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Biochemical Pharmacology 68 (2004) 1309–1320
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Insulin and glucose induced changes in expression level of nucleoside transporters and adenosine transport in rat T lymphocytes

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Received 7 May 2004; accepted 14 June 2004

Abstract

Adenosine is an endogenous agent exerting potent action on the immune system including regulation of lymphocyte functioning. Impaired T lymphocyte functioning is a common feature of diabetes. The aims of this study were to examine the effects of glucose and insulin on nucleoside transporters (NT) expression level and adenosine (Ado) transport in rat T lymphocytes cultured under the defined concentrations of glucose and insulin. Performed experiments revealed that rat T lymphocytes expressed the equilibrative nucleoside transporter type 1 and 2 (rENT1, rENT2) and concentrative nucleoside transporter type 2 (rCNT2). The mRNA levels of rENT2 and rCNT2 were highly dependent on insulin but were not affected by changes in extracellular glucose concentration. Exposition of T cells to 10 nM insulin resulted in 73% increase in rENT2 mRNA and 50% decrease in the rCNT2 mRNA level. The level of rENT1 mRNA was sensitive to extracellular glucose concentration but not to insulin. The highest differences among cells cultured in high (20 mM) and low (5 mM) glucose were observed in equilibrative nitrobenzylthioinosine sensitive adenosine transport, which was lowered by 65% in cells cultured at high glucose. Alterations in adenosine transport were accompanied by changes in adenosine accumulation in the cell. These results indicate that adenosine transport in rat T lymphocytes is independently and differentially regulated by glucose and insulin by means of changes in the nucleoside transporters expression level. Altered adenosine transport has a great impact on its intracellular level. This suggests that under diabetic conditions adenosine action on T lymphocytes might be altered.

Keywords: Insulin; Glucose; Nucleoside transporters; Adenosine; T lymphocytes

1. Introduction

Adenosine is an endogenous agent exerting a potent action on a wide variety of physiological systems, including the cardiovascular, respiratory, gastrointestinal, urogenital, nervous, and lymphatic system. Long-standing observations of patients with severe combined immunodeficiency (SCID) point to the importance of adenosine (Ado) for the development and function of the immune system [1]. Numerous experimental data indicate that adenosine can affect several events such as T lymphocyte activation, proliferation, IL-2 production, and lymphocyte-mediated cytolysis [2–5]. Impaired lymphocyte functioning is a common feature of human diabetes. The proliferative response of T cells derived from type-1 diabetes patients to sperm whale myoglobin was reported to be significantly reduced [6]. It was demonstrated that activated diabetic T cells could survive even severe IL-2 deprivation, which killed activated normal T cells [7]. Diabetic lymphocytes have defective TCR mediated signaling which may result in aberrant T cell activation and proliferation [8]. The adenosine metabolism in tissues of diabetic rats including lymphocytes is altered due to lowered expression of adenosine kinase [9,10]. Moreover, our studies showed that decreased activity of adenosine kinase was associated with increased adenosine concentration in some but not all diabetic tissues [11].

Adenosine is generated intracellularly and in extracellular space during normal metabolic activity [12–14].

Abbreviations: ENT, equilibrative nucleoside transporter; CNT, concentrative (Na^+-dependent) nucleoside transporter; NT, nucleoside transporter; Ado, adenosine; NBTI, nitrobenzylthioinosine; es, equilibrative nucleoside transport system sensitive to inhibition by NBTI; ei, equilibrative nucleoside transport system insensitive to inhibition by NBTI; ci, concentrative nucleoside transport insensitive to NBTI; cif, purine preferring concentrative transport insensitive to NBTI; EHNA, citidine erythro-9-(2-hydroxy-3-nonyl)adenine; STZ, streptozotocin.

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doi:10.1016/j.bcp.2004.06.020

Action of extracellular adenosine is mediated by adenosine receptors linked to a variety of signal transduction systems [15,16]. In the cell adenosine may act through the occupancy of an intracellular P site [17] or modify several metabolic pathways including inhibition of SAH hydrolase [18], which may lead to impairment of methylation reactions necessary for cell viability [19–21]. Extracellular and intracellular concentrations of adenosine depend on metabolism and transport across plasma membrane. Thus, currier mediated transport of this nucleoside is likely to play an important role in modulating cell function, because efficiency of the transport processes may determine adenosine availability either to receptors or to metabolizing enzymes.

Two types of nucleoside transporters are known to mediate nucleoside transport across the plasma membrane, the equilibrative facilitated-diffusion type and the concentrative Na^+-dependent one. The equilibrative transporters (ENT) are subdivided into two types based on sensitivity to inhibition by the nitrobenzylthioinosine (NBTI). ENT1 is sensitive to inhibition by nanomolar concentration of NBTI, whereas ENT2 is barely inhibited even by 1 μM concentration of NBTI [22]. Based on kinetic data, the existence of five concentrative transport systems (N1, N2, N3, N4, N5) has been postulated [23]. To date N1-, N2- and N3-related cDNA (CNT2, CNT1, and CNT3, respectively) has been cloned and functionally characterized [24–26].

The expression level of particular nucleoside transporter varies depending on cell type and physiological state [27]. Cell specific changes in NBTI-sensitive transport of adenosine in cultured human diabetic cells were reported. It was demonstrated that in endothelial cells isolated from human diabetic umbilical vein, NBTI-sensitive adenosine transport was reduced [28], whereas in smooth muscle cells isolated from diabetic umbilical artery, adenosine transport was significantly elevated [29]. Our previous studies showed that in kidney, liver and heart of diabetic rat the expression level of nucleoside transporters was altered in a tissue-specific manner [30]. Unfortunately, our knowledge about status of nucleoside transporters in cells of immune system in diabetes is very limited. In this report we described the changes occurring in expression level of nucleoside transporters in diabetic rat T lymphocytes. We present evidence that in rat T lymphocytes the expression level of rCNT2 and rENT2 transporters depends on insulin and that glucose affects the expression level of rENT1 transporter. The impact of occurring changes in expression level of nucleoside transporters on adenosine transport in rat T lymphocytes is also demonstrated.

2. Materials and methods

2.1. Materials

Fluorescein conjugate mouse anti rat CD2[LFA-2] clone OX-34 was from Chemicon International. Fluorescein conjugate mouse anti rat CD3 clone G4.18 was from BD Biosciences. Insulin, thiobutabarbital sodium (Inactin), Histopaque-1077, penicillin, streptomycin, RPMI-1640 medium, inulin, nitrobenzyltioinosine (NBTI), adenosine, inosine, thymidine, cytidine erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) streptozotocin were obtained from Sigma-Aldrich. Oligo(dT) and dNTP were from Roche Diagnostics GmbH. Glucose Hexokinase Reagent Set was from Pointe Scientific, Inc. [^{3}H] adenosine was from Amersham. All primers used were from Integrated Technologies, Inc. Total RNA Prep Plus Kit was from A&A Biotechnology. Tth DNA polymerase, Tfl DNA polymerase, and RNasin were from Promega.

2.2. Animals

Male Wistar rats (200–240 g) fed on Altromin C 1000 diet (Altromin GmbH) were used for all experiments. All animals had access to food and water ad lib.

2.3. Experimental diabetes

Diabetes was induced by a single intravenous injection of 75 mg/kg body weight streptozotocin (STZ). STZ was dissolved in 10 mM citrate buffer, pH 4.5. Control rats (hereafter referred to as normal rats) were injected with citrate instead of STZ. On the 1st, 5th, 10th day after STZ injection and on the day of the experiment, blood glucose levels were measured from tail blood. Only rats with the glucose level of 20–30 mM were used for further experiments. On the day of the experiment randomly selected rats were anesthetized with pentobarbital (40 mg/kg of body weight), the spleen was removed and the splenocytes were isolated.

2.4. Cells and culture conditions

Single cell suspension of splenocytes was prepared by pressing spleens isolated from normal rats through sterilized 20 μm pore size nylon mesh gauze in the presence of sterile saline. Mononuclear cells were isolated by centrifugation of the cell suspension through Histopaque-1077 at 700 × *g* for 30 min at room temperature. Mononuclear cells found at the saline/Histopaque interface were washed and suspended in RPMI-1640 medium supplemented with 3% BSA. The cells were then separated into adhesive and nonadhesive by the panning method [31] relying on incubation (1 h at 37 °C) of cell suspension in the presence of 3% BSA in plastic bottles with surface for adhesive cells (Sarstedt AG & Co.). Following incubation, the nonadhesive cells were collected by centrifugation (700 × *g* for 10 min) and suspended in RPMI-1640 medium supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum. The purity of isolated cell fractions was examined by flow cytometry. The nonadherent fraction (T cells) contained 92–95% CD2

(OX-34) and 86–89% CD3 (G4.18) positive cells. The number of viable cells was determined by Trypan Blue dye exclusion. Only cell preparations with a 95% viability or greater were used. Cells were cultured in flat-bottomed culture bottles in humidified atmosphere containing 5% CO_2 at 37 °C at a density of (0.5–1) × 10^6 cells/ml in a total volume of 6 ml RPMI-1640 medium supplemented with antibiotics (at concentrations as stated above) and 10% fetal bovine serum and containing glucose and insulin at concentration and for the time detailed in the figure legends.

2.5. Transport measurements

Cells were harvested by centrifugation (700 × *g* for 10 min), washed twice in 15 ml of the appropriate transport buffer containing 20 mM Hepes-Tris, pH 7.4, 130 mM (NaCl or choline chloride), 3 mM K_2HPO_4, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 μM EHNA (adenosine deaminase inhibitor) and suspended to a final density of 50 × 10^4 cells/ml. After suspension in the transport buffer cells were incubated for 0.5 h at 24 °C. The nucleoside transport was determined by the oil stop procedure [32]. The uptake process was initiated by mixing 200 μl of the cell suspension with 10 μl of the ^{3}H-labeled adenosine (1–2 μCi/nmol). Examination of the time course of adenosine transport in rat T lymphocytes revealed that both Na^+-dependent and Na^+-independent adenosine uptake was linear at least throughout the 25 s incubation (not shown); therefore in our transport experiments 20 s time point was routinely used. The adenosine uptake was terminated by transferring an aliquot of the transport mixture on top of 0.2 ml oil (silicone fluid with a final density of 1.032 g/ml) in a 0.4 ml microcentrifuge tube (0.4 cm × 4.5 cm), and immediately centrifuged (5000 × *g* for 1 min) on Beckman Microfuge™ 11. The tip of the tube containing the cell pellet was cut off and placed into the scintillation vial containing 5 ml of the Sigma-Fluor Universal LSC cocktail (Sigma–Aldrich). The scintillation vials were left for 12 h in the dark and the radioactivity was determined using a Wallac 1409 liquid-scintillation counter. In the transport mixture the [^{14}C]-labeled inulin (0.25 μCi/ml) was included to correct for the extracellular medium trapped in the pellet (usually 3–6% of inulin was found in the pellet).

2.6. Measurement of adenosine accumulation

Cultured cells were harvested by centrifugation, washed twice in 15 ml of transport buffer containing 20 mM Hepes-Tris, pH 7.4, 130 mM NaCl, 3 mM K_2HPO_4, 2 mM $MgCl_2$, 1 mM $CaCl_2$, and suspended to a final density of 1.5 × 10^6 cells/ml. After suspension in the transport buffer cells were incubated for 0.5 h at 24 °C. At the 25th minute to the incubation mixture adenosine was added to reach final concentration of 0.5 μM. At the 30th minute the nucleoside transport was terminated by transferring the incubation mixture (typically 8 ml) on top of 5 ml silicone oil layered over 0.2 ml of 5% $HClO_4$ in a 15 ml conical tube (2.8 cm × 11.5 cm), and immediately centrifuged (5000 × *g* for 2 min). Obtained perchloric acid extract was neutralized and adenosine and its metabolites were measured by the chemiluminescent method with a BioOrbit 1250 luminometer as described in detail previously [11]. The assay relied on the determination of hydrogen peroxide formed by sequential catabolism of adenosine, inosine and hypoxanthine/xanthine to uric acid [33].

2.7. RNA extraction and reverse transcription

Total RNA was extracted from cells with the use of Total RNA Prep Plus Kit, and stored at −80 °C. Reverse transcription was performed in 20 μl final volume of 50 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 1 mM dNTPs, 250 ng oligo(dT), 14 U of reverse transcriptase (MMLV-RT), 10 U of RNasin, and 1–5 μg of RNA. Reactions were incubated for 45 min at 42 °C and 5 min at 95 °C.

2.8. Generation of probes by PCR

Probes for nucleoside transporters and β-actin used in RNase protection assay were prepared by PCR as described previously [30]. Amplified DNA fragments were 406, 404, 399, 390 and 511 bp for rENT1, rENT2, rCNT1, rCNT2 and β-actin, respectively. Digoxigenin labeled rENT1/2, rCNT1/2 and β-actin antisense probes (ssDNA) were obtained by running PCR with an antisense primer and appropriate DNA fragment as a template in the presence of digoxigenin labeled dUTP.

2.9. RNase protection assay

Changes in the mRNA level of each nucleoside transporter were analyzed by ribonuclease protection technique using Multi Nuclease Protection Assay (Ambion) with β-actin as a reference template. Usually 10–15 μg of total RNA was hybridized to the appropriate nucleoside transporter and β-actin probes according to the manufacturer's protocol. Protected RNA fragments were fractionated by electrophoresis on 8 M urea/6% polyacrylamide gel and transferred to a positively charged nylon membrane. The hybridized probes were immunodetected, visualized and analyzed using the Gel Doc 2000 system (Bio-Rad) and the computer program Quantity One (Bio-Rad). The relative expression level of given nucleoside transporter (NT) gene was presented as a ratio of NT/β-actin probe.

2.10. Analytical

Protein concentrations were determined by the method of Bradford [34] with bovine serum albumin as a standard.

The DNA and RNA concentrations were determined by measuring the absorbance at 260 nm. Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecylsulfate (SDS) was performed according to Leammli [35]. Glucose was measured with the hexokinase method using the Pointe Scientific Kit.

2.11. Statistical analysis

The statistical analysis was carried out using the STATISTICA 5PL statistical package (StatSoft). Statistical significance was determined using the *t*-test. *P* values below 0.05 were considered as significant.

3. Results

3.1. Insulin and glucose effect on expression of nucleoside transporters in T lymphocytes

Previously reported data showed that the activity and mRNA level of adenosine kinase (AK), a key enzyme metabolizing adenosine in the cell was significantly lowered in splenocytes isolated from diabetic rats [10]. Considering the decreased rate of adenosine phosphorylation resulted from diminished activity of AK it would be expected that under such conditions, adenosine may accumulate in the cell, unless the nucleoside transport is not altered. In order to examine the expression level of nucleoside transporters (NT) we isolated total RNA from T lymphocytes prepared from spleens of normal and streptozotocin (STZ)-induced diabetic rats. Relative mRNA level of investigated NT was assessed based on RNase protection assay. Performed experiments revealed that on the 10th day after STZ administration, mRNA levels of rENT1 and rENT2 in diabetic lymphocytes were lowered by 50% as compared with mRNA levels in normal T cells (Fig. 1). The level of rCNT2 mRNA was two-fold increased in diabetic T cells. The mRNA for rCNT1 transporter was absent or its level was very low and our probe gave no detectable signal in RNase protection assay performed on RNA isolated from normal or diabetic T cells (not shown).

In order to discriminate the effect of glucose and insulin on expression level of NT the experiments were performed on T lymphocytes cultured in medium containing defined concentrations of glucose and insulin. Conducted experiments showed that at 5 mM glucose the mRNA levels of rENT2 and rCNT2 were highly dependent on insulin, whereas the rENT1 expression was not affected by insulin (Fig. 2). Character of insulin-induced changes in the expression of rENT2 and rCNT2 differed significantly. Exposition of lymphocytes to 10 nM insulin resulted in 73% increase in rENT2 mRNA and 50% decrease in the rCNT2 mRNA level (Fig. 2). The same insulin-induced alterations in mRNA level of rENT2 and rCNT2 were observed in cells cultured at 20 mM glucose (not shown).

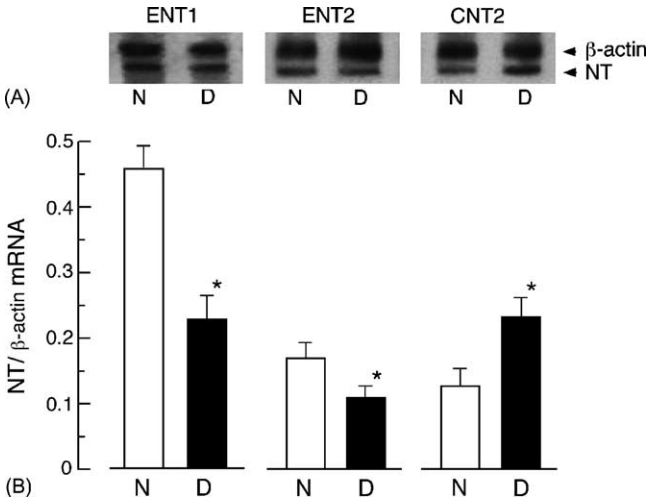

Fig. 1. Expression level of nucleoside transporters in T lymphocytes isolated from spleens of normal and STZ-induced diabetic rats. Total RNA was extracted and RNase protection assay was performed as described under Section 2. (A) The presented RNase protection assays are representative of those obtained in three independent experiments performed on RNA isolated from lymphocytes of normal (N) and diabetic (D) rats. The positions of β-actin and nucleoside transporter (NT) bands are indicated. (B) The quantified results of RNase protection assays normalized to β-actin mRNA. The data represent the mean ± S.D. from three independent experiments. $^{*}P < 0.03$ D vs. N.

These data indicate that the insulin effect on mRNA level of rENT2 and rCNT2 do not depends on glucose level. Examination of the insulin dose response effect on NT expression revealed that maximal effect could be observed

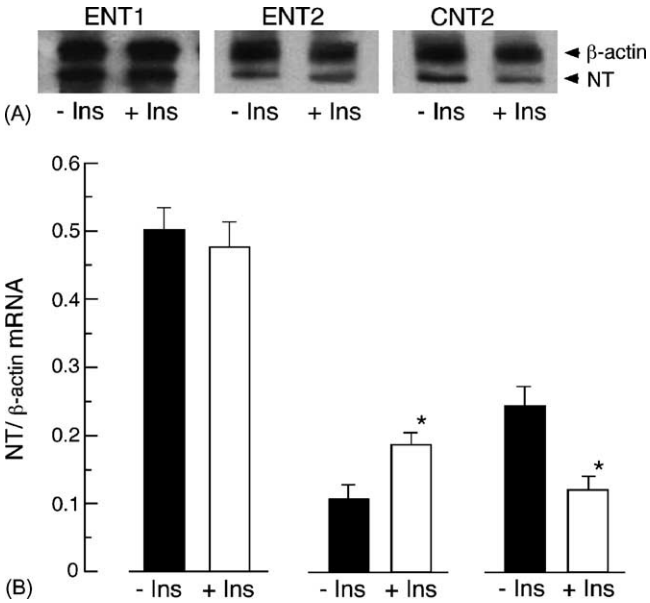

Fig. 2. Effect of insulin on nucleoside transporters mRNA level in cultured rat T lymphocytes. T cells isolated from spleens of normal rats were cultured as described under Section 2 for 3 days in the presence of 5 mM glucose and the absence (−Ins) or presence (+Ins) of 10 nM insulin. (A) The presented RNase protection assays are representative of those obtained in four independent experiments. The positions of β-actin and nucleoside transporter (NT) bands are indicated. (B) The quantified results of RNase protection assays normalized to β-actin mRNA. The data represent the mean ± S.D. from four independent experiments. $^{*}P < 0.002$ for (+Ins) vs. (−Ins).

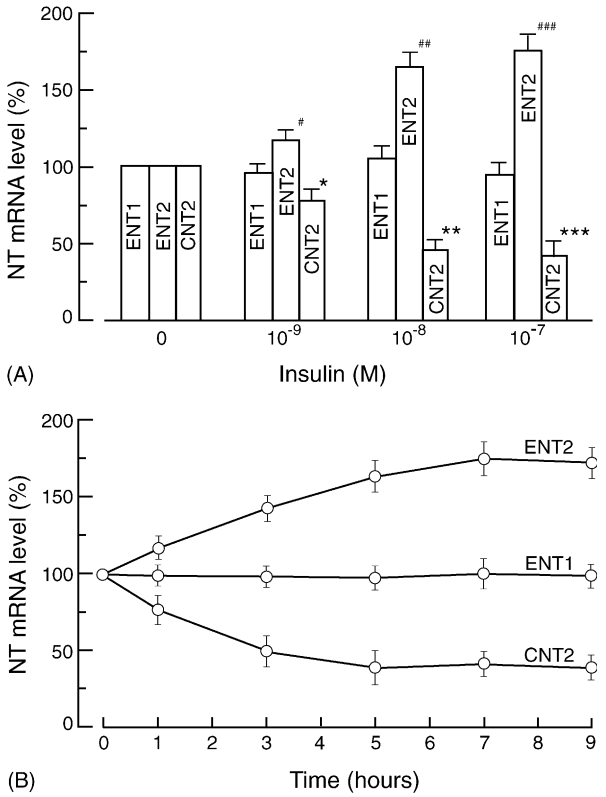

Fig. 3. Dose- and time-dependent courses of insulin action on the nucleoside transporters mRNA levels in cultured rat T lymphocytes. Cells were cultured as described under Section 2 for 2 days in the presence of 5 mM glucose and the absence of insulin. (A) On the third day to the culture medium insulin at the indicated concentrations was added and cells were cultured for 10 h, and after harvesting, total RNA was isolated and NT mRNA was determined by RNase protection assays. Concentration of insulin in RPMI-1640 medium supplemented with 10% fetal bovine serum varied in the range of (5–8) $\times$ 10^{-11} M. Insulin in this concentration had no measurable effect on NT mRNA level (not shown). (B) Cells were cultured for 2 days in the presence of 5 mM glucose and on the third day 10 nM insulin was added to the culture medium (time 0). Cells were harvested at times indicated and NT mRNAs were quantified by RNase protection assays. The data represent the mean $\pm$ S.D. from three independent experiments. $^{*}P < 0.003$ for 10^{-9} M vs. 0 M; $^{**}P < 0.003$ for 10^{-8} M vs. 10^{-9} M; $^{***}P < 0.0002$ for 10^{-7} M vs. 0 M; $^{\#}P < 0.002$ for 10^{-9} M vs. 0 M; $^{\#\#}P < 0.002$ for 10^{-8} M vs. 10^{-9} M; $^{\#\#\#}P < 0.0003$ for 10^{-7} M vs. 0 M.

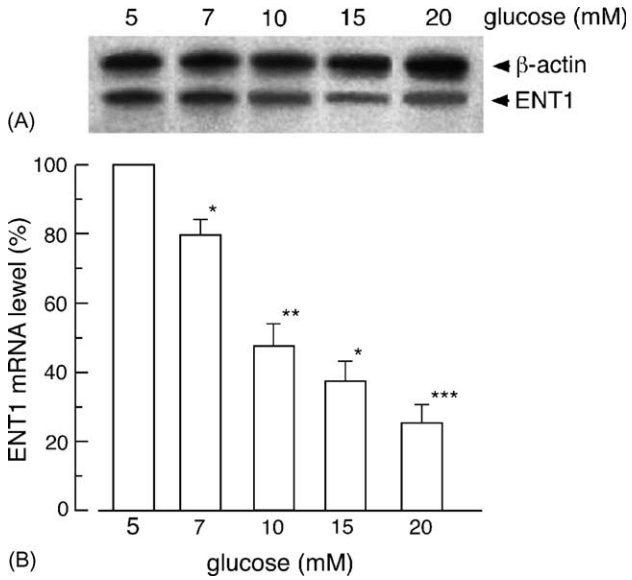

Fig. 4. Dose-dependent course of glucose action on the rENT1 mRNA level in cultured rat T lymphocytes. Cells were cultured as described under Section 2 for 3 days in the presence of glucose at the concentrations indicated. On the fourth day cells were harvested, total RNA was extracted and RNase protection assay was performed. (A) The presented RNase protection assays are representative of those obtained in four independent experiments. The positions of β-actin and rENT1 bands are indicated. (B) The quantified results of RNase protection assays normalized to β-actin mRNA. The data represent the mean $\pm$ S.D. from four independent experiments. $^{*}P < 0.002$ relative to 5 mM glucose; $^{**}P < 0.004$ for 10 mM glucose vs. 7 mM glucose; $^{***}P < 0.005$ for 20 mM glucose vs. 10 mM glucose.

at 10 nM insulin, and only slight increase was seen with 100 nM insulin (Fig. 3A). The maximal effect of 10 nM insulin on mRNA levels of rENT2 and rCNT2 was observed at the 7th and 5th hour, respectively (Fig. 3B). These experiments showed that insulin does not affect the expression level of nitrobenzylthioinosine (NBTI)-sensitive transporter (ENT1) in rat T cells. Therefore, we assumed that decreased mRNA level of rENT1 in lymphocytes isolated from diabetic rats was caused by elevated glucose level.

To address the question of whether changes in glucose level affect the rENT1 mRNA level we cultured the cells in the presence of various concentrations of glucose. Results obtained from performed experiments confirmed fully our supposition. Increase of glucose concentration from 5 to 20 mM caused decrease in the rENT1 mRNA level by 80% (Fig. 4). The effect of high glucose on rENT1 expression level was reversible and changing the culture medium to low glucose (5 mM) medium resulted in restoration of the rENT1 mRNA level seen at 5 mM glucose. Maximal effect of 20 mM glucose on rENT1 mRNA level was visible on the third day after transferring the cells to the high glucose medium (Fig. 5). The glucose effect was not dependent on insulin and the same glucose-induced changes in rENT1 mRNA level were observed in the presence or absence of 10 nM insulin (not shown).

3.2. Characterization of adenosine transport system in rat T lymphocytes

In order to characterize the adenosine transport in rat T cells the nucleoside uptake was measured in Na^+ and Na^+ free buffers in the presence or absence of 1 μM NBTI. In rat T lymphocytes adenosine appeared to be transported via both Na^+-dependent and Na^+-independent routes (Fig. 6A). Obtained data revealed that Na^+-independent (equilibrative) transport in T cells cultured in the presence of 5 mM glucose and 10 nM insulin was composed of two discrete transporters, NBTI-sensitive (*es*) and -insensitive (*ei*). At 10 μM adenosine the equilibrative uptake accounts for 74% of overall adenosine transport in T cells. Most (68%) of this transport activity was the *es* type, whereas the *ei* transport accounted for 32% (Fig. 6A). Calculations

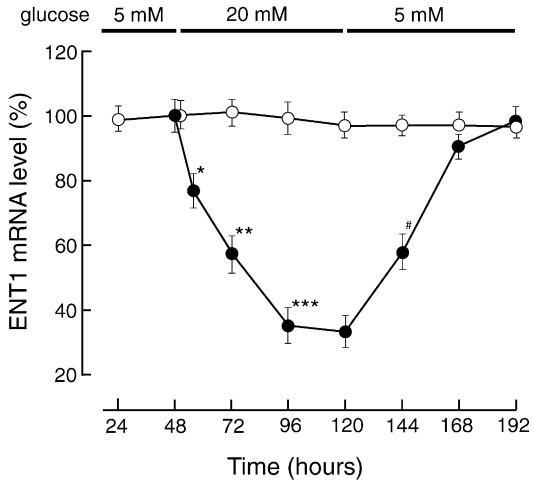

Fig. 5. Time course of glucose action on the abundance of rENT1 mRNA in cultured rat lymphocytes. Cells were cultured as described under Section 2 for 2 days in the presence of 5 mM glucose. On the third day cells were harvested and half of the cells was transferred to the culture medium containing 20 mM glucose and cultured for time as indicated (●). Other half was cultured in medium containing 5 mM glucose (○). On the fifth day cells cultured at 20 mM glucose were harvested and transferred to the medium containing 5 mM glucose. At indicated time-points from cell culture ~10^6 cells were withdrawn, RNA was extracted and RNase protection assay was performed. The data represent the mean ± S.D. from three independent experiments. $^{*}P < 0.003$ relative to mRNA level at 48; $^{**}P < 0.009$ for mRNA level at 72 h vs. mRNA level at 58 h; $^{***}P < 0.004$ for mRNA level at 96 h vs. mRNA level at 72 h; $^{\#}P < 0.002$ for mRNA level at 144 h vs. mRNA level at 120 h.

made to evaluate the Na^+-dependent (concentrative) adenosine uptake indicated that in rat T cells adenosine is transported by NBTI-insensitive (*ci*) transport system (Fig. 6B).

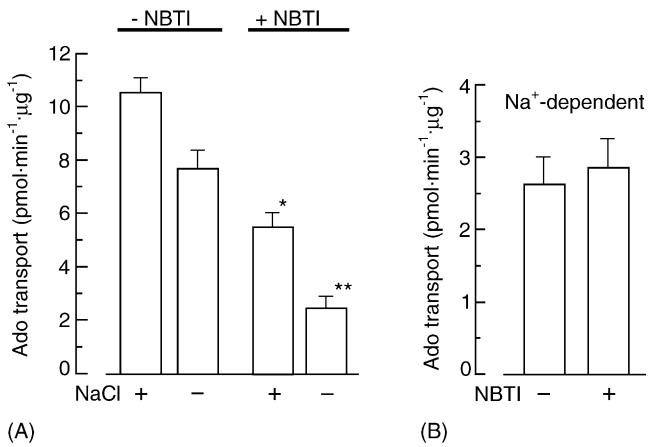

Fig. 6. Adenosine transport in cultured rat T lymphocytes as a function of Na^+ and NBTI presence. Cells were cultured for 2 days at 5 mM glucose and 10 nM insulin. (A) Transport of 10 μM adenosine was measured as described under Section 2 in the presence (+NBTI) or absence (−NBTI) of 1 μM NBTI. (B) The Na^+-dependent adenosine transport was calculated by subtracting those rates measured in the Na^+-free buffer from those measured in the Na^+ buffer. The data represent the mean ± S.D. from five independent experiments. $^{*}P < 0.0002$ relative to Ado transport in the presence of Na^+ and the absence of NBTI; $^{**}P < 0.0001$ relative to Ado transport in the absence of NBTI and Na^+.

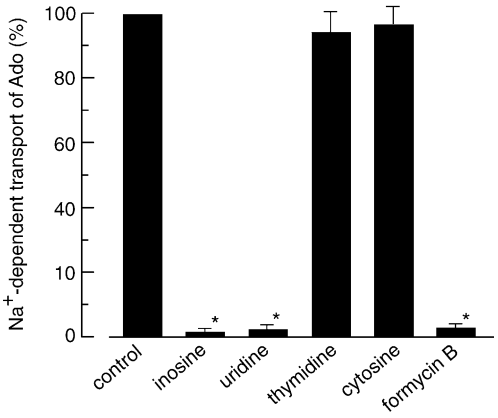

Fig. 7. Effect of various nucleosides and nucleoside analogues on Na^+-dependent adenosine transport in cultured rat T lymphocytes. Cells were cultured for 2 days at 5 mM glucose and 10 nM insulin. Transport of 10 μM adenosine was measured as described under Section 2 in the absence (control) or presence of 200 μM competing compounds in Na^+-free (130 mM choline chloride) and Na^+-containing (130 mM NaCl) transport buffer. The Na^+-dependent adenosine uptake was calculated as described in legend to Fig. 6 and expressed as a percentage of adenosine flux in the absence of competing compounds. The bars represent the mean ± S.D. of data from at least three experiments. $^{*}P < 0.0001$ relative to control.

There are at least three major *ci* transport systems with varying substrate specificities [36]. The substrate specificity of the T cell *ci* transport system was examined by measuring the adenosine (10 μM) uptake in the presence of other nucleosides and nucleoside analogs at concentration of 200 μM. Inosine, uridine and formycin B almost totally inhibited the Na^+-dependent adenosine *ci* transport, whereas thymidine and cytosine were unable to significantly affect the *ci* adenosine uptake (Fig. 7). These data indicate that Na^+-dependent adenosine uptake in rat T cells is mediated by *cif* transport system.

In summary, these results suggest that rat T cells possess at least two equilibrative transporters (*ei* and *es*) and one purine preferring concentrative transporter (*cif*).

3.2.1. Insulin and glucose effect on adenosine transport in T lymphocytes

In order to examine the impact of glucose and insulin-induced changes in expression of rENT1, rENT2 and rCNT2 on adenosine transport in T cells, we performed kinetic studies. The adenosine transport was measured at nucleoside concentrations ranging from 1 to 300 μM in cells cultured in the presence of various concentrations of glucose and insulin. Results presented in Fig. 8 indicate that overall adenosine transport was 30% lower in cells cultured in the presence of 20 mM glucose and the absence of insulin comparing to adenosine transport in cells cultured in the presence of 5 mM glucose and 10 nM insulin. However, the Na^+-dependent *cif* transport of adenosine increased two-fold in cells cultured under high glucose and the absence of insulin (Fig. 8B). The highest differences among cells cultured in high and low glucose were observed in equilibrative adenosine transport system,

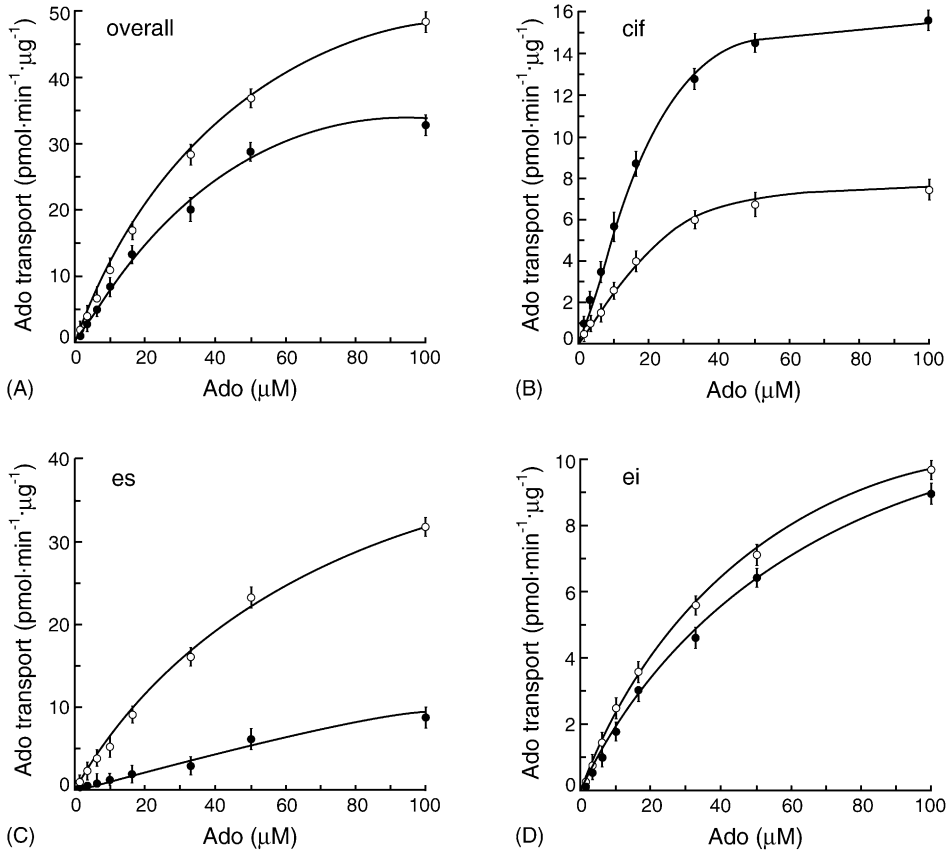

Fig. 8. Concentration dependence of adenosine transport in cultured rat T lymphocytes. Cells were cultured for 3 days at 5 mM glucose and 10 nM insulin (○) or at 20 mM glucose and the absence of insulin (●). Adenosine transport was measured as described under Section 2 in Na^+-free, and Na^+-containing transport buffers. (A) Measured overall (Na^+ buffer) transport of adenosine in cultured T cells. (B) The Na^+-dependent (*cif*) adenosine transport calculated as described in legend to Fig. 6. (C) Equilibrative NBTI-sensitive (*es*) adenosine transport was calculated by subtracting those rates measured in choline buffer and the presence of 1 μM NBTI from those measured in the choline buffer and the absence of NBTI. (D) Equilibrative NBTI-insensitive (*ei*) adenosine transport was measured in the choline buffer and the presence of 1 μM NBTI. The data represent the mean ± S.D. from at least three independent experiments.

which was 65% lower in cells cultured at high glucose (Fig. 8C). Analysis of the kinetic parameters of adenosine transport in T cells cultured under various conditions indicated that alterations in the glucose and insulin level induced changes in the V_{max} but not in the K_m value (Table 1). This suggested that insulin and glucose influenced the number of nucleoside transporters in the cell but not the affinity for adenosine. Analysis of the adenosine transport in cells cultured at high and low glucose and in the absence or presence of insulin indicated that insulin affects the Na^+-dependent *cif* transporter and NBTI-insensitive *ei* transporter (Table 1). On the other hand, the NBTI-sensitive *ei* transporter appeared to be sensitive to changes in glucose level. Cells cultured in the presence of 10 nM insulin and increased concentrations of glucose showed diminished adenosine *es* transport (Fig. 9). Examination of the time course of the glucose-induced alterations in T cell adenosine transport showed that maximal changes in adenosine *es* transport were observed on the third day after change of glucose concentration in cell culture medium (not shown). These data are consistent with the changes observed in NT mRNA levels in T cells (Fig. 5).

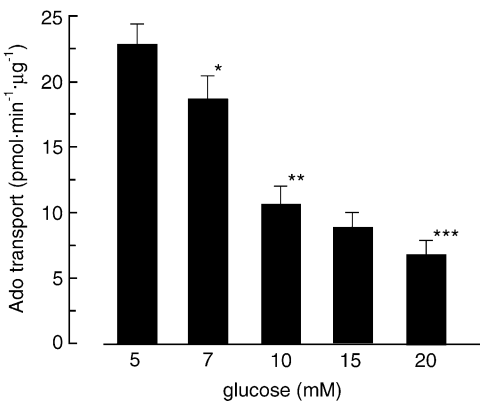

Fig. 9. Dose-dependent course of glucose action on equilibrative NBTI-sensitive (*es*) adenosine transport in cultured rat T lymphocytes. Cells were cultured for 3 days at glucose concentrations as indicated. Adenosine (50 μM) uptake was measured as described under Section 2. Equilibrative NBTI-sensitive (*es*) adenosine transport was calculated as described in Fig. 8 legend. The data represent the mean ± S.D. from three independent experiments. $^*P < 0.02$ relative to Ado transport in cells cultured at 5 mM glucose; $^{**}P < 0.001$ relative to Ado transport in cells cultured at 7 mM glucose; $^{***}P < 0.02$ relative to Ado transport in cells cultured at 10 mM glucose.

Table 1
Kinetic parameters of adenosine transport in cultured rat T lymphocytes

Cell culture conditions	Adenosine transport	K_m (μM)	V_{max} (pmol min^{-1} μg^{-1})
5 mM glucose, 10 nM insulin	Overall transport	71.4 ± 8.7	100.3 ± 12.3
	Na^+-dependent (*cif*)	31.2 ± 6.3	10.1 ± 1.6
	NBTI-sensitive (*es*)	54.3 ± 12.6	68.4 ± 7.6
	NBTI-insensitive (*ei*)	96.9 ± 9.4	22.3 ± 2.1
5 mM glucose, no insulin	Overall transport	69.2 ± 9.6	99.4 ± 11.8
	Na^+-dependent (*cif*)	29.1 ± 6.8	23.8 ± 2.7[*]
	NBTI-sensitive (*es*)	48.7 ± 10.3	65.1 ± 6.6
	NBTI-insensitive (*ei*)	91.8 ± 11.2	16.4 ± 2.2[**]
20 mM glucose, no insulin	Overall transport	70.1 ± 9.2	69.1 ± 8.2[#]
	Na^+-dependent (*cif*)	36.7 ± 7.5	25.3 ± 2.9
	NBTI-sensitive (*es*)	57.1 ± 14.5	23.8 ± 2.4[†,¥]
	NBTI-insensitive (*ei*)	93.3 ± 7.8	17.5 ± 1.9[††]
20 mM glucose, 10 nM insulin	Overall transport	74.5 ± 9.3	56.4 ± 7.2
	Na^+-dependent (*cif*)	33.3 ± 8.1	11.1 ± 1.8[§]
	NBTI-sensitive (*es*)	55.0 ± 17.1	22.4 ± 3.1
	NBTI-insensitive (*ei*)	89.4 ± 8.8	23.6 ± 2.3[§§]

Cells were cultured for 3 days at concentrations of glucose and insulin as indicated in the table. Adenosine transport was measured as described under Section 2 and the transport components were calculated as described in the legend of Fig. 8. Values of apparent K_m and V_{max} were determined by non-linear regression of the Eadie-Hoftsee plots (*v* vs. *v*/*s*). The data represent the mean ± S.D. from at least three independent experiments.

[*] $P < 0.0001$ relative to *cif* transport in cells cultured at 5 mM glucose and 10 nM insulin.
[**] $P < 0.008$ relative to *ei* transport in cells cultured at 5 mM glucose and 10 nM insulin.
[#] $P < 0.03$ relative to overall transport in cells cultured at 5 mM glucose and the absence of insulin.
[†] $P < 0.0006$ relative to *es* transport in cells cultured at 5 mm glucose and the presence of 10 nM insulin.
[¥] $P < 0.0008$ relative to *es* transport in cells cultured at 5 mM glucose and the absence of insulin.
[††] $P < 0.04$ relative to *ei* transport in cells cultured at 5 mM glucose and the presence of 10 nM insulin.
[§] $P < 0.002$ relative to *cif* transport in cells cultured at 20 mM glucose and the absence of insulin.
[§§] $P < 0.02$ relative to *ei* transport in cells cultured at 20 mM glucose and the absence of insulin.

3.2.2. Insulin and glucose effect on adenosine accumulation in T lymphocytes

Adenosine level in the cell depends mainly on its metabolism and transport across plasma membranes. Changes induced by glucose and insulin in expression level of nucleoside transporters would suggest that intracellular adenosine level might vary depending on adenosine transport kinetic. To resolve this question, we have measured adenosine content of cells incubated for 5 min in medium containing 500 nM adenosine. Such adenosine concentration is close to its physiological plasma level, which was reported to be in 100–300 nM range [37]. Our measurements showed that in cells cultured under 20 mM glucose concentration and in the absence of insulin adenosine accumulated to 3.1-fold lower level than in cells cultured under low glucose and the absence of insulin, whereas there were no differences in the inosine level in these two types of cells (Table 2). There were no significant differences in adenosine level in cells cultured under high glucose concentration and the presence or absence of 10 nM insulin. On the other hand, inosine accumulated to the slightly higher level in cells cultured at 20 mM glucose and the presence of insulin comparing to cells cultured under high glucose and the absence of insulin (Table 2). Comparison of data presented in Tables 1 and 2 indicates that at 20 mM glucose changes in accumulation of adenosine were accompanied by alterations in *es* transport systems, whereas inosine level was related to status of

Table 2
Intracellular accumulation of adenosine and its metabolites in cultured rat T lymphocytes

Cell culture condition	Adenosine (pmol/10^6 cells)	Inosine (pmol/10^6 cells)	Hypoxanthine + xanthine (pmol/10^6 cells)
5 mM glucose, 10 nM insulin	1.71 ± 0.32	2.47 ± 0.25	3.89 ± 0.51
5 mM glucose, no insulin	2.23 ± 0.34	2.11 ± 0.37	4.32 ± 0.48
20 mM glucose, no insulin	0.71 ± 0.16[*,†]	2.01 ± 0.19	4.13 ± 0.49
20 mM glucose, 10 nM insulin	0.55 ± 0.14[**]	2.53 ± 0.20[††]	3.91 ± 0.42

Cells were cultured for 2 days at concentrations of glucose and insulin as indicated in the table. On the third day cells were transferred to transport buffer and incubated for 5 min in medium containing 0.5 μM adenosine and intracellular nucleosides were measured by the chemiluminescent method as described under Section 2. The data represent the mean ± S.D. from at least three independent experiments.

[*] $P < 0.002$ relative to Ado level in cells cultured at 5 mM glucose and the absence of insulin.
[†] $P < 0.008$ relative to Ado level in cells cultured at 5 mM glucose and the presence of 10 nM insulin.
[**] $P < 0.005$ relative to Ado level in cells cultured at 5 mM glucose and the presence of 10 nM insulin.
[††] $P < 0.03$ relative to inosine level in cells cultured at 20 mM glucose and the absence of insulin.

ei transport system. Lack of significant changes in hypoxanthine/xanthine levels in the lymphocytes cultured under various glucose and insulin concentrations may suggest that diabetic like conditions does not alter the activity of xanthine oxidase.

In summary, results from performed experiments showed that adenosine transport in rat T lymphocytes is differentially regulated by glucose and insulin by means of alteration of the nucleoside transporters expression level and that altered adenosine transport has a great impact on its intracellular level.

4. Discussion

Data collected and presented in this report demonstrate that at least three nucleoside transport systems are present in rat T lymphocytes, an *es* transport system, combined with rENT1, an *ei* transport system which appears to be associated with rENT2, and the concentrative transport mediated by rCNT2. Moreover, insulin and glucose differentially and independently regulate the expression level of these transporters and have a great impact on adenosine uptake by T cells. To our knowledge, these findings provide the first evidence that adenosine transport in T lymphocytes is modulated by glucose and insulin, implying that under diabetic conditions the biological actions of adenosine and its metabolites in T cells might be altered.

Cells of immune system belong to broad and heterogeneous family of cell types, which are activated by a wide variety of signals including the agents transported by nucleoside transporters [38–40]. However, our knowledge about the regulatory properties of nucleoside transport in lymphocytes especially in T cells is very limited. Studies performed on B cell lines and murine bone marrow macrophages indicate that these cells express both the concentrative (Na^+-dependent), and equilibrative (Na^+-independent) nucleoside transport systems [41]. The presence of mRNA for ENT1, CNT1 and CNT2 transporters was demonstrated in murine bone marrow macrophages [42]. However, in several human hematological cell lines and normal leukocytes the mRNA for CNT1 was not detected, whereas these cells showed expression of ENT1, ENT2 and CNT2 transporter [43]. Our study demonstrated that the mRNA for CNT1 in rat T cells was absent or its level was very low and undetectable. Data gathered in the last few years indicate that the nucleoside transporters are not constitutively expressed but their expression is a highly regulated event. Studies on rat liver parenchymal and hepatoma cells provided evidence for dependence of nucleoside transporter expression on cell cycle progression and differentiation [44,45]. In Raji and BLS-1 cells the level of ENT1 mRNA was reported to be affected by various B cell activators such as phorbol 12-myristate 13-acetate (PMA), tumor necrosis factor-α (TNF-α) and lipopolysaccharide (LPS) [46]. Changes in CNT1 and CNT2 mRNA levels were observed in murine bone macrophages treated with LPS and TNF-α [42]. Hormonal modulation of the concentrative nucleoside transport activity in hepatocytes was also reported. In those studies Na^+-dependent uptake of uridine was up regulated by glucagon and insulin in rat liver parenchymal cells [47]. In other epithelial cells the expression level of CNT1 was demonstrated to be dependent on the nutritional status. Increased expression of CNT1 was observed in plasma membrane vesicles isolated from jejunum of rats starved for 48 h [48]. Changes in adenosine transport mediated by NBTI-sensitive (*es*) transporter were observed in human umbilical artery smooth muscle cells and human umbilical vein endothelial cells isolated from gestational diabetic pregnancies [24,29]. Moreover, insulin in non-diabetic human umbilical artery smooth muscle cells increased NBTI-sensitive adenosine transport, whereas in diabetic cells the increased adenosine uptake was inhibited by insulin [29]. On the other hand results reported from experiments performed on human umbilical vein endothelial cells showed that the NBTI-sensitive transporter is down regulated by elevated glucose [49,50]. Our results presented in this report indicate that the expression level of rENT1 transporter and the NBTI-sensitive equilibrative adenosine transport in rat T lymphocytes was affected by changes in glucose concentration but was insensitive to insulin. Whereas, the expression level of rENT2 transporter and NBTI-insensitive equilibrative adenosine transport was up regulated by insulin and was not affected by glucose. In addition, insulin but not glucose decreased mRNA level of rCNT2 transporter and the Na^+-dependent adenosine transport. Diminished transport capacity of T cells cultured at high glucose and the absence of insulin was associated with over two-fold reduction in intracellular adenosine level comparing to cells cultured in the presence of 5 mM glucose and 10 nM insulin (Table 2), whereas, the level of inosine in these cells did not differ significantly. This data may indicate that adenosine and inosine levels in T cell are mainly dependent on membrane transport processes and not on metabolism. Previously we demonstrated that the activities of adenosine deaminase, 5′-nucleosidase and AMP deaminase in various rat tissues including splenocytes were not changed during diabetes [9,10]. Here we showed that the hypoxanthine/xanthine level in rat T cells was insensitive to variation in glucose and insulin concentrations (Table 2) what may indicate that the activity of xanthine oxidase is not changed under diabetic-like conditions. On the other hand, the activity of adenosine kinase in rat lymphocytes cultured under the absence of insulin was decreased by 75% comparing with its activity in cells cultured in the presence of insulin [10]. Therefore, difference in the adenosine level observed in cells cultured under low and high glucose is more likely to be the result of changes in nucleoside transporter systems. In experiments performed to determine intracellular accumulation of adenosine the inhibitor of adenosine

deaminase (EHNA) was not present in incubation medium, therefore during 5-min incubation significant level of inosine could be generated by extracellular activity of adenosine deaminase. The small differences observed in level of inosine in T cells cultured under high and low glucose and the presence or absence of insulin corresponded to small alterations in capacity of *ei* transport and rENT2 expression level. Previous studies performed on kidney epithelial cell line (PK15) stably transfected with cloned hENT2 demonstrated that although ENT2 is a low-affinity adenosine transporter, it has four-fold higher affinity for inosine [51]. The relevance of *ei* transport system for cellular inosine uptake supports data showed in Tables 1 and 2, which indicate that exposition of cells cultured under high glucose concentration to 10 nM insulin up regulated *ei* transport system and was associated with increased accumulation of inosine in the cells.

Based on presented results, it might be assumed that reduced function of the nucleoside transporters during hyperglycemia is likely to be involved in controlling the nucleoside levels in T lymphocytes. In hematopoietic cells de novo synthesis is limited and the salvage via membrane transporters is likely to be crucial for maintaining intracellular level of nucleosides [23,52]. Reduced intracellular level of adenosine may have a significant role in modulating lymphocyte excitability under diabetic conditions. Modulation of cellular immunity by insulin and glucose has been demonstrated. Increased production of IL-2 was observed in response to exposition of murine T cells to insulin [53]. Elevated glucose level in primary culture of human mononuclear cells was shown to suppress cytokine (IL-2, IL-6, IL-10) production and inhibit cell proliferation [54]. This is in line with experiments on cultured human peripheral blood mononuclear cells from diabetic patients, which demonstrated decreased basal production of cytokines (Il-6, TNFα) [55]. The proliferative response of CD4+ T cells derived from type-1 diabetic patients to the primary protein antigens was reported to be significantly reduced [56]. Inability of IL-2 to restore impaired thymidine uptake in mononuclear cells from non-insulin-dependent diabetic patients was also showed [57]. These findings and our data presented here are in line with recently reported dependence of macrophage proliferation and activation on nucleoside transporters functioning [58]. Pharmacological blockade of the *es* transport system inhibited macrophage-colony stimulating factor (M-CSF)-dependent cell proliferation. On the other hand interferon γ induced expression of CNT1 and CNT2 and blocked M-CSF-induced proliferation.

The relevance of adenosine for the development and function of the immune system is widely accepted. Adenosine has been reported to be involved in the regulation of T cell-stimulated antibody production, T cell proliferation and differentiation, cytokines production, and lymphocyte-mediated cytolysis [2–5]. Most of these adenosine immunomodulatory effects are mediated through binding to specific surface receptors. However, some actions of adenosine seemed to require adenosine uptake by the cell. It has been reported that adenosine needs to be transported into mouse macrophages to induce NO production and inhibit of LPS-induced TNF-α expression [59]. Similar results were reported for human peripheral blood mononuclear cells [60]. Studies on mice deficient for the A_{2a} receptor subtype showed that adenosine inhibits IL-12 and TNF-α production via receptor-dependent and independent mechanisms [61].

In summary, our study demonstrates that glucose and insulin differentially and independently regulate the expression level of nucleoside transporters in rat T lymphocytes. Alterations in nucleoside transporters mRNA levels were accompanied by changes in capacity of nucleoside transport systems without alteration in affinity for adenosine. Under diabetic-like conditions, i.e. hyperglycemia and the absence of insulin, the changes occurring in nucleoside transport system of rat T lymphocyte reduced the adenosine uptake leading to diminished accumulation of this nucleoside in the cell. It may be assumed that adenosine action requiring its transport into the cell might be impaired under diabetic conditions.

Acknowledgments

We are greatly indebted to Gabriela Dzierzko from the department of clinical biochemistry AMG for technical assistance with adenosine measurements. This work was supported by the State Committee for Scientific Research (KBN) grant No. 3 P05A 05524.

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