{2A}, A_{2B} and A_3 receptors [1]. Caffeine can block all the adenosine receptors, although the affinity of caffeine is much higher for the A_1, A_{2A} and A_{2B} receptors than for the A_3 receptor [2]. Several, but not all, epidemiological studies have concluded that coffee consumption decreases the risk of developing non-insulin-dependent diabetes (type 2 diabetes) [3,4]. Coffee contains several thousand active components including chlorogenic acid and magnesium [2]. Nevertheless it is tempting to speculate that the inverse association between coffee and type 2 diabetes may be due to the effects of caffeine, and hence that adenosine acting on one or more of the caffeine sensitive receptors is important in the regulation of glucose

* *Corresponding author*. Tel.: +46 8 524 8 7937; fax: +46 8 34 12 80.
E-mail address: Stina.M.Johansson@ki.se (S.M. Johansson).

[1] These authors contributed equally.

0006-2952/$ – see front matter
doi:10.1016/j.bcp.2007.08.006

homeostasis. It is not certain which receptor is involved and how it transmits its signal although the results of previous studies using pharmacological approaches have suggested that the A_1R is the adenosine receptor most involved in metabolism [5–8].

Adenosine and adenosine agonists have also been shown to decrease insulin secretion [9–11] and increase glucagon secretion [12–14] in pancreas. Regarding skeletal muscle, some studies suggest that adenosine has positive effects on glucose uptake [15–18], whereas others indicate negative effects [19–22]. It is still unclear which adenosine receptor underlies these changes.

In the present study, we used A_1 knock out [A_1R (−/−)] mice to examine the role of A_1 receptors in glucose homeostasis. The results indicate that the A_1R is not critical for regulating muscle glucose uptake, but strongly influences pancreatic islet function.

2. Materials and methods

2.1. Materials

Midazolam was from Hoffmann-La Roche (Nutley, NJ). Fentanyl was from Janssen Pharmaceuticals (Neuss, Germany). Trasylol was from Bayer (Leverkusen, Germany). 2-Deoxy-D-[1,2-^{3}H]glucose (2-DG) and carboxy-[^{14}C]inulin were from Amersham Bioscience (Buckinghamshire, UK). Human insulin (Actrapid) was from Novo Nordisk (Bagsvaerd, Denmark). 2-Chloro-N^6-cyclopentyladenosine (CCPA) and tribromoethanol were from Sigma (St. Louis, MO). Scintillation liquid Ultima Gold was from Packard (Meriden, CT). Qiagen RNeasy kit was from Qiagen GmbH (Hilden, Germany). High-Capacity cDNA Archive Kit, primers and probes for real time RT-PCR and TaqMan Universal PCR Master Mix No AmpErase UNG were from Applied Biosystems (Foster City, CA). The kit for determination of insulin was obtained from Diagnostica (Falkenberg, Sweden) and the kit for glucagon determination was from Eurodiagnostica (Malmö, Sweden). Glucose reagent strips were from Medisense, Baxter Travenol (Deerfield, IL). Hemoglobin A1c (HbA1c) kit was from Roche Diagnostics GmbH (Mannheim, Germany). All other reagents and enzymes were from Sigma (St. Louis, MO), Boehringer Mannheim, GmbH (Mannheim, Germany) or Merck (Darmstadt, Germany).

2.2. Animals

Wild type A_1R (+/+) and knock out A_1R (−/−) mice with a C57BL/6 background and wild type mice (NMRI strain) were used. Male mice were used in all experiments except in the real-time RT-PCR experiments. In these experiments, both male and female mice were used. The A_1R (−/−) mice were generated as previously described [23] and back-crossed to a C57BL/6 congenic strain by Jackson Laboratory (Bar Harbor, ME) according to their general procedures for back-crossing, until the mice were determined to be congenic by 140 genomic markers. PCR-based genotyping was used to identify A_1R (−/−) offspring [24]. The mice (3–4 months old) were housed at a constant temperature (22–23 °C) and 12-h light/dark cycles with free access to standard pellet food and tap water ad libitum. All the experimental protocols were evaluated and approved by the local ethical committees in Stockholm, Uppsala or Malmö/Lund, Sweden.

2.3. In vivo glucose challenges

For the in vivo studies, glucose (1 g/kg body weight) was dissolved in 0.9% NaCl and delivered to freely fed mice by an intraperitoneal (i.p.) injection. Blood samples were taken by the retrobulbar approach as previously described [25]. In other experiments in freely fed mice, glucose (3 g/kg body weight) was delivered by intravenous injection (i.v.) into the tail vein and thereafter blood samples were taken at different time-points from the cut tip of the tail [26].

2.4. In situ pancreatic perfusion

The pancreatic perfusion experiments were performed as described previously [27]. Briefly, the mice were anesthetized with midazolam (0.4 mg/25 g body weight) and fentanyl (0.02 mg/25 g body weight) and kept on a heating pad during the entire experiment. The abdominal cavity was opened and arteries (renal, hepatic and splenic) were ligated and the aorta was tied off above the level of pancreatic artery. The pancreas was perfused with Krebs-Ringer HEPES buffer (1 ml/min) supplemented with glucose (3.3 mM) and 0.20% bovine serum albumin via a silicone catheter placed in aorta. After 10 min of infusion, the medium glucose concentration was changed to 16.7 mM. The perfusate was collected via a silicone catheter from the portal vein in tubes at different time points for further measurements of insulin and glucose levels.

2.5. Muscle stimulation

Skeletal muscles from A_1R (+/+) and A_1R (−/−) mice were used in all experiments except those with the A_1 agonist, CCPA, where muscles from NMRI mice were used. The mice were sacrificed and both extensor digitorum longus (EDL; glycolytic) and soleus (oxidative) muscles were rapidly removed. The tendons of the muscles were tied with nylon thread to stainless steel hooks and each muscle was transferred to a stimulation chamber (World Precision Instruments), which contained a Tyrode solution consisting of (in mM): NaCl, 121; KCl, 5; $CaCl_2$, 1.8; NaH_2PO_4, 0.4; $MgCl_2$, 0.5; $NaHCO_3$, 24; EDTA, 0.1; glucose, 5.5; 0.1% fetal calf serum, gassed continuously with 5% CO_2/95% O_2, yielding a pH of 7.4 [28]. The temperature was set to 25 °C. In the chamber, the muscles were mounted between a force transducer and an adjustable holder. The muscles were stretched to the length where maximum tetanic force was obtained. After a 25 min recovery period, the muscles were stimulated with current pulses (0.5 ms duration; ~150% of the voltage required for maximum force response) via plate electrodes lying parallel to the fibers. The muscles were stimulated at 50 Hz (tetanic duration 100 ms, 2 trains/s) for 10 min and immediately transferred to vials for glucose uptake measurements. In experiments where insulin-mediated glucose uptake was measured, insulin was added to the vials after a 30-min preincubation period. When CCPA (100 nM) was used, it was included in the Tyrode solution prior to addition of the muscles.

2.6. 2-Deoxy-D-[1,2-^{3}H]glucose uptake

The 2-Deoxy-D-[1,2-^{3}H]glucose (2-DG) uptake was measured both in contraction- and insulin-stimulated muscles as described elsewhere [28,29]. Briefly, the muscles were incubated in continuously gassed (as above) vials containing 1.5 ml Tyrode solution without glucose but supplemented with 2 mM pyruvate in a shaking water bath (100 oscillations/min) at 35 °C for a total of 80 (insulin, 2 or 20 mU/ml) or 40 min (contraction). 2-DG (final concentration 1 mM; 1 mCi/mmol) and carboxy-[^{14}C]inulin (0.2 μCi/ml medium, for assessment of extracellular space) were present during the last 20 min. Thereafter, muscles were blotted, frozen and added to tubes containing 0.5 ml of 1N NaOH. The muscles were weighed, digested by heating at 70 °C for 15 min, cooled, centrifuged (23,000 × *g* for 5 min) and duplicate 200 μl aliquots of the supernatant were added to 4 ml scintillation liquid (Ultima Gold) and counted for ^{3}H and ^{14}C with a scintillation counter.

2.7. Real time reverse transcription polymerase chain reaction (RT-PCR)

The muscles were dissected out and rapidly frozen. They were homogenized in a lysis buffer (Qiagen) and RNA was isolated from the muscles with Qiagen RNeasy kit according to the manufacturer's protocol (Qiagen GmbH). The cDNA synthesis was carried out with a High-Capacity cDNA Archive Kit with random primers and multiscribe reverse transcriptase enzyme according to the manufacturer's instructions (Applied Biosystems). The Applied Biosystem 2720 Terminal cycler was used for the reverse transcription and the incubation conditions were set to 25 °C for 10 min followed by 37 °C for 2 h. Detection of A_1R, A_{2A}R, A_{2B}R and A_3R mRNA was performed by using real time RT-PCR [30]. The real time RT-PCR reactions were run in an ABI Prism 7500 Sequence Detector System (Applied Biosystems, Foster City, CA). Each run consisted of 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Primers (900 nM of each primer per reaction) and probes (200 nM per reaction) used for the adenosine receptors are described elsewhere [31]. All the reactions were performed in triplicate. TaqMan Universal PCR No AmpErase UNG master mix was used in all reactions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin were used as endogenous controls. The difference in the number of cycles needed to reach the detection threshold (ct = cycle at threshold) using GAPDH and β-actin as reference ($\Delta ct = ct_{adenosine\ receptor} - ct_{endogenous\ control}$) was calculated. ΔΔct was calculated as ($\Delta\Delta ct = (\Delta ct_{adenosine\ receptor})_{sample} - (\Delta ct_{adenosine\ receptor})_{calibrator\ (A1R\ (+/+)\ mouse)}$) in the experiments where the mRNA expression between A_1R (+/+) and A_1R (−/−) mice was compared. The ΔΔct-value was finally expressed as relative fold change in gene expression (mean ± 95% confidence interval) by using the $2^{-(\Delta\Delta ct)}$ formula.

2.8. Body weight and hemoglobin A1c (HbA1c)

The body weights of the mice were measured before experiments were performed. For the HbA1c determination, the mice were decapitated and blood samples were collected from the site of decapitation and heparinized.

2.9. Analyses of blood samples

Plasma glucose (i.p. glucose tolerance test) was determined enzymatically and insulin and glucagon with a radioimmunoassay (RIA) as described previously [32–34]. Blood glucose concentrations (i.v. glucose tolerance test) were measured with glucose reagent strips. HbA1c levels were determined with a kit according to the manufacturer's instructions (Roche Diagnostics GmbH).

2.10. Statistics

Levels of significance ($p < 0.05$) between means were assessed using Student's t-test for unpaired data or an ANOVA followed by Tukey–Kramer's multiple comparisons test where appropriate. Values are presented as mean ± S.E.M. unless otherwise stated.

3. Results

3.1. Body weight and HbA1c levels

Body weight and HbA1c values were not significantly different between A_1R (+/+) and A_1R (−/−) mice (Table 1). The HbA1c data thus do not indicate an abnormal blood glucose status over a longer period of time in the A_1R (−/−) mice. The A_1R (−/−) mice fed normal lab chow had a normal body weight despite the fact that a major antilipolytic factor (the A_1R) has been eliminated [5,35,36]. The weight of the abdominal adipose tissue was also not significantly different between genotypes (data not shown).

3.2. In vivo glucose tolerance and pancreatic hormone response after glucose challenge

In vivo glucose tolerance was investigated by a glucose challenge in freely fed mice. There were no significant differences in blood/plasma glucose concentrations in the basal state. The changes in the glucose concentrations measured at different time-points after glucose injection in A_1R (−/−) mice (i.p., Fig. 1A or i.v., Fig. 1B) were not appreciably different from those measured in A_1R (+/+) animals. Thus glucose tolerance was not significantly altered in A_1R (−/−) mice.

The pancreatic hormone responses were measured when glucose was administered i.p. to freely fed mice. The basal plasma insulin levels did not differ between the A_1R (+/+) and A_1R (−/−) mice. In A_1R (+/+) mice, glucose injection resulted in a rapid increase of plasma insulin concentration. However, by 10 min after the glucose challenge the insulin levels had

Table 1 – Body weight and HbA1c levels in A_1R (+/+) and A_1R (−/−) mice. Values are mean ± S.E.M. for 8–11 mice of each genotype

Genotype	A_1R (+/+)	A_1R (−/−)
Body weight (g)	30.9 ± 0.2	31.3 ± 1.5
HbA1c (%)	2.7 ± 0.1	2.7 ± 0.1

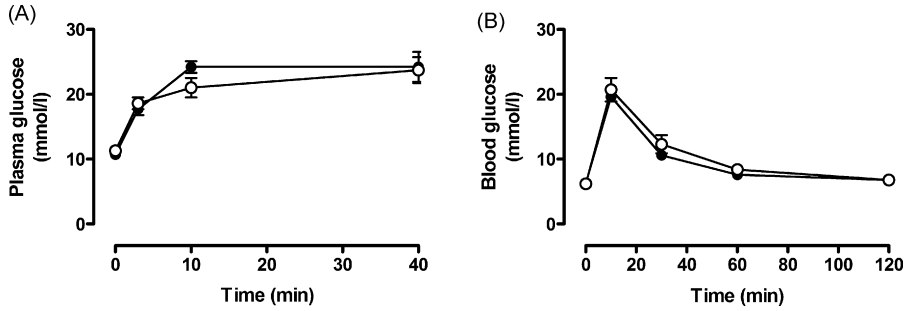

Fig. 1 – Circulating glucose levels in A_1R (+/+) (●) and A_1R (−/−) (○) freely fed mice at different time points after i.p. glucose injection (1 g/kg body weight) (A) or i.v. glucose injection (3 g/kg body weight) (B). Values are mean ± S.E.M., *n* = 8–10 mice of each genotype.

returned to basal values. In A_1R (−/−) mice, the glucose-induced increase in plasma insulin was maintained until the end of the 40 min period of measurement (Fig. 2A). The basal glucagon levels were also similar in A_1R (+/+) and A_1R (−/−) mice. In A_1R (+/+) mice, as expected, the glucose load suppressed plasma glucagon levels, but paradoxically glucagon levels increased continuously in A_1R (−/−) mice (Fig. 2B).

3.3. *Phasic insulin release from adenosine A_1R (−/−) mice measured by in situ pancreatic perfusion*

To assess the role of A_1R in the dynamics of insulin release, we performed in situ pancreatic perfusions with fractionated sampling. Fig. 3 illustrates that basal insulin release (first 10 min with 3.3 mM glucose) from perfused pancreas of A_1R (−/−) mice was slightly higher compared to A_1R (+/+) controls. Glucose stimulated insulin release showed a typical biphasic pattern when the glucose concentration in perfusate was raised from 3.3 to 16.7 mM in the A_1R (+/+) mice. Thus after the glucose concentration increased to 16.7 mM (Fig. 3B), first-phase insulin release was initiated and it lasted for 2–3 min. When the same experiment was repeated in adenosine A_1R (−/−) pancreata (Fig. 3), the peak in first-phase insulin secretion was of the same magnitude as that seen in A_1R (+/+) mice (*n* = 4). However, ablation of A_1R brought about a significant marked increase in second-phase insulin secretion representing a 100% increase compared to the A_1R (+/+) mice ($P < 0.005$). There were no differences in the response to a high potassium pulse (results not shown).

3.4. *Adenosine receptor expression and glucose uptake in muscle tissue*

The expression of the adenosine receptors in skeletal muscles was measured. All adenosine receptors were present at mRNA level in both EDL and soleus muscles in A_1R (+/+) mice. The Δct values for the different receptors in the A_1R (+/+) mouse were: 11.2 (A_1R), 9.1 ($A_{2A}R$), 10.9 ($A_{2B}R$) and 14.9 (A_3R) in soleus and 11.6 (A_1R), 9.2 ($A_{2A}R$), 10.6 ($A_{2B}R$) and 13.4 (A_3R) in EDL. The A_1R was not present in A_1R (−/−) mice (ct value—not detectable). When the mRNA levels of the adenosine receptors that were present in both the A_1R (+/+) and A_1R (−/−) mice were compared, no significant differences in mRNA levels of the $A_{2A}R$, $A_{2B}R$ and A_3R in EDL and soleus between the A_1R (+/+) and A_1R (−/−) mice were found (Fig. 4). These results are in agreement with previous studies, where it was shown that our A_1R (−/−) mice did not have an altered expression of the other adenosine receptors in heart tissue [38].

The observation that the glucose tolerance test (GTT) elicited a normal blood glucose response, but an increased plasma insulin response in A_1R (−/−) mice suggested a peripheral insulin resistance in these mice. Therefore, glucose uptake measurements were performed on isolated muscles. Glucose uptake in soleus and EDL muscles was similar in A_1R (−/−) and A_1R (+/+) mice in the basal state and after exposure to insulin (2 or 20 mU/ml) (Fig. 5). There was also no difference in contraction-mediated glucose uptake between groups (Fig. 5). The effect of the A_1R agonist CCPA (100 nM) on glucose uptake in the presence of saturating insulin was also studied in wild type (NMRI) mice. CCPA did not significantly

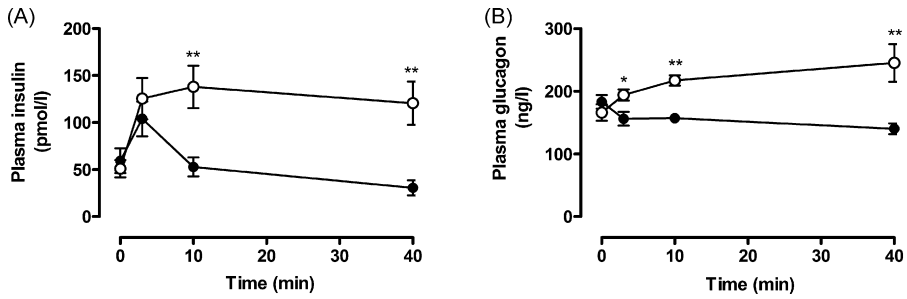

Fig. 2 – Plasma insulin (A) and glucagon (B) response to i.p. glucose injection (1 g/kg body weight). Values are mean ± S.E.M. for A_1R (+/+) (●) and A_1R (−/−) (○) mice. *n* = 8–10 mice of each genotype. $^*p < 0.05$, $^{**}p < 0.01$ between groups.

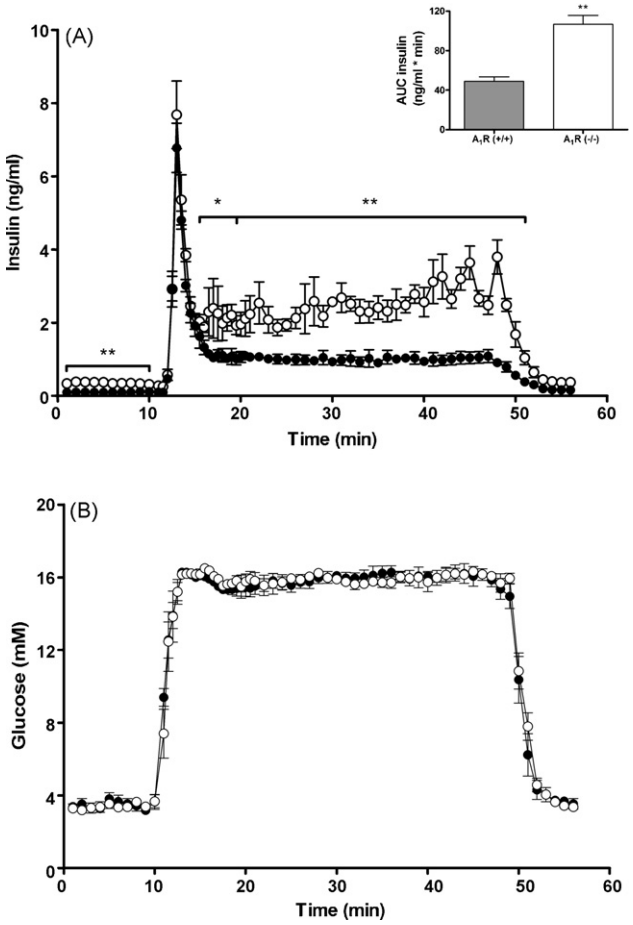

Fig. 3 – Dynamics of insulin release from perfused pancreata in A_1R (+/+) and A_1R (−/−) mice. (A) Insulin release measured in adenosine A_1R (+/+) and A_1R (−/−) pancreata before and after the glucose concentration in the perfusate was increased from 3.3 to 16.7 mM. Samples were taken at 60-s intervals, except during the first 10 min after increasing the glucose concentration (t = 11–21 min, when the sample interval was 30 s. Values are mean ± S.E.M. for A_1R (+/+) (●) and A_1R (−/−) (○) mice. n = 4 mice of each genotype. The insert shows AUC of the data. (B) An illustration of glucose levels in the same samples shown in A. Statistical significance is provided for comparisons between A_1R (+/+) and A_1R (−/−) mice are denoted by asterisks (*) $^{*}p < 0.05$, $^{**}p < 0.01$.

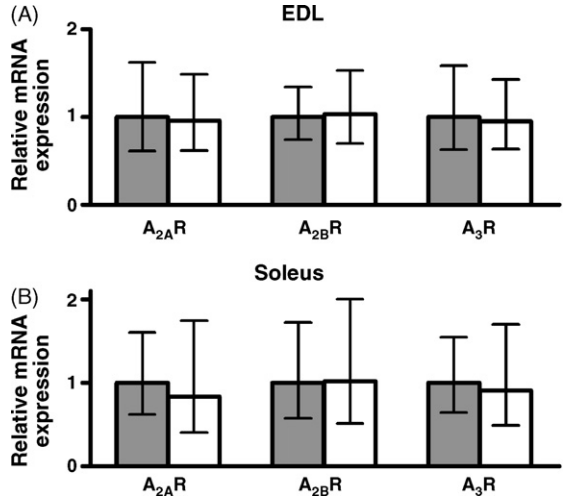

Fig. 4 – Relative mRNA levels of the adenosine A_1, A_{2A}, A_{2B} and A_3 receptors in EDL (A) and soleus (B) muscles in A_1R (+/+) (▩) mice compared to A_1R (−/−) (□) mice. Values are means (mean values in A_1R (+/+) mice are set to 1) and the error bars represent 95% confidence intervals, n = 3–8 mice in each group.

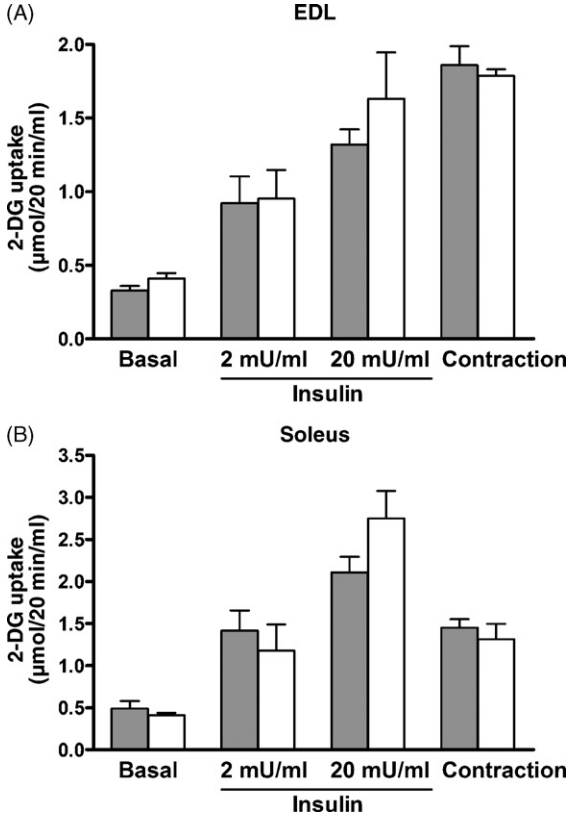

Fig. 5 – 2-DG uptake in EDL and soleus muscles. Values are mean ± S.E.M., n = 6 mice of each genotype. Data were obtained in the basal state, after exposure to insulin or after repeated contractions in A_1R (+/+) (▩) and A_1R (−/−) (□) mice.

affect insulin-mediated 2-DG uptake (see Supplemental material, Fig. S1). Nor did CCPA have a significant effect when the muscles were stimulated with intermediate concentrations of insulin (0.2 and 0.6 mU/ml) (data not shown).

4. Discussion

The major findings of the present study are: (1) an essentially normal glucose homeostasis and (2) altered pancreatic hormone responses to a glucose challenge in A_1R (−/−) mice fed normal lab chow. That glucose levels were normal despite elevated plasma insulin responses to the glucose challenge in

the A_1R (−/−) mice suggested the presence of peripheral insulin resistance. Since skeletal muscle is quantitatively the most important organ for glucose disposal during hyperinsulinemia [39], glucose uptake experiments were performed on isolated skeletal muscles. However, the A_1R agonist, CCPA, did not affect insulin-mediated glucose uptake. This is in apparent conflict with several previous studies on rat muscle [16,17], and could perhaps be due to high endogenous levels of adenosine. However, this possibility is less likely, as there were no significant differences between A_1R (−/−) and A_1R (+/+) mice in terms of basal or insulin-mediated glucose uptake in either EDL or soleus muscles. Nor were there any significant differences between the genotypes in contraction-mediated glucose uptake. Thus both genetic and pharmacological approaches yielded consistent results to indicate that A_1Rs do not play a significant role in the control of muscle glucose uptake under several physiological conditions (basal, exercise and insulin) in muscle from young fed mice.

Pharmacological studies indicate that adenosine receptors are present in skeletal muscles [15,16,18,37], although the expression of the different adenosine receptors in skeletal muscle (EDL and soleus) in mice at mRNA level has not been clarified. Therefore the expression of the adenosine receptors was measured and we verified that mRNA for all adenosine receptors were present in EDL and soleus muscles of mice. This suggests that the receptor proteins are also present. We do not know, however, if all receptors are present in the same type of cell.

If the muscles are not insulin resistant, then the higher plasma insulin levels in the A_1R (−/−) mice during the GTT should be associated with higher rates of peripheral (i.e. muscle) glucose utilization. This should result in lower blood glucose concentrations during the GTT. One possible reason why this was not observed could be a compensatory release of glucose from the liver. There are two potential mechanisms whereby this could have occurred: the elevated glucagon levels in the A_1R (−/−) mice stimulate glycogenolysis in the liver; or insulin-mediated inhibition of hepatic gluconeogenesis [40] is defective in the A_1R (−/−) mice. These explanations are not mutually exclusive and thus both mechanisms may be involved. The net effect would be a maintained blood glucose level in the face of a higher glucose turnover in the A_1R (−/−) mice.

These data do not rule out the possibility that A_1Rs play a significant role in glucose transport in tissues other than skeletal muscle. It has for example been shown that adenosine stimulates and adenosine deaminase inhibits glucose uptake in adipose tissue [41,42] and heart [43,44].

The increased glucose-stimulated insulin release and the paradoxical glucagon response after glucose injection in the A_1R (−/−) mice suggested a role for the A_1R in pancreatic islet function. Therefore we examined insulin secretion in the perfused pancreas. Previous studies have shown that adenosine and its analogues can affect insulin secretion [45]. For example, it has been shown that the adenosine analogue phenylisopropyladenosine (PIA) can inhibit glucose-stimulated insulin secretion [10,46] and the A_1R has been postulated to be the adenosine receptor involved [11,46]. One hypothesis that has been proposed to explain the adenosine-mediated inhibition of insulin secretion is that the A_1R is coupled to the inhibitory G_i protein and a stimulation of A_1R thereby will inhibit adenylyl cyclase, which will result in lower cellular cAMP levels. cAMP stimulates the release of insulin [47]. Thus deletion of the A_1R would be expected to result in an increased insulin secretion. Recently, however, Rüsing et al. [48] showed that another second messenger system must also be involved in the adenosine-mediated inhibition of insulin secretion since their results showed that agonists to both G_i-coupled adenosine receptors (A_1R and A_3R) and G_s-coupled adenosine receptor ($A_{2A}R$) were able to decrease the plasma insulin levels in rats and reduce insulin secretion in INS-1 cells. Antagonists to the G_s-coupled $A_{2B}R$ were also able to counteract the inhibitory effect of the adenosine analogue, 5′-*N*-ethylcarboxamidoadenosine (NECA). Our current findings are consistent with the idea that the A_1R is involved in insulin secretion. The function of the other adenosine receptors and the intracellular signal transduction pathways behind the involvement of the adenosine receptors in insulin secretion await further evaluation.

Glucose-induced insulin release follows a biphasic time course. A transient first phase is followed by a more or less pronounced second phase depending on the amount of glucose given. Our present results both in vivo and in situ show that the A_1R is involved in the second phase, but not in the first phase of insulin secretion. The mechanisms behind the two phases of release are still unclear but it has been suggested that the rapid first phase reflects the release of a limited pool of readily releasable granules in close proximity to the L-type Ca^{2+} channels in the plasma membrane, while the second phase is regulated by replenishment of this pool by granules originating from a ''reserve pool'' situated at a greater distance from the channels [49]. Hence the A_1Rs seem to influence the mobilization of the granules from this ''reserve pool''.

Glucagon is known as an amplifier of insulin secretion [50]. It is thus possible that the elevated glucagon levels after the initial phase of the GTT (i.e. >5 min) enhanced insulin secretion by the β cells. High cAMP levels in the α cells can increase the secretion of glucagon and therefore we also consider a role for the A_1R in glucagon release. Previous studies [12,13] have shown that adenosine can stimulate glucagon release, but this effect is probably mediated by A_2 receptors (unknown whether A_{2A} or A_{2B}) that couple to stimulatory G proteins [14]. If A_1Rs are present in α cells, stimulation of these receptors could be expected to inhibit glucagon release. Conversely, if the A_1R is lacking, glucagon release would be expected to be stimulated.

These studies thus suggest that although the basal plasma glucose and insulin levels are essentially normal in the A_1R (−/−) mice, there are major perturbations that could influence the animal's ability to cope with metabolic stress. Future studies with animals that are older than our mice (>3–4 months) or stressed with a high fat diet would be a good complement to this study. There is also good reason to perform studies under fasting conditions. Even an overnight fast represents a major metabolic stress in mice and there is evidence (H. Edlund, Umeå University, personal communication) that 12 h fasting leads to lower basal glucose levels and improved glucose tolerance in A_1R (−/−) mice. Furthermore, investigation of the mechanisms behind the pancreatic

hormone response in the A_1R (−/−) mice would also give more information about the role of the A_1R in metabolism. These studies should also be of value to assess the feasibility of using a drug acting on the A_1R in treatment of metabolic disorders such as type 2 diabetes mellitus.

In conclusion, this study suggests that A_1Rs are important in regulating pancreatic islet function, but have less influence on muscle glucose transport. Furthermore, our results raise the question whether altered function of A_1Rs in the pancreas is involved in the pathogenesis of type 2 diabetes.

Acknowledgements

We thank Astrid Nordin and Britt-Marie Nilsson for skilled technical assistance. We thank Dr. Eva Björkstrand for help with the HbA1c determinations, Dr Shi-Jin Zhang for help with dissecting out muscles and Dr Elisabetta Daré for help with the real time RT-PCR. The studies were supported by grants from the Swedish Research Council, Novo Nordisk Foundation, the Swedish Diabetes Foundation, Albert Påhlsson Foundation, Crafoord Foundation and by Biovitrum.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2007.08.006.

REFERENCES

[1] Fredholm BB, IJzerman AP, Jacobson KA, Klotz KN, Linden J. International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. Pharmacol Rev 2001;53:527–52.
[2] Fredholm BB, Bättig K, Holmén J, Nehlig A, Zvartau EE. Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. Pharmacol Rev 1999;51:83–133.
[3] van Dam RM, Hu FB. Coffee consumption and risk of type 2 diabetes: a systematic review. J Am Med Assoc 2005;294:97–104.
[4] van Dam RM, Feskens EJ. Coffee consumption and risk of type 2 diabetes mellitus. Lancet 2002;360:1477–8.
[5] Schwabe U, Schonhofer PS, Ebert R. Facilatation by adenosine of the action of insulin on the accumulation of adenosine 3′,5′-monophosphate lipolysisand glucose oxidation in isolated fat cells. Eur J Biochem 1974;46:537.
[6] Crist GH, Xu B, Lanoue KF, Lang CH. Tissue-specific effects of in vivo adenosine receptor blockade on glucose uptake in Zucker rats. FASEB J 1998;12:1301–8.
[7] Dong Q, Ginsberg HN, Erlanger BF. Overexpression of the A1 adenosine receptor in adipose tissue protects mice from obesity-related insulin resistance. Diabetes Obes Metab 2001;3:360–6.
[8] Xu B, Berkich DA, Crist GH, LaNoue KF. A1 adenosine receptor antagonism improves glucose tolerance in Zucker rats. Am J Physiol 1998;274:E271–9.
[9] Ismail NA, El Denshary EE, Montague W. Adenosine and the regulation of insulin secretion by isolated rat islets of Langerhans. Biochem J 1977;164:409–13.
[10] Campbell IL, Taylor KW. Effects of adenosine, 2-deoxyadenosine and N6-phenylisopropyladenosine on rat islet function and metabolism. Biochem J 1982;204:689–96.
[11] Hillaire-Buys D, Gross R, Loubatières-Mariani MM, Ribes G. Effect of pertussis toxin on A1-receptor-mediated inhibition of insulin secretion. Br J Pharmacol 1989;96:3–4.
[12] Petrack B, Czernik AJ, Ansell J, Cassidy J. Potentiation of arginine-induced glucagon secretion by adenosine. Life Sci 1981;28:2611–5.
[13] Chapal J, Loubatières-Mariani MM, Roye M, Zerbib A. Effects of adenosine, adenosine triphosphate and structural analogues on glucagon secretion from the perfused pancreas of rat in vitro. Br J Pharmacol 1984;83:927–33.
[14] Chapal J, Loubatières-Mariani MM, Petit P, Roye M. Evidence for an A2-subtype adenosine receptor on pancreatic glucagon secreting cells. Br J Pharmacol 1985;86:565–9.
[15] Vergauwen L, Hespel P, Richter EA. Adenosine receptors mediate synergistic stimulation of glucose uptake and transport by insulin and by contractions in rat skeletal muscle. J Clin Invest 1994;93:974–81.
[16] Han DH, Hansen PA, Nolte LA, Holloszy JO. Removal of adenosine decreases the responsiveness of muscle glucose transport to insulin and contractions. Diabetes 1998;47:1671–5.
[17] Cheng JT, Chi TC, Liu IM. Activation of adenosine A1 receptors by drugs to lower plasma glucose in streptozotocin-induced diabetic rats. Auton Neurosci 2000;83:127–33.
[18] Thong FS, Derave W, Kiens B, Graham TE, Ursø B, Wojtaszewski JF, et al. Caffeine-induced impairment of insulin action but not insulin signaling in human skeletal muscle is reduced by exercise. Diabetes 2002;51:583–90.
[19] Leighton B, Lozeman FJ, Vlachonikolis IG, Challiss RA, Pitcher JA, Newsholme EA. Effects of adenosine deaminase on the sensitivity of glucose transport, glycolysis and glycogen synthesis to insulin in muscles of the rat. Int J Biochem 1988;20:23–7.
[20] Budohoski L, Challiss RA, McManus B, Newsholme EA. Effects of analogues of adenosine and methyl xanthines on insulin sensitivity in soleus muscle of the rat. FEBS Lett 1984;167:1–4.
[21] Espinal J, Challiss RA, Newsholme EA. Effect of adenosine deaminase and an adenosine analogue on insulin sensitivity in soleus muscle of the rat. FEBS Lett 1983;158:103–6.
[22] Challis RA, Budohoski L, McManus B, Newsholme EA. Effects of an adenosine-receptor antagonist on insulin-resistance in soleus muscle from obese Zucker rats. Biochem J 1984;221:915–7.
[23] Johansson B, Halldner L, Dunwiddie TV, Masino SA, Poelchen W, Giménez-Llort L, et al. Hyperalgesia, anxiety, and decreased hypoxic neuroprotection in mice lacking the adenosine A1 receptor. Proc Natl Acad Sci USA 2001;98:9407–12.
[24] Turner CP, Seli M, Ment L, Stewart W, Yan H, Johansson B, et al. A1 adenosine receptors mediate hypoxia-induced ventriculomegaly. Proc Natl Acad Sci USA 2003;100:11718–22.
[25] Rerup C, Lundquist I. Blood glucose level in mice. 1. Evaluation of a new technique of multiple serial sampling. Acta Endocrinol 1966;52:357–67.
[26] Johansson M, Jansson L, Carlsson PO. Improved vascular engraftment and function of autotransplanted pancreatic islets as a result of partial pancreatectomy in the mouse and rat. Diabetologia 2007;50:1257–66.
[27] Jing X, Li DQ, Olofsson CS, Salehi A, Surve VV, Caballero J, et al. CaV2.3 calcium channels control second-phase insulin release. J Clin Invest 2005;115:146–54.

[28] Sandström ME, Zhang SJ, Bruton J, Silva JP, Reid MB, Westerblad H, et al. Role of reactive oxygen species in contraction-mediated glucose transport in mouse skeletal muscle. J Physiol 2006;575:251–62.
[29] Shashkin P, Koshkin A, Langley D, Ren JM, Westerblad H, Katz A. Effects of CGS 9343B (a putative calmodulin antagonist) on isolated skeletal muscle. Dissociation of signaling pathways for insulin-mediated activation of glycogen synthase and hexose transport. J Biol Chem 1995;270:25613–8.
[30] Halldner L, Lopes LV, Daré E, Lindström K, Johansson B, Ledent C, et al. Binding of adenosine receptor ligands to brain of adenosine receptor knock-out mice: evidence that CGS 21680 binds to A1 receptors in hippocampus. Naunyn Schmiedebergs Arch Pharmacol 2004;370:270–8.
[31] Chunn JL, Young HW, Banerjee SK, Colasurdo GN, Blackburn MR. Adenosine-dependent airway inflammation and hyperresponsiveness in partially adenosine deaminase-deficient mice. J Immunol 2001;167:4676–85.
[32] Heding L. A simplified insulin radioimmunoassaymethod. In: Donato L, Milhaud G, Sirchis J, editors. Labelled proteins in tracer studies. Brussels: Euratom; 1966. p. 345–50.
[33] Panagiotidis G, Salehi AA, Westermark P, Lundquist I. Homologous islet amyloid polypeptide: effects on plasma levels of glucagon, insulin and glucose in the mouse. Diabetes Res Clin Pract 1992;18:167–71.
[34] Salehi A, Chen D, Håkanson R, Nordin G, Lundquist I. Gastrectomy induces impaired insulin and glucagon secretion: evidence for a gastro-insular axis in mice. J Physiol 1999;514:579–91.
[35] Vannucci SJ, Klim CM, Martin LF, LaNoue KF. A1-adenosine receptor-mediated inhibition of adipocyte adenylate cyclase and lipolysis in Zucker rats. Am J Physiol 1989;257:E871–8.
[36] Fredholm BB. Effect of adenosine, adenosine analogues and drugs inhibiting adenosine inactivation on lipolysis in rat fat cells. Acta Physiol Scand 1978;102:191–8.
[37] Cheng JT, Liu IM, Chi TC, Shinozuka K, Lu FH, Wu TJ, et al. Role of adenosine in insulin-stimulated release of leptin from isolated white adipocytes of Wistar rats. Diabetes 2000;49:20–4.
[38] Yang JN, Tiselius C, Daré E, Johansson B, Valen G, Fredholm BB. Sex differences in mouse heart rate and body temperature and in their regulation by adenosine A1 receptors. Acta Physiol (Oxf) 2007;190:63–75.
[39] DeFronzo RA, Gunnarsson R, Björkman O, Olsson M, Wahren J. Effects of insulin on peripheral and splanchnic glucose metabolism in noninsulin-dependent (type II) diabetes mellitus. J Clin Invest 1985;76:149–55.
[40] Claus TH, Pilkis SJ. Regulation by insulin of gluconeogenesis in isolated rat hepatocytes. Biochim Biophys Acta 1976;421:246–62.
[41] Joost HG, Steinfelder HJ. Modulation of insulin sensitivity by adenosine. Effects on glucose transport, lipid synthesis, and insulin receptors of the adipocyte. Mol Pharmacol 1982;22:614–8.
[42] Martin SE, Bockman EL. Adenosine regulates blood flow and glucose uptake in adipose tissue of dogs. Am J Physiol 1986;250:H1127–35.
[43] Law WR, Raymond RM. Adenosine potentiates insulin-stimulated myocardinal glucose uptake in vivo. Am J Physiol 1988;254:970–5.
[44] Angello DA, Berne RM, Coddington NM. Adenosine and insulin mediate glucose uptake in normoxic rat hearts by different mechanisms. Am J Physiol 1993;265:H880–5.
[45] Hillaire-Buys D, Chapal J, Bertrand G, Petit P, Loubatières-Mariani MM. Purinergic receptors on insulin-secreting cells. Fundam Clin Pharmacol 1994;8:117–27.
[46] Hillaire-Buys D, Bertrand G, Gross R, Loubatières-Mariani MM. Evidence for an inhibitory A1 subtype adenosine receptor on pancreatic insulin-secreting cells. Eur J Pharmacol 1987;136:109–12.
[47] Hashiguchi H, Nakazaki M, Koriyama N, Fukudome M, Aso K, Tei C. Cyclic AMP/cAMP-GEF pathway amplifies insulin exocytosis induced by Ca2+ and ATP in rat islet beta-cells. Diabetes Metab Res Rev 2006;22:64–71.
[48] Rüsing D, Müller CE, Verspohl EJ. The impact of adenosine and A(2B) receptors on glucose homoeostasis. J Pharm Pharmacol 2006;58:1639–45.
[49] Olofsson CS, Göpel SO, Barg S, Galvanovskis J, Ma X, Salehi A, et al. Fast insulin secretion reflects exocytosis of docked granules in mouse pancreatic B-cells. Pflugers Arch 2002;444:43–51.
[50] Samols E, Marri G, Marks V. Interrelationship of glucagon, insulin and glucose. The insulinogenic effect of glucagon. Diabetes 1966;15:855–66.