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The Effect of Insulin on Expression Level of Nucleoside Transporters in Diabetic Rats

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ABSTRACT

Evidence that the time course of insulin-induced changes in adenosine level in diabetic rats is different from that observed for expression of adenosine kinase prompted us to study the insulin effect on expression of nucleoside transporters in tissues of diabetic rats. RNase protection assay demonstrated that mRNA levels of equilibrative (rENT) and Na^+-dependent nucleoside transporters (rCNT) were altered in diabetic tissues. The rENT1 mRNA level with respect to values obtained in age- and sex-matched nondiabetic rats was decreased by 45, 32, and 10% in diabetic heart, liver, and kidney, respectively. The level of rENT2 mRNA was lowered by 40% in diabetic kidney and heart, and by 24% in diabetic liver. Changes in the expression pattern of rCNT1 and rCNT2 in diabetic tissues differed significantly from that observed for rENT. The levels of rCNT1 and rCNT2 mRNA did not change significantly in diabetic kidney. In diabetic heart, the mRNA levels of rCNT1 and rCNT2 increased 1.7- and 2-fold, respectively. Changes in expression of nucleoside transporters were accompanied by alterations in adenosine content. Administration of insulin to diabetic rats resulted in a drop in adenosine concentration in examined tissues and return of the rCNT1, rCNT2, and rENT2 but not rENT1 mRNA levels to values observed in nondiabetic rats. In summary, these data demonstrate that insulin affects expression of nucleoside transporters in a cell-specific manner. We conclude that change in the expression level of the nucleoside transporters occurring in tissues of diabetic rat is an important factor influencing adenosine levels in the cell.

Adenosine plays an important role in physiology of several organs (Clare and Coupe, 1989; Mubagwa et al., 1996). Several enzymes and transport processes control adenosine turnover inside and outside the cell. In the cell, besides de novo synthesis, dephosphorylation of AMP and hydrolysis of *S*-adenosylhomocysteine can form adenosine. Once generated, adenosine could be deaminated to inosine by adenosine deaminase, phosphorylated to AMP by adenosine kinase, or transported into extracellular fluid. Extracellular metabolism of nucleotides produces adenosine, which is taken up by the cell or deaminated to inosine. Under normal conditions, most of the adenosine formed in the cell is phosphorylated to AMP by adenosine kinase (Kroll et al., 1993). Performed experiments showed that pharmacological blockade of adenosine kinase leads to massive increases in adenosine concentration (Ely et al., 1992). This means that under normal conditions, there is a high rate of intracellular adenosine formation and that generated adenosine is efficiently phosphorylated by adenosine kinase. Hence, adenosine kinase would be considered as a very sensitive control point for adenosine level in the cell.

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Adenosine exerts its physiological effect by coupling to cell-surface receptors (A_1, A_{2A}, A_{2B}, or A_3) (Olah and Stiles, 2000). The affinity for adenosine varies between receptors; thus, its activation depends on adenosine concentration. On the other hand, the level of adenosine depends on its metabolism and transport across plasma membranes. Thus, carrier-mediated transport of adenosine 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 transporters are known to mediate nucleoside transport across the plasma membrane, the equilibrative facilitated-diffusion (ENT) type and the concentrative Na^+-dependent type (Baldwin et al., 1999). The equilibrative transporters mediate nucleoside transport in both directions depending on nucleoside concentration gradient across the plasma membrane, whereas Na^+-dependent transporters couple the movement of nucleoside regardless its concentration gradient to sodium transport (Griffith and Jarvis, 1996).

Equilibrative transporters are subdivided into two types based on sensitivity to inhibition by the nitrobenzylmercaptopurine riboside (NBMPR). ENT1 is sensitive to inhibition by nanomolar concentrations of NBMPR, whereas ENT2 is

ABBREVIATIONS: CNT, concentrative Na^+-dependent nucleoside transporter; ENT, equilibrative nucleoside transporter; STZ, streptozotocin; NBMPR, nitrobenzylmercaptopurine riboside; PCR, polymerase chain reaction; AK, adenosine kinase.

resistant to this compound up to 1 μM (Jarvis and Young, 1987). Both transporters exhibit broad substrate specificities for purine and pyrimidine nucleosides, although ENT2 might also transport hypoxanthine. Equilibrative transporters are widely distributed in different cell types and tissues; however, the expression level varies between examined tissues (Hyde et al., 2001).

Based on kinetic data, the existence of five concentrative transporters has been postulated (Griffith and Jarvis, 1996). To date CNT1, CNT2, and CNT3 has been cloned and functionally characterized (Huang et al., 1994; Fang et al., 1996; Ritzel et al., 2001). The substrate specificity of concentrative transporters varied much more comparing with equilibrative ones. CNT1 and CNT2 show specificity for pyrimidine and purine nucleosides, respectively, whereas CNT3 transports purine and pyrimidine nucleosides equally well (Baldwin et al., 1999).

Recently, we have reported that expression of adenosine kinase is significantly reduced in tissues of diabetic rat, which is associated with increased adenosine concentration in the cells (Pawelczyk et al., 2000; Sakowicz and Pawelczyk, 2002). Administration of insulin to diabetic animals restores normal expression of adenosine kinase; within 24 h after recovery of enzyme activity, however, the level of adenosine remains elevated, indicating that some changes in transport processes have occurred (Sakowicz and Pawelczyk, 2002). Therefore, the present study was undertaken to evaluate the insulin effect on expression level of nucleoside transporters in diabetic rats.

Materials and Methods

Materials. Xanthine oxidase, adenosine deaminase, peroxidase, and nucleoside phosphorylase were from Roche Applied Science (Mannheim, Germany). Luminol, adenosine, ATP, and AMP were obtained from Sigma-Aldrich (Poznan, Poland). Glucose hexokinase reagent set was from Pointe Scientific, Inc. (Lincoln Park, MI). All primers used were from Integrated Technologies, Inc. (Coralville, IA). Total RNA Prep plus kit was from A & A Biotechnology (Gdansk, Poland). Oligo(dT) and dNTP were from Invitrogen (Carlsbad, CA). Moloney murine leukemia virus reverse transcriptase was from Epicentre Technologies (Madison, WI). *Tth* DNA polymerase, *Tfl* DNA polymerase, and RNasin were from Promega (Madison, WI). Multinuclease protection assay kit and Bright Stars-Plus membrane were from Ambion (Austin, TX). Digoxigenin-labeled 18 S probe was purchased at GENSET Corp. (La Jolla, CA). All other reagents were of analytical grade. Male Wistar rats (200–240 g) fed an Altromin C 1000 diet (Altromin GmbH, Lage, Germany) were used for all experiments. All animals had access to food and water ad libitum.

Experimental Diabetes. Diabetes was induced by a single intravenous injection of 75 mg/kg of body weight of 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 days 1, 5, and 10 after STZ injection and on the day of the experiment, blood glucose levels were measured from tail blood. Only rats with glucose levels of 20 to 30 mM were used for further experiments. To examine insulin effect on expression of nucleoside transporters and adenosine level on day 10 after STZ treatment, rats were injected with long-acting insulin (10 units/kg) once a day for 4 days. On the day of the experiment, randomly selected rats were anesthetized with pentobarbital (40 mg/kg of body weight), and the tissue of interest was removed and immediately frozen in liquid nitrogen.

Preparation of Tissue Extracts for Adenosine Measurement. Rats were anesthetized and the tissue of interest was freeze-clamped in aluminum tongs precooled in liquid nitrogen, removed, and placed in liquid nitrogen. Frozen tissue was weighed and then thoroughly pulverized in frozen 5% perchloric acid. Tissues were thawed during constant grinding, centrifuged to remove cellular debris, and neutralized with K_2CO_3 to pH 7.0. Perchlorate crystals were removed by centrifugation. Tissues were extracted to the final concentration of 100 mg (wet weight)/ml of extract.

Adenosine Measurement. Adenosine was measured by the chemiluminescent method with a BioOrbit 1250 luminometer as described previously (Sakowicz and Pawelczyk, 2002). The assay relies on the determination of hydrogen peroxide formed by sequential catabolism of adenosine, inosine, and hypoxanthine/xanthine to uric acid (Kather et al., 1987). For assay, usually 50 μl of neutralized perchloric acid extracts were made up with H_2O or adenosine standard to yield a final volume of 0.1 ml. Adenosine was determined by a two-step procedure. During the first step, inosine, hypoxanthine, and xanthine were removed by adding an equal volume of 0.1 M bicarbonate buffer, pH 8.2, containing 1 mM $MgCl_2$, 25 μM luminol, 1.5 U/ml peroxidase, 1 U/ml xanthine oxidase, and 0.3 U/ml nucleoside phosphorylase. When light emission decayed to baseline level (usually within 1–2 min), the determination of adenosine was initiated by addition of 0.1 ml of bicarbonate buffer containing 25 μM luminol, 1.5 U/ml peroxidase, 2.5 U/ml adenosine deaminase, 1 U/ml xanthine oxidase, and 0.3 U/ml nucleoside phosphorylase. The peak of light emission (usually between 20 and 30 s) was taken as a measure of adenosine content. Each batch of peroxidase, xanthine oxidase, and nucleoside phosphorylase was checked for contamination by adenosine deaminase before use. The recovery of standards averaged 98 $\pm$ 3%.

RNA Extraction and Reverse Transcription. Total RNA was extracted from tissues frozen in liquid nitrogen with the use of Total RNA Prep Plus kit and stored at -80°C. RNA was stored as a pellet under ethanol at -40°C. Reverse transcription was performed in a 20-μl final volume of 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM dNTPs, 250 ng of oligo(dT), 14 units of reverse transcriptase (Moloney murine leukemia virus reverse transcriptase), 10 units of RNasin, and 1 to 5 μg of RNA. Reactions were incubated for 45 min at 42°C and 5 min at 95°C.

Generation of Probes by PCR. Probes for nucleoside transporters and β-actin used in Northern blot and RNase protection assay were prepared by PCR.

For rat equilibrative nucleoside transporters (rENT1, rENT2), the reaction mixtures contained 50 mM Tris-HCl, pH 9.0, 20 mM ammonium sulfate, 300 ng of cDNA, and 0.50 μM concentrations of 5′ and 3′ primers, 0.25 mM concentrations of each dNTP, 2.50 mM $MgCl_2$, and 1 U of *Tfl* DNA polymerase. The PCR consisted of an initial denaturation at 95°C for 3 min and 34 cycles of 30 s at 95°C, 30 s at 60°C (for rENT1), or 30 s at 61°C (for rENT2), 50 s at 72°C, and a final extension of 10 min at 72°C. For rENT1 cDNA amplification, TadoW1 [CCCTGGTAAGGTGGAGATGG (forward)] and TadoW2 [TCCTCCTCTTGGCTCCTCCC (reverse)] primers were used. For rENT2 cDNA amplification, TadoNW1 [CTTCTTCATTA CCGCCATCCCG (forward)] and TadoNW2 [GCCACTGAGGAA-GAGGGTGCTG (reverse)] primers were used. The rENT1 and rENT2 primers were based on cDNA sequences (GenBank accession numbers AF15304 and AF05305, respectively).

For rat concentrative nucleoside transporters (rCNT1, rCNT2), the reaction mixtures contained 50 mM Tris-HCl, pH 9.0, 20 mM ammonium sulfate, 300 ng of cDNA, 0.50 μM concentrations of each of the 5′ and 3′ primers, 0.25 mM concentrations of each dNTP, 2.50 mM $MgCl_2$, and 1 U of *Tfl* DNA polymerase. The PCR consisted of an initial denaturation at 95°C for 3 min and 34 cycles of 30 s at 95°C, 30 s at 62°C (for rCNT1), or 30 s at 64°C (for rCNT2), 50 s at 72°C, and a final extension of 10 min at 72°C. For rCNT1 cDNA amplification, TadoN1 [GCACCGGCAGCTGTTTGGA (forward)] and TadoN2 [CCCCAGGACACAGCTCGCC (reverse)] primers were used. For rCNT2 cDNA amplification, TadoC1 [GGAGCTC ATG-

GAAGTCGGAAC (forward)] and TadoC2 [CCCATGAACACCCTCT-TAAGCCA (reverse)] primers were used. The rCNT1 and rCNT2 primers were based on cDNA sequences (GenBank accession numbers U10279 and U66723, respectively). Primers for β-actin amplification were as described previously (Pawelczyk et al., 2000). 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 (single-stranded DNA) were obtained by running PCR with antisense primer and appropriate DNA fragment as template in the presence of digoxigenin-labeled dUTP.

Northern Blot Analysis. The expression levels of nucleoside transporters in tissues of normal rats were examined by Northern blot. Total RNA (10 μg) isolated from examined tissue was separated on 3% formaldehyde/1% agarose gel and transferred onto a Bright Stars-Plus positively charged nylon membrane. Transferred RNA was cross-linked by irradiation with UV light. Membrane was treated, prehybridized, and hybridized according to Ambion manual using digoxigenin-labeled rENT1, rENT2, rCNT1, rCNT2, and 18 S probes. Hybridized probes were immunodetected with antidigoxigenin, Fab fragments conjugated to alkaline phosphatase, and then visualized with the chemiluminescence substrate disodium 3-(methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate. Light emission released after dephosphorylation of disodium 3-(methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro) tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate by alkaline phosphatase was recorded on X-ray film. Developed bands were quantified with the use of Gel Doc 2000 system (Bio-Rad, Hercules, CA). The relative amounts (optical density per square millimeter) of probes were compared using computer program Quantity One (Bio-Rad).

RNase Protection Assay. Changes in the mRNA level of each nucleoside transporter were analyzed by ribonuclease protection technique using multinuclease protection assay (Ambion) with β-actin as a reference template. Usually, 10 to 20 μg of total RNA was hybridized to the appropriate nucleoside transporter and β-actin probes were prepared according to the manufacturer's protocol. Protected RNA fragments were fractionated by electrophoresis on a 8 M urea/6% polyacrylamide gel and transferred to a positively charged nylon membrane. The hybridized probes were immunodetected, visualized, and analyzed as described above. The relative expression level of given nucleoside transporter gene was presented as a ratio of nucleoside transporter/β-actin probe.

Analytical. Protein concentrations were determined by the method of Bradford (1976) with bovine serum albumin as a standard. The DNA and RNA concentrations were determined by measuring the absorbance at 260 nm. Polyacrylamide gel electrophoresis in the presence of SDS was performed according to Laemmli (1970). Glucose was measured with the hexokinase method using the Pointe Scientific Kit.

Results

The expression level of nucleoside transporters in normal rat tissues was examined by Northern blot. Detectable amounts of equilibrative and Na^+-dependent nucleoside transporter mRNA were present in kidney, heart, and liver of healthy Wistar rats. The expression levels of rENT1 and rENT2 were highest in heart, slightly lower in liver, and the lowest in kidney (Fig. 1). The highest levels of mRNA for rCNT1 were detected in liver and kidneys. In heart, the level of rCNT1 mRNA was the lowest (Fig. 2). Liver contained the highest level of rCNT2 mRNA. The level of rCNT2 mRNA in kidney and heart was low.

Changes in nucleoside transporter mRNA level in tissues of STZ-induced diabetic rats were assessed based on RNase protection assay. Performed experiments revealed that on

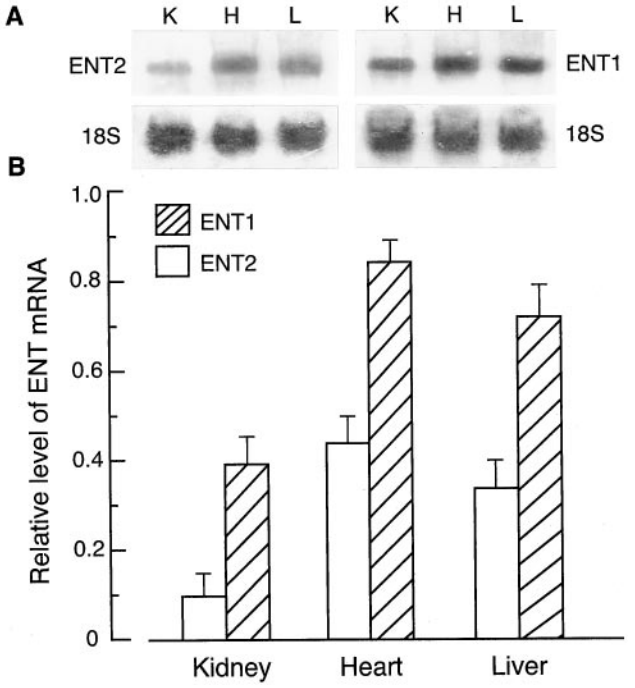

Fig. 1. The expression patterns of ENT1 and ENT2 mRNA in normal rat tissues. Total RNA was extracted and Northern blot analysis was performed as described under *Materials and Methods*. A, the presented Northern blots are representative of those obtained in at least four independent experiments. B, the quantified results of Northern blot analysis normalized to 18 S ribosomal RNA. The data represent the mean ± S.D. from four independent experiments.

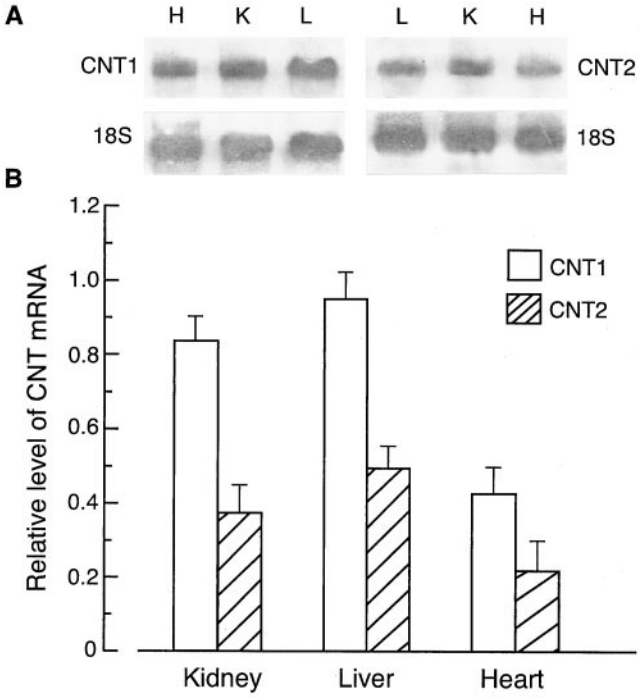

Fig. 2. The expression patterns of CNT1 and CNT2 mRNA in normal rat tissues. Total RNA was extracted and Northern blot analysis was performed as described under *Materials and Methods*. A, the presented Northern blots are representative of those obtained in at least three independent experiments. B, the quantified results of Northern blot analysis normalized to 18 S ribosomal RNA. The data represent the mean ± S.D. from at least three independent experiments.

day 10 after STZ administration, mRNA levels of rENT1 and rENT2 in diabetic tissues were lowered compared with normal tissues. The highest decrease in the level of rENT1 mRNA was observed in diabetic heart (45%) and liver (32%), whereas in diabetic kidney, the mRNA level of rENT1 was only slightly (10%) lowered (Fig. 3). The level of rENT2 mRNA was lowered by 40% in diabetic kidney and heart and by 24% in diabetic liver (Fig. 4). Changes in the expression pattern of rCNT1 and rCNT2 in diabetic tissues differed significantly from that observed for rENT. The levels of rCNT1 and rCNT2 mRNA did not change significantly in diabetic kidney compared with normal tissues (Figs. 5 and 6). In diabetic liver, the level of rCNT1 mRNA increased by 25% (Fig. 5) and the level of rCNT2 mRNA decreased by 22% (Fig. 6). The highest changes in expression level of Na^+-dependent nucleoside transporters were observed in diabetic heart. On day 10 after STZ administration, the levels of rCNT1 (Fig. 5) and rCNT2 (Fig. 6) mRNA in diabetic heart increased 1.7- and 2-fold, respectively.

To determine whether alterations in expression of nucleoside transporters observed in diabetic tissues were reversible on day 10 after STZ treatment, rats were given long-acting insulin (10 U/kg) once a day for 4 days. Administration of insulin to diabetic rats resulted in normalization of the mRNA levels for rCNT1 (Fig. 5) and rCNT2 (Fig. 6) in heart, liver, and kidney. Similarly, normalization of mRNA level for rENT2 was observed in kidney, liver, and heart, although the level of rENT2 mRNA was slightly (20%) elevated in heart (Fig. 4). On the other hand, insulin treatment had no effect on expression level of nitrobenzylthioinosine-sensitive transporter (ENT1) in diabetic tissues (Fig. 3). The changes in mRNA level for rENT2 were fast. In diabetic liver, within 6 h from insulin administration, the level of mRNA for rENT2 reached 95% of the level observed in normal tissue, whereas the level of rENT1 mRNA remained unchanged during 4 days of insulin administration (Fig. 7). During insulin treatment, the same time-dependent course of changes in mRNA levels of rENT1 and rENT2 were observed for diabetic heart and kidney (not shown). In diabetic heart, the time required for normalization of the rCNT1 and rCNT2 mRNA level in response to insulin administration was 10 h (not shown).

The data from previous studies on activity of adenosine kinase in diabetic rats (Pawelczyk et al., 2000), together with present findings indicating alterations in expression level of nucleoside transporters, would suggest that adenosine levels in diabetic tissues might be altered. To resolve this question, we have measured adenosine concentration in normal and diabetic rat tissues using the chemiluminescent method (Kather et al., 1987). Our assays showed that adenosine content in diabetic tissues elevated slowly over 5 days after STZ treatment and reached a steady state between days 5 and 10 of the experiment. On day 10 after STZ treatment, there was 3.5- and 2-fold increases in adenosine content in heart and liver, respectively (Fig. 8). In contrast, the adenosine level in diabetic kidney was only slightly (25%) elevated. Administration of insulin (10 U/kg) to diabetic rats for 4 days resulted in a normalization of adenosine level in diabetic heart, whereas adenosine level in liver and kidney dropped by 30 and 50% below the level seen in normal tissues, respectively (Fig. 8).

Discussion

Our previous studies showed that expression of adenosine kinase (AK) is decreased in tissues of diabetic rats (Pawelczyk et al., 2000). The alterations in AK expression were associated with elevated level of adenosine, but the time course of insulin-induced changes in adenosine level was different from that observed for expression of AK, suggesting that the same changes in nucleoside transporters might occur (Sakowicz and Pawelczyk, 2002). Data collected and presented in this report indicate that the metabolic and functional changes observed in diabetic rats include alterations in mRNA level of nucleoside transporters and increase in adenosine concentration in some tissues.

The distribution patterns of mRNA for rCNT1 and rCNT2 obtained in our studies showed some degree of tissue specificity. The high level of rCNT1 message found in liver and kidneys is in good agreement with reported localization of rCNT1 protein, which has been reported high in rat kidney and liver (Valdes et al., 2000; Hamilton et al., 2001). The mRNA for rCNT2 was less abundant compared with rCNT1 mRNA, and the highest level of rCNT2 transcript was de-

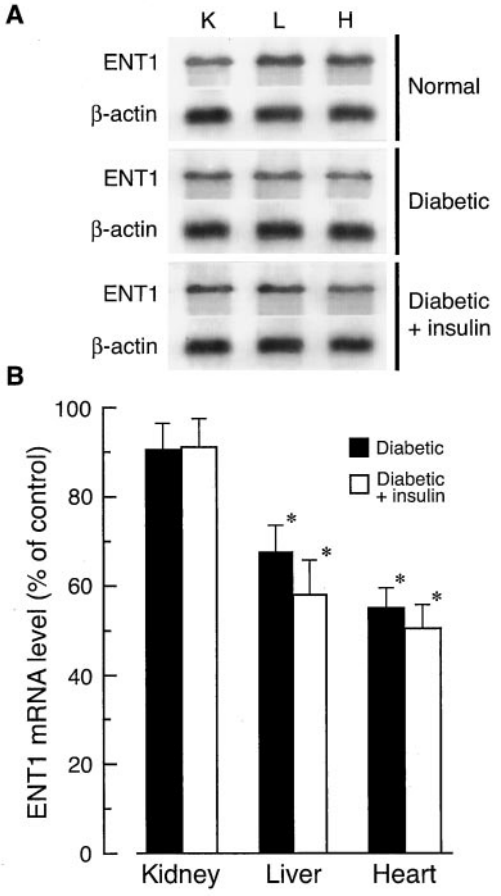

Fig. 3. Changes in ENT1 mRNA level during STZ-induced diabetes and insulin administration. Total RNA was extracted and RNase assay was performed as described under *Materials and Methods*. On day 10 after STZ injection, the rats were injected with a dose of 10 units of insulin/kg once a day for 4 days. Total RNA was extracted from diabetic rats on days 10 and 14 after STZ injection. A, the presented RNase assays are representative of those obtained in three independent experiments. B, the quantified results of RNase assays normalized to β-actin mRNA. The data represent the mean ± S.D. from three independent experiments. *, $P < 0.001$ from control

tected in liver (Fig. 2). Similarly, the level of rCNT2 protein in rats was reported to be higher in liver and lower in kidney (Valdes et al., 2000). The rank order of rENT1 and rENT2 mRNA abundance in rat tissues in the current study was heart > liver > kidney (Fig. 1). Similar distribution of ENT1 and ENT2 mRNA was reported for mouse tissues (Choi et al., 2000).

Examination of mRNA levels for rENT1 and rENT2 in diabetic rats showed that the messages for these two types of transporters were decreased. Consequently, adenosine transport in diabetic rats might be impaired if it were assumed that changes in functional protein level corresponded to changes in mRNA level. The gene expression in eukaryotes is controlled to a large extent at the transcriptional level; over the last several years, however, numerous data have been gathered indicating complex mechanisms of translational regulation (Rhoads, 1999). Nevertheless, for the purposes of this discussion, we have made an assumption that the alterations in nucleoside transporter mRNA levels reflect changes in protein level. Decrease in the level of equilibrative transporters may lead to impaired adenosine movement across the plasma membrane. Considering the decreased rate of adenosine phosphorylation caused by lowered activity of adenosine kinase (Pawelczyk et al., 2000) it may be assumed that under such conditions, adenosine may accumulate in the cell. The observed changes in adenosine concentration in diabetic heart and liver partially support such an assumption. The highest increase in adenosine concentration and most distinct decrease in mRNA level for rENT1 and rENT2 were observed in diabetic heart. On the other hand, the lack of significant changes in mRNA level for rENT1 and the most profound (compared with other tissues) decrease in adenosine kinase activity (Pawelczyk et al., 2000) in diabetic kidneys were not accompanied by any significant changes in adenosine concentration. Such a discrepancy between observed expression level of rENT1 and lack of significant changes in adenosine concentration in diabetic kidneys would be explained by translational alterations or by action of an unidentified equilibrative nucleoside transporter(s). Analysis of kinetic data for ENT in various tissues indicates the possible existence of at least four types of equilibrative transporters (Griffith and Jarvis, 1996). Th current work of Stephen Baldwin's groups identified a new cDNA clone in mouse kidney (designated mENT3) homologous to but different from published ENT1 and ENT2 sequences (Hyde et al., 2001). The possibility of increased nucleoside efflux in diabetic kidney is supported by the observations indicating

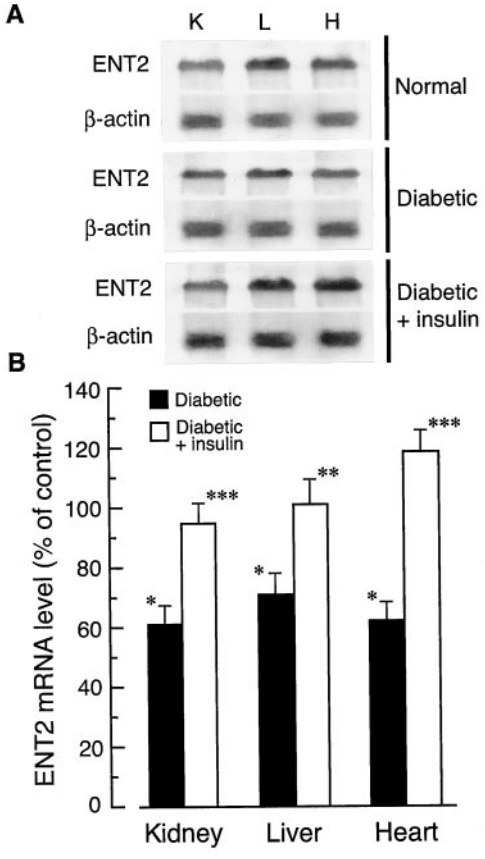

Fig. 4. Changes in ENT2 mRNA level during STZ-induced diabetes and insulin administration. Total RNA was extracted and RNase assay was performed as described under *Materials and Methods*. Total RNA was extracted from diabetic rats on day 10 after STZ injection and on day 14 (after 4 days of insulin administration). A, the presented RNase assays are representative of those obtained in three independent experiments. B, the quantified results of RNase assays normalized to β-actin mRNA. The data represent the mean ± S.D. from three independent experiments. *, $P < 0.002$ from control; **, $P < 0.01$ diabetic + insulin versus diabetic; ***, $P < 0.004$ diabetic + insulin versus diabetic.

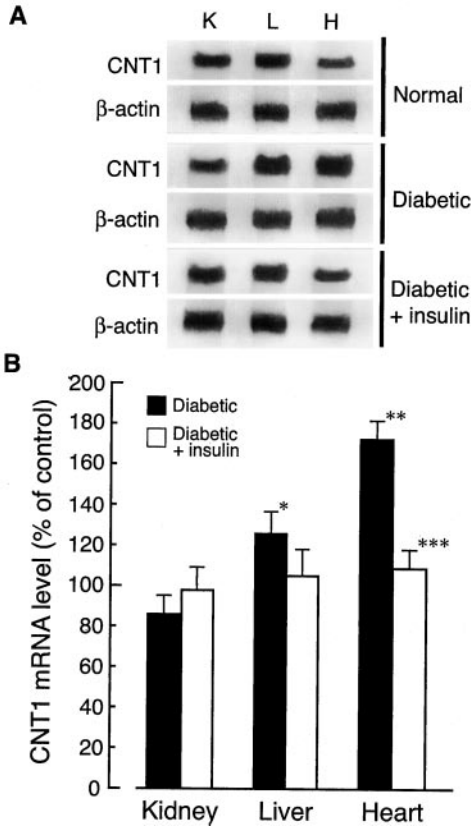

Fig. 5. Changes in CNT1 mRNA level during STZ-induced diabetes and insulin administration. Total RNA was extracted and RNase assay was performed as described under *Materials and Methods*. Total RNA was extracted from diabetic rats on day 10 after STZ injection and on day 14 (after 4 days of insulin administration). A, the presented RNase assays are representative of those obtained in three independent experiments. B, the quantified results of RNase assays normalized to β-actin mRNA. The data represent the mean ± S.D. from three independent experiments. *, $P < 0.01$ from control; **, $P < 0.0001$ from control; ***, $P < 0.0005$ diabetic + insulin versus diabetic.

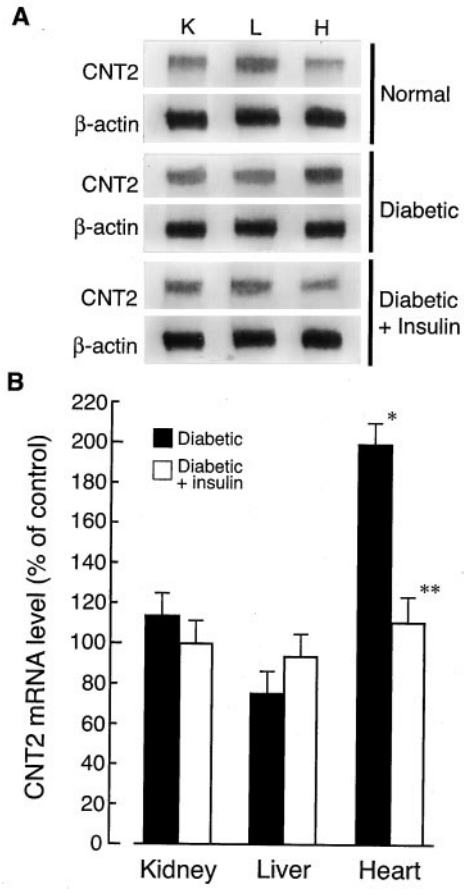

Fig. 6. Changes in CNT2 mRNA level during STZ-induced diabetes and insulin administration. Total RNA was extracted and RNase assay was performed as described under *Materials and Methods*. Total RNA was extracted from diabetic rats on day 10 after STZ injection and on day 14 (after 4 days of insulin administration). A, the presented RNase assays are representative of those obtained in three independent experiments. B, the quantified results of RNase assays normalized to β-actin mRNA. The data represent the mean ± S.D. from three independent experiments. *, $P < 0.0001$ from control; **, $P < 0.0003$ diabetic + insulin versus diabetic

higher concentration of adenosine in renal vein than in arterial blood of diabetic rats (Angielski et al., 1989).

The possible mechanisms for regulation of adenosine transport also include changes in internalization of the nucleoside transporters. Recycling of nitrobenzylthioinosine-sensitive transporter (ENT1) has been demonstrated to occur in cultured chromaffin cells and reticulocytes (Liang and Johnstone, 1992; Torres et al., 1992). Cell-specific changes in NBMPR-sensitive transport of adenosine in cultured human diabetic cells have been also reported. It was demonstrated that in endothelial cells isolated from human diabetic umbilical vein, NBMPR-sensitive adenosine transport was reduced (Sobrevia et al., 1994), whereas in smooth muscle cells isolated from diabetic human umbilical artery, adenosine transport was significantly elevated (Aguayo et al., 2001).

The differences in adenosine levels observed in diabetic tissues could also result from alterations in expression of concentrative transporters if it were assumed that changes in mRNA level corresponded to functional protein level. The highest increases in rCNT1 and rCNT2 mRNA level were observed in diabetic heart, whereas there were no alterations in rCNT1 and rCNT2 transcripts in diabetic kidneys (Figs. 5 and 6). This might indicate that under diabetic conditions, there is increased Na^+-dependent influx of adenosine into heart cells but not into kidney cells. Considering the decreased expression of rENT1 and rENT2 in diabetic heart, it may be assumed that under diabetic conditions, increased Na^+-dependent influx of adenosine into heart cells is associated with decreased efflux of this nucleoside. Such changes in

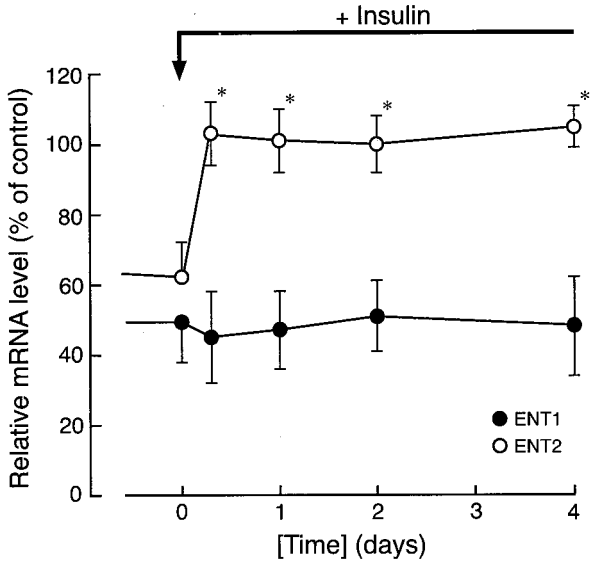

Fig. 7. Time course of insulin action on the abundance of ENT1 and ENT2 mRNA in rat liver. On day 10 after STZ injection, the rats were injected with a dose 10 units of insulin/kg once a day. At the times indicated, total RNA was extracted and RNase assay was performed as described under *Materials and Methods*. The data represent the mean ± S.D. from three independent experiments. *, $P < 0.007$ diabetic + insulin versus diabetic

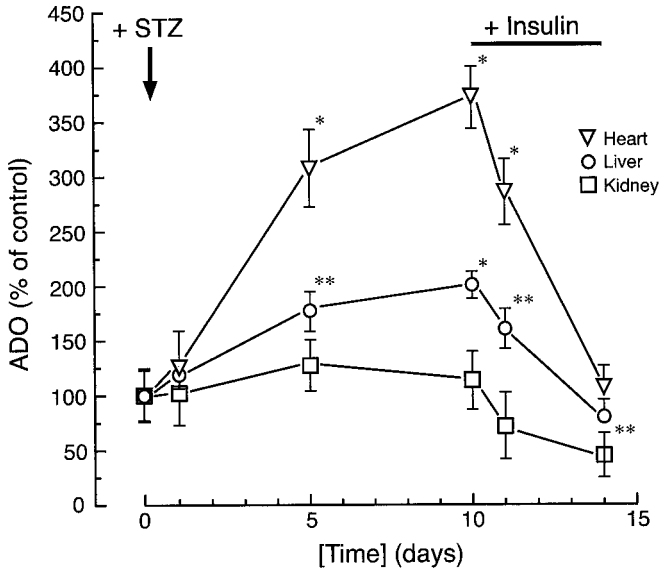

Fig. 8. Adenosine level in rat tissues during diabetes development and insulin administration. On day 10, STZ-induced diabetic rats were injected intramuscularly with insulin (long-acting) at a dose of 10 units/kg once a day for 4 days. At the times indicated, rats were killed between 7:00 and 8:00 AM and the tissue extract for adenosine measurement was prepared as described under *Materials and Methods*. The values are mean ± S.D. ($n = 5$). *, $P < 0.0001$ from control; **, $P < 0.002$ from control.

expression pattern of nucleoside transporters together with decreased activity of adenosine kinase may be the major contributors of adenosine elevation in diabetic heart.

Administration of insulin to diabetic rats resulted in normalization of adenosine level in heart, whereas in liver and kidneys, the adenosine content decreased below values observed in normal rats (Fig. 8). The stimulatory action of insulin on adenosine transport in nondiabetic cells has been reported. This includes the Na^+-dependent uridine transport in rat liver parenchymal cells (Gomez-Angelats et al., 1996), human astrocytoma cell line U-373 MG (Kum et al., 1989), and cultured rat intestinal epithelial cells IEC-6 (Jacobs et al., 1990). Herein, we showed that insulin has little or no effect on mRNA level of rCNT1 and rCNT2 in diabetic kidney and liver. On the other hand, administration of insulin to diabetic rats resulted in decrease of mRNA level of rCNT1 and rCNT2 in heart. Insulin was also without effect on decreased level of rENT1 mRNA in diabetic heart, liver, and kidneys.

The different changes in adenosine content observed in heart, liver, and especially in kidneys after insulin treatment might also result from differences in the status of insulin receptors. Studies on insulin binding in STZ-induced diabetic rats showed that the number of insulin surface receptors in liver and kidneys is increased in diabetes (Thompson et al., 1990; Sato et al., 1991). In contrast, the number of insulin receptors in diabetic heart has been reported to be unchanged (Thompson et al., 1990) or decreased (Bikhazi et al., 2000) compared with normal rat heart.

The physiological actions of adenosine depend on its extracellular concentration and are mediated by cell surface receptors. Thus, transport across the plasma membrane would be considered the important factor influencing the adenosine concentration in the immediate vicinity of its receptor. Such an assumption is supported by the experimental evidence indicating that under physiological conditions, pharmacological blockade of nucleoside transporters leads to an increase in plasma adenosine concentration (Saito et al., 1999). Data presented in this report indicate that under diabetic conditions, the adenosine transport may be altered by changes in expression level of nucleoside transporters. The most profound alterations in adenosine level and expression of nucleoside transporters have been found in heart of diabetic rat (Figs. 6 and 8). In heart tissue, reduction of extracellular adenosine concentration because of increased Na^+-dependent uptake and decreased efflux rate caused by decreased equilibrative transporters may have very important physiological significance. Such changes may result in impaired regulation of coronary blood flow by adenosine and its ability to exert a cardioprotective effect during ischemia and reperfusion. Because extracellular ATP stimulates (Erlinge, 1998) and adenosine inhibits (Dubey et al., 1998) proliferation of vascular smooth muscle cells, reduction of extracellular adenosine concentration may also affect rate of cell growth. Therefore, the above-mentioned alterations in adenosine handling may play an important role in development of such diabetic cardiovascular complications as an increase in basement membrane thickness of the microvasculature (Aageneas and Moe, 1961), impaired ventricular performance with decreased cardiac output (Fein et al., 1980), or diminished ability of diabetic myocardium to preconditioning (Ghosh et al., 2001).

Increased level of adenosine in diabetic cells accompanied by decreased activity of adenosine kinase and diminished efflux may drive the metabolism of adenosine toward deamination to inosine and hypoxanthine. The latter product serves as a substrate for xanthine oxidase that oxidizes xanthine into uric acid with concomitant generation of $O_2^{\cdot-}$. It has been demonstrated that increased purine degradation results in altered radical formation (Siems et al., 1989; Jabs et al., 1995). On the other hand, alterations in both the rates of formation and extent of scavenging of the oxygen free radicals have been implicated in vascular dysfunction seen in diabetes (Ammar et al., 2000; Singal et al., 2001). There is a growing body of evidence suggesting that an antioxidative treatment might be helpful to reduce cardiac risk in diabetes (Laight et al., 2000; Beckman et al., 2001).

In conclusion, the data presented in this report indicate that expression of nucleoside transporters in tissues of diabetic rats is altered and that changes in expression level of nucleoside transporters in diabetic heart and liver correlate well with changes in adenosine concentration in these tissues. Thus, it may be assumed that in diabetic rats, nucleoside transporters are important factors influencing adenosine level in the cell. The major challenge for future studies is to resolve the relationship of adenosine transport processes, cellular adenosine level, and adenosine metabolism in diabetic kidney, especially in each compartment of this organ. The mechanism leading to insulin-induced alterations in the mRNA level for rENT and rat concentrative Na^+-dependent nucleoside transporter requires further investigation.

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