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Regulation of Adenosine Receptors Expression in Rat B Lymphocytes by Insulin

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ABSTRACT

Development of diabetes is associated with altered expression of adenosine receptors (ARs). Some of these alterations might be attributed to changes in insulin concentration. This study was undertaken to investigate the possible insulin effect on ARs level, and to determine the signaling pathway utilized by insulin to regulate the expression of ARs in rat B lymphocytes. Western blot analysis of B lymphocytes protein extracts indicated that all four ARs were present at detectable levels in the cells cultured for 24 h without insulin ($\leq 10^{-11}$ M), although the protein band of A_{2A}-AR was barely visible. Inclusion of insulin (10^{-8} M) in the culture medium resulted in an increase of A_1-AR and A_{2A}-AR protein levels and a significant decrease of A_{2B}-AR protein, whereas the protein level of A_3-AR remained unchanged. Alterations in the ARs protein content were accompanied by changes in the ARs mRNA levels. Increase of the insulin concentration from 10^{-11} to 10^{-8} M resulted in 50% decrease of A_{2B}-AR mRNA level and two-, and threefold increase of A_1-AR and A_{2A}-AR mRNA levels, respectively. Pretreatment of B cells with cycloheximide completely blocked the insulin action on A_1-AR and A_{2A}-AR mRNA, but not on A_{2B}-AR expression. Detailed pharmacological analysis demonstrated that insulin-induced A_1-AR and A_{2A}-AR mRNA expression through the Ras/Raf-1/MEK/ERK pathway. The insulin effect on A_{2B}-AR expression was blocked by p38 MAP kinase inhibitor (SB 203580). Concluding, elevated insulin concentration differentially affects the expression of ARs in B lymphocytes in a fashion that might enhance the various immunomodulatory effects of adenosine. J. Cell. Biochem. 109: 396–405, 2010.

KEY WORDS: B-CELL; INSULIN; ADENOSINE RECEPTORS

Development of diabetes mellitus results from the defects in insulin secretion, insulin action on peripheral tissues, or both. In type 1 diabetes, there is an absolute deficiency of insulin due to a cellular-mediated autoimmune destruction of the insulin-secreting cells, whereas patients with type 2 diabetes display combination of insulin resistance and relative insulin deficiency [American Diabetes Association, 2008]. Progression of diabetes results in the development of specific complications including altered function of immune system. Patients with diabetes are generally more prone to infection and resulting complications [McMahon and Bistrian, 1995; Joshi et al., 1999]. However, the pathomechanisms behind the apparent immunosuppression observed in diabetes are not fully understood. It was demonstrated that peripheral blood mononuclear cells from diabetic patients are reduced in their ability to produce cytokines [Kaye et al., 1986; Pickup et al., 2000] and the proliferative response of T cells to primary protein antigens are significantly reduced [Eibl et al., 2002]. The mechanisms responsible for the impaired lymphocyte function in diabetes are largely unclear, however, some clue point to adenosine as a factor involved in the pathomechanism leading to altered function of lymphocytes in diabetes [Sakowicz-Burkiewicz et al., 2006; Nemeth et al., 2007].

Adenosine is an endogenous nucleoside exerting a potent action on the immune cells. Under in vitro conditions adenosine has the ability to alter events such as lymphocyte activation, proliferation, cytokines production, and lymphocyte-mediated cytolysis [Wolberg et al., 1975; Sandberg, 1983; Dos Reis et al., 1986; Antonysamy et al., 1995; Hasko et al., 2000]. These adenosine actions result from ligation of cell surface adenosine receptors (ARs) and subsequent activation of the downstream intracellular pathways. There are four types of known ARs namely A_1-AR, A_{2A}-AR, A_{2B}-AR, and A_3-AR [Fredholm et al., 2001]. Development of diabetes results in altered expression of ARs in many types of cells [Grden et al., 2005, 2007]. Moreover, it appears that diabetes-induced changes

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in ARs expression are tissue and cell type specific [Pawelczyk et al., 2005].

B lymphocytes are the core constituent of immunological system, therefore, the mechanisms that affect function of these cells have important impact on the humoral response. Previously we have documented that insulin specifically regulate the expression of nucleoside transporters in rat B cells [Sakowicz-Burkiewicz et al., 2004]. Moreover, the alterations of nucleoside transporters expression occurred in B lymphocytes under diabetic conditions result in diminished uptake of adenosine. Therefore, the adenosine concentration in the near vicinity of B cell plasma membrane might increase resulting in higher bioavailability of this nucleoside at receptor sites. However, there are no data on the insulin effect on ARs expression level in B lymphocytes. Therefore, the goal of our study was to investigate the expression level of ARs in B lymphocytes cultured at different insulin concentrations. We evaluated the signaling pathways utilized by insulin to regulate the ARs expression in rat B lymphocytes.

MATERIALS AND METHODS

REAGENTS

Histopaque-1077, insulin, penicillin, streptomycin, 2′-amino-3′-methoxyflavone (PD 98059), glucose, rapamycin, wortmannin, cycloheximide, manumycin A, leupeptin, geldanamycin, and RPMI-1640 medium, were obtained from Sigma–Aldrich Sp. z o.o. (Poznan, Poland). Bisindolylmaleimide I (Bis I) and 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole (SB 203580), were obtained from Calbiochem-Merck Sp. z o.o. (Warsaw, Poland). All primers used were from Integrated DNA Technologies, Inc. (Coralville, IA). Total RNA Prep Plus Kit was from A&A Biotechnology (Gdansk, Poland).

ANTIBODIES

Primary rabbit polyclonal antibodies to A_1-AR (A-268) and β-actin, were from Sigma–Aldrich Sp. z o.o. Rabbit polyclonal antibody to A_{2B}-AR (AB1589P) was from Chemicon International (Temecula, CA). Goat polyclonal antibodies to A_{2A}-AR (R-18), A_3-AR (C-17), phospho-ERK-1/2 (Tyr-204), phospho-MEK-1 (Ser-218/Ser-222), and phospho-Raf-1 (Ser-338) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal antibodies to Raf-1 (C-20), MAPK kinase (MEK)-1 (C-18), c-Jun (H-79), c-Fos (H-125), Elk-1 (I-20), extracellular signal-regulated protein kinase (ERK)-1/2 (K-23), p38 MAPK (C-20), phospho-p38 MAPK (Tyr-182)-R, $p70^{S6K}$ (C-18), phospho-$p70^{S6K}$ (Thr 421/Ser-424), phospho-Elk-1 (Tyr-204), and agarose-conjugated rabbit polyclonal antibodies to PI3K (330-430) were from Santa Cruz Biotechnology, Inc. Rabbit anti-goat IgG alkaline phosphatase conjugate (A4187) and goat anti-rabbit IgG alkaline phosphatase conjugate (A3687) were from Sigma–Aldrich Sp. z o.o. Fluorescein conjugate mouse anti rat CD2[LFA-2] (clone OX-34) was from Chemicon International (Hofheim, Germany). Fluorescein conjugate Armenian hamster anti rat CD40 (clone HM40-3), and mouse anti rat CD19 (clone 1D3) were from BD Biosciences (Heidelberg, Germany).

ANIMALS

Male Wistar rats (200–240 g) fed on Altromin C 1000 diet (Altromin GmbH, Lage, Germany) were used for all experiments. All animals had access to food and water ad lib. The experiments on animals were conducted in accordance with the protocol approved by the Regional Bioethical Commission at the Medical University of Gdansk (permission-NKEBN/24/2003).

CELLS AND CULTURE CONDITIONS

Rats were killed by decapitation and the spleen was removed. Single cell suspension of splenocytes was prepared by pressing spleens through sterilized 20 mm 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. 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 non-adhesive by the panning method as described previously [Sakowicz-Burkiewicz et al., 2004]. The purity of isolated cell fractions was examined by flow cytometry. The adherent fraction (B cells) contained 95–97% CD2 (OX-34) negative cells, 89–93% CD40 (HM40-3) and 85–90% CD19 (1D3) 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 $2–4 \times 10^6$ cells/ml in RPMI-1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 10% fetal bovine serum. For the first 24 h cells were cultured without insulin (insulin $\leq 10^{-11}$ M). After 24 h the compounds examined (insulin and inhibitors) were added to the lymphocyte culture in the concentration and the order and for the time detailed in the figure legends. Insulin was dissolved in saline, and inhibitors were dissolved in a small volume (<0.2% of the total volume of culture medium) of DMSO.

REAL-TIME PCR ANALYSIS

The levels of ARs transcripts were analyzed by real-time PCR performed in a Light Cycler 2.0 (Roche Diagnostics GmbH, Mannheim, Germany) using the Light Cycler DNA SYBR Green I Kit. The reaction mixture contained 1 μl Master Mix, 5 pmol of each primer and 2 μl of cDNA. The primers for A_1-AR, A_{2A}-AR, A_{2B}-AR, A_3-AR, and β-actin cDNA amplification were as described previously [Pawelczyk et al., 2005]. As a negative controls water was run with every PCR. The specificity of product was controlled by melting curve analysis, and by agarose gel electrophoresis. The ratio of AR/β-actin was calculated for each sample. Analysis of the data was done using Light Cycler software 4.0.

WESTERN BLOT ANALYSIS

The extract of B cells was obtained by sonication (3 × 15 s) of cell suspension in 20 mM Tris–HCl, pH 7.2, 1 mM dithiothreitol, 0.2 mM Pefabloc SC, and 5 μM leupeptin. The proteins from obtained extract were separated by 12% SDS–polyacrylamide gel electrophoresis, and electrophoretically transferred to Immobilon poly-(vinylidene difluoride) transfer membrane. The membrane was incubated at 4°C (overnight) with 3% BSA in Tris-buffered saline (TBS). The

membrane was then cut horizontally at appropriate position (based on positions of prestained molecular mass markers), and incubated with appropriate primary antibodies. Next the membrane strips were incubated with alkaline phosphatase-conjugated secondary antibodies. Membrane bound antibodies were visualized with 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium. For A_1-AR and A_{2B}-AR the β-actin was used as a reference protein. Since, A_{2A}-AR is located at 45 kDa position, and rat A_{2B}-AR protein band migrate at position ranging 52–32 kDa (depending on tissue of origin), we used a p14-3-3 as a reference protein for these two receptors [Grden et al., 2005, 2007; Pawelczyk et al., 2005].

PHOSPHATIDYLINOSITOL 3-KINASE (PI3K) ASSAY

The PI3K activity was assayed by a radioenzymatic method as described in details previously [Pawelczyk et al., 2003]. Briefly, lymphocyte lysates were immunoprecipitated with an agarose-conjugated polyclonal anti-PI3K antibody and immune complexes were washed and incubated with phosphatidylinositol liposomes and [γ^{32}]ATP for 15 min at 37°C. The reaction was stopped by the addition of 20 μl 8 M HCl and extracted with $CHCl_3$:CH_3OH (1:1). The compounds in organic phase were separated by thin layer chromatography and visualized by autoradiography.

STATISTICAL ANALYSIS

The statistical calculation was performed with ANOVA or Dunnett's test for comparison to control group. Paired Student's *t*-test was performed when two groups were analyzed. *P* values below 0.05 were considered as significant.

RESULTS

THE INSULIN EFFECT ON ADENOSINE RECEPTORS EXPRESSION LEVEL IN RAT B LYMPHOCYTES

Western blot analysis of rat B lymphocytes protein extracts indicated that all four ARs were present at detectable levels in cells cultured for 24 h without insulin ($\leq 10^{-11}$ M), although the protein band of A_{2A}-AR was barely visible (Fig. 1). Inclusion of insulin (10^{-8} M) in the culture medium resulted in an increase of A_1-AR and A_{2A}-AR protein levels and a significant decrease of A_{2B}-AR protein, whereas the protein level of A_3-AR remained unchanged. Alterations in the ARs protein content were accompanied by changes in the ARs mRNA levels. Detailed analysis of dose- and time-dependent courses of insulin action on A_1-AR mRNA level showed that maximal effect of insulin was observed in 24th hour of incubation, and at insulin concentration of 10^{-8} M (Fig. 2). Change the insulin concentration from 10^{-11} to 10^{-8} M resulted in twofold increase of A_1-AR mRNA level. Similar effect of insulin was observed on A_{2A}-AR mRNA level. An increase of insulin concentration from 10^{-11} to 10^{-8} M led to 3.5-fold raise in A_{2A}-AR mRNA level (Fig. 3). Exposition of B cells to insulin (10^{-8} M) resulted in 50% decrease of A_{2B}-AR mRNA level. The maximal insulin-induced suppression of A_{2B}-AR mRNA was observed at 36 h (Fig. 4). Examination of A_3-AR mRNA in B cells cultured at different insulin concentrations indicated no changes in A_3-AR transcript level (not shown). These results together with data from Western blot analysis indicated that insulin did not affect the expression level of A_3-AR in rat B lymphocytes.

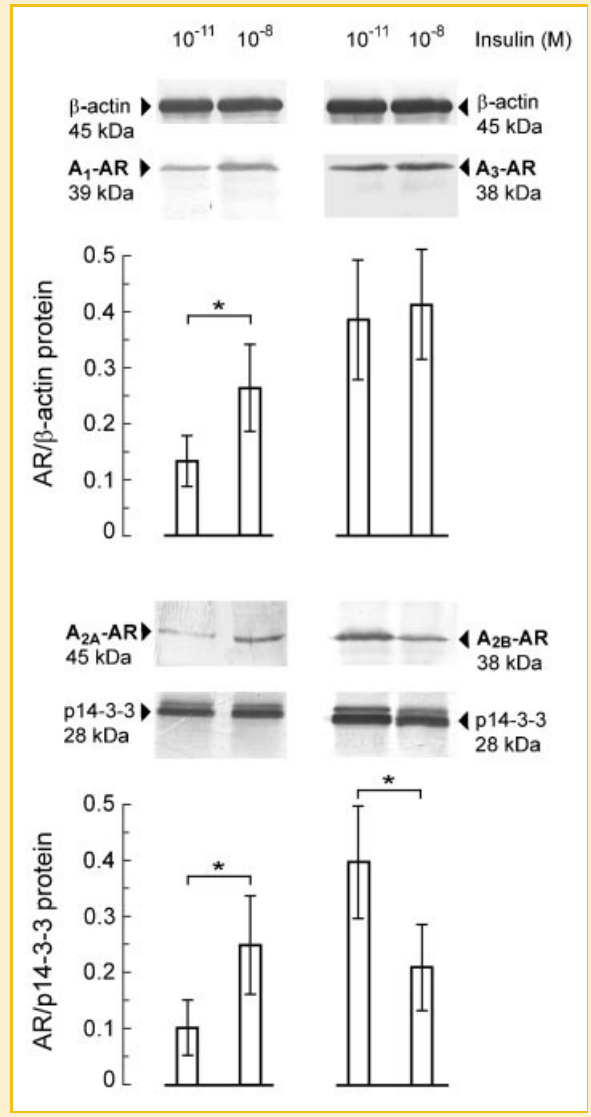

Fig. 1. The protein level of adenosine receptors in rat B lymphocytes cultured at various insulin concentrations. B lymphocytes isolated from rat spleen were cultured for 48 h at 10^{-11} or 10^{-8} M insulin and the protein extracts were prepared as described under the Materials and Methods Section. The proteins were separated on 12% SDS–PAGE and immunoblotted with appropriate antibodies. The presented blots are representative of those obtained in at least three independent experiments. The blots were scanned and quantified. The bars represent mean values normalized to appropriate reference protein (β-actin or p14-3-3 protein) ± SD (n = 3). $^*P < 0.05$.

To determine whether insulin regulates ARs expression by transcriptional or posttranscriptional mechanisms, we treated B cells with actinomycin D, an inhibitor of transcription. Following addition of actinomycin D the decline of insulin-induced ARs mRNAs was fast, but not significantly different from that observed in unstimulated cells. The calculated half-lives of A_1-AR, A_{2A}-AR, A_{2B}-AR, and A_3-AR mRNAs were 9.5 ± 0.5, 7,6 ± 0.3, 8.2 ± 0.5, and 14 ± 0.7 h, respectively (not shown). Thus, although AR's genes transcriptions

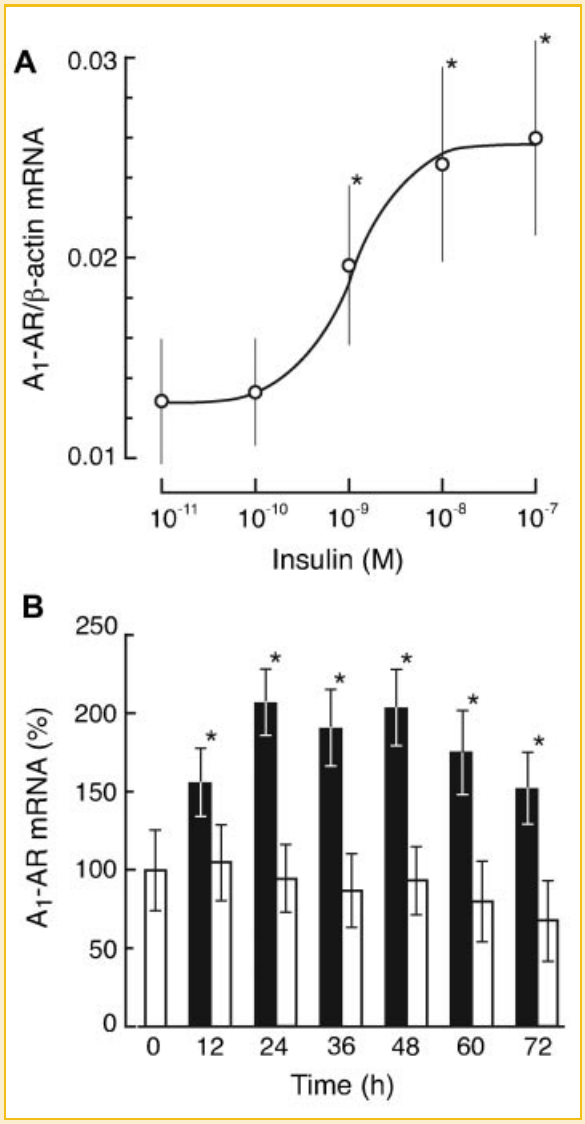

Fig. 2. The insulin effect on the abundance of A_1-AR mRNA in rat B lymphocytes. A: Dose-dependent course of insulin action on the A_1-AR mRNA level. Cells were cultured for 24 h in the presence of insulin at the concentrations indicated. Next, cells were harvested, total RNA was extracted, and the A_1-AR mRNA level was determined by real-time PCR as described under the Materials and Methods Section. The data represent the mean ± SD from three experiments. $^*P < 0.05$ versus 10^{-11} M insulin. B: Time course of insulin action on the A_1-AR mRNA level. Cells were cultured in the presence of 10^{-11} M insulin for 24 h. On the second day (time 0) cells were transferred to the culture medium containing 10^{-8} M insulin (black bars) or were maintained at low insulin (open bars). At time indicated, cells were harvested and the A_1-AR mRNA level was determined as described above. The data represent the mean ± SD from three experiments. $^*P < 0.05$ versus 0 time.

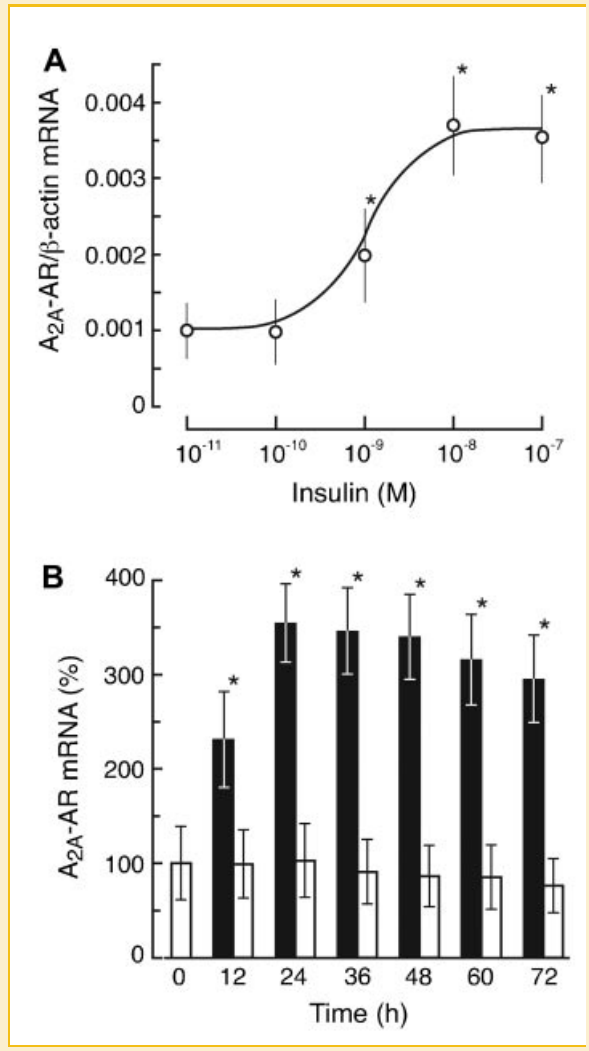

Fig. 3. The insulin effect on the abundance of A_{2A}-AR mRNA in rat B lymphocytes. A: Dose-dependent course of insulin action on the A_{2A}-AR mRNA level. Cells were cultured for 24 h in the presence of insulin at the concentrations indicated. Next, cells were harvested, total RNA was extracted, and the A_{2A}-AR mRNA level was determined by real-time PCR as described under the Materials and Methods Section. The data represent the mean ± SD from three experiments. $^*P < 0.05$ versus 10^{-11} M insulin. B: Time course of insulin action on the A_{2A}-AR mRNA level. Cells were cultured in the presence of 10^{-11} M insulin for 24 h. On the second day (time 0) cells were transferred to the culture medium containing 10^{-8} M insulin (black bars) or were maintained at low insulin (open bars). At time indicated, cells were harvested and the A_{2A}-AR mRNA level was determined as described above. The data represent the mean ± SD from three experiments. $^*P < 0.05$ versus 0 time.

were not directly measured, we assumed that change in the particular AR mRNA level was not due to change in mRNA stability, but reflected alteration in the rate of gene transcription.

THE INSULIN-SIGNALING PATHWAYS IN RAT B LYMPHOCYTES

To define the key steps of insulin signaling involved in regulation of the ARs expression in rat B lymphocytes we used specific inhibitors of distinct steps of cellular-signaling pathways. Optimal concentrations of the inhibitors were determined by pilot studies and are in accordance with previous reports [Yano et al., 1993; Alessi et al., 1995; Cuenda et al., 1995; Abraham and Wiederrecht, 1996; Nagase et al., 1996; Davies et al., 2000]. The mitogen-activated protein kinase (MAPK) pathway and phosphatidylinositol 3-kinase (PI3K) pathway are the main routes downstream of the insulin receptor. The key components of MAPK pathway are small G protein Ras, kinase Raf-1, MEK, and ERK 1 and 2. Effective Ras signaling requires attachment of this protein to the plasma membrane mediated by the

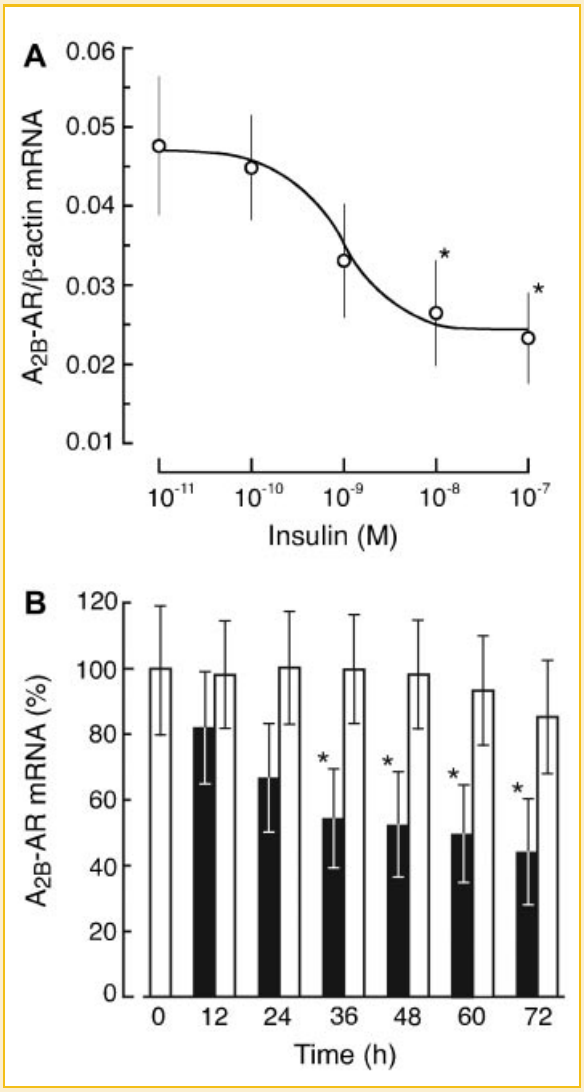

Fig. 4. The insulin effect on the abundance of A_{2B}-AR mRNA in rat B lymphocytes. A: Dose-dependent course of insulin action on the A_{2B}-AR mRNA level. Cells were cultured for 48 h in the presence of insulin at the concentrations indicated. Next, cells were harvested, total RNA was extracted, and the A_{2B}-AR mRNA level was determined by real-time PCR as described under the Materials and Methods Section. The data represent the mean ± SD from three experiments. $^{*}P < 0.05$ versus 10^{-11} M insulin. B: Time course of insulin action on the A_{2B}-AR mRNA level. Cells were cultured in the presence of 10^{-11} M insulin for 24 h. On the second day (time 0) cells were transferred to the culture medium containing 10^{-8} M insulin (black bars) or were maintained at low insulin (open bars). At time indicated, cells were harvested and the A_{2B}-AR mRNA level was determined as described above. The data represent the mean ± SD from three experiments. $^{*}P < 0.05$ versus 0 time.

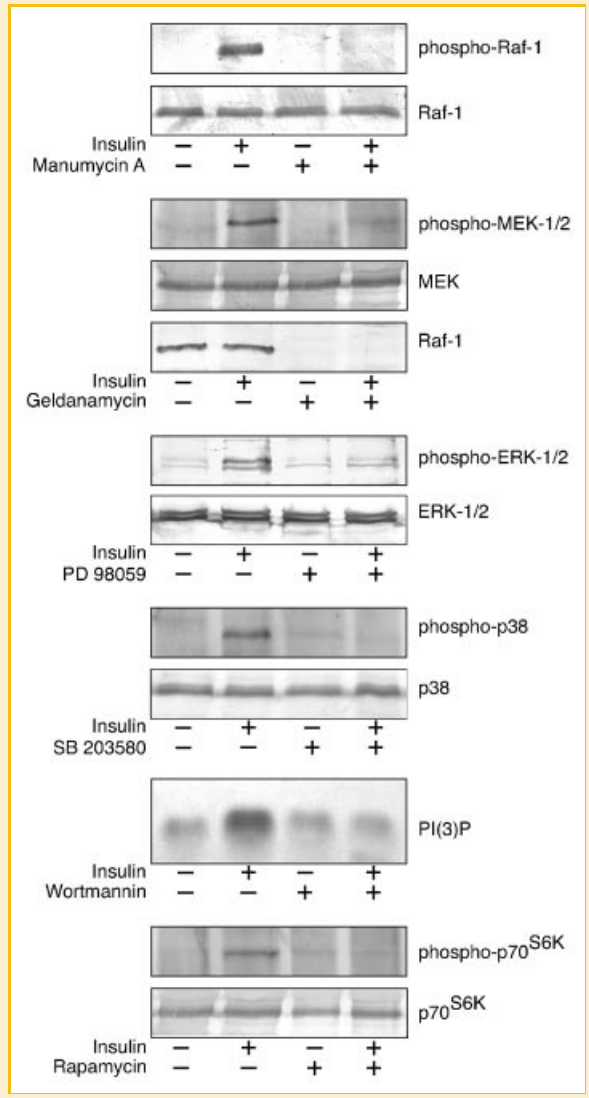

Fig. 5. Activation status of MAPK family members, PI3K and $p70^{S6K}$ after insulin treatment of B lymphocytes. B cells isolated from rat spleen were cultured in the presence of 10^{-11} M insulin for 24 h (−). Next, the cells were transferred to the culture medium containing 10^{-8} M insulin for 24 h (+) and at the 48 h the protein extracts were prepared and analyzed by immunoblotting with antibodies specific for the unphosphorylated and phosphorylated proteins (indicated on the right). The PI3K activity was assayed as described under the Materials and Methods Section by a radioenzymatic assay and visualized by an autoradiography of incorporated [γ^{32}]ATP into phosphatidylinositol (PI(3)P). The inhibitors investigated (indicated on the left) were added to the culture medium 1 h before insulin addition at concentrations as indicated in the Figure 6. Geldanamycin (5 μM) was included into the culture medium 24 h before addition of 10^{-8} M insulin. The presented immunoblots are representative of those obtained in at least three independent experiments.

farnesyl residue. Inhibition of the farnesyl protein transferase prevents the attachment of Ras protein to the plasma membrane and adversely affects the signal propagation by Raf-1/MEK/ERK pathway. We observed that pretreatment of B lymphocytes with farnesyl protein transferase inhibitor, manumycin A suppressed the phosphorylation of Raf-1, an immediate downstream signaling molecule of Ras (Fig. 5). Raf-1 kinase exists as a multiprotein complex composed of hsp90, p50, and other proteins [Stancato et al., 1993]. It was demonstrated that disruption of the Raf-1-hsp90 molecular complex by the benzoquinone ansamycin geldanamycin results in destabilization of Raf-1 and accelerated destruction of the protein [Schulte et al., 1996]. Pretreatment of the B cells for 24 h with geldanamycin resulted in a decrease of Raf-1 protein to undetectable level and suppression of insulin-induced

phosphorylation of MEK (Fig. 5). MEK is the known mediator of Raf-1 on ERK phosphorylation and activation. Pretreatment of the B lymphocytes for 60 min with MEK inhibitor (PD 98059) resulted in suppression of insulin-induced ERK 1/2 phosphorylation, but did not alter the total levels of ERK 1/2 proteins (Fig. 5). We observed that exposition of rat B lymphocytes to insulin (10^{-8} M) resulted in phosphorylation of p38 MAP kinase, which was blocked by SB 203580 an selective inhibitor of the a and b isoforms of p38 MAP kinase. Incubation of the B cells with insulin resulted in activation of PI3K (Fig. 5). PI3K is efficiently inhibited by wortmannin, which binds covalently to its p110 catalytic subunit [Yano et al., 1993]. Prior exposure of the B cells (60 min before addition of insulin) to 100 nM wortmannin totally suppressed the insulin-induced activation of PI3-K (Fig. 5). Incubation of the B lymphocytes with insulin stimulated another element downstream of the PI3-K pathway, namely p70 ribosomal S6 kinase ($p70^{S6K}$). The phosphorylation of $p70^{S6K}$ was seen as early as 30 min after exposition of the cells to insulin and was prevented by prior pretreatment of the cells with rapamycin (Fig. 5). Rapamycin, by inactivating mTOR (target of rapamycin), potently inhibits phosphorylation and activation of $p70^{S6K}$ [Abraham and Wiederrecht, 1996].

INSULIN STIMULATES EXPRESSION OF A_1-AR AND A_{2A}-AR IN RAT B LYMPHOCYTES IN A Ras/Raf-1/MEK/ERK-DEPENDENT MANNER

Pretreatment of rat B lymphocytes with cycloheximide (a translational inhibitor) completely blocked the insulin action on A_1-AR and A_{2A}-AR mRNA expression (Fig. 6). This would suggests that in rat B cells protein synthesis is required for the insulin-induced expression of A_1-AR and A_{2A}-AR. Inhibition of PI3-K with wortmannin and inhibition of mTOR with rapamycin did not affect the insulin action on A_1-AR and A_{2A}-AR transcripts level (Fig. 6). Exposition of the cells to Bis I, an isozyme non-selective protein kinase C (PKC) inhibitor did not effect the insulin-induced expression of ARs transcripts. We observed that incubation of B cells for 24 h with geldanamycin rendered them unresponsive to insulin in term of A_1-AR and A_{2A}-AR mRNA expression. Pretreatment of rat B cells with manumycin A or with PD 98059 completely blocked the insulin effect on A_1-AR and A_{2A}-AR mRNA level. Incubation of the cells with SB 203580 for 60 min before the exposition to insulin had no effect on insulin-induced expression of A_1-AR and A_{2A}-AR mRNA (Fig. 6). Taken together our data indicate that intact Ras/Raf-1/MEK/ERK pathway is required for insulin to induce the expression of A_1-AR and A_{2A}-AR mRNA in rat B lymphocytes.

The common effect of insulin action observed in several cell types including lymphocytes is stimulation of activating protein-1 (AP-1) complex [O'Brien and Granner, 2004]. AP-1 is ubiquitously expressed transcription factor composed of the Fos and Jun proteins families. We found that the level of Fos and Jun proteins increased on addition of insulin to the cultured B cells (Fig. 7). A detectable increase of Fos and Jun proteins levels was observed at 4–8 h, with a peak around 12 h. The major mediator of *c-fos* gene induction by insulin and other growth factors is transcription factor Elk-1 [Karin, 1994]. In response to mitogen stimulation Elk-1 undergoes phosphorylation on several serine residues. We observed that in rat B cells insulin-induced transient phosphorylation of Elk-1

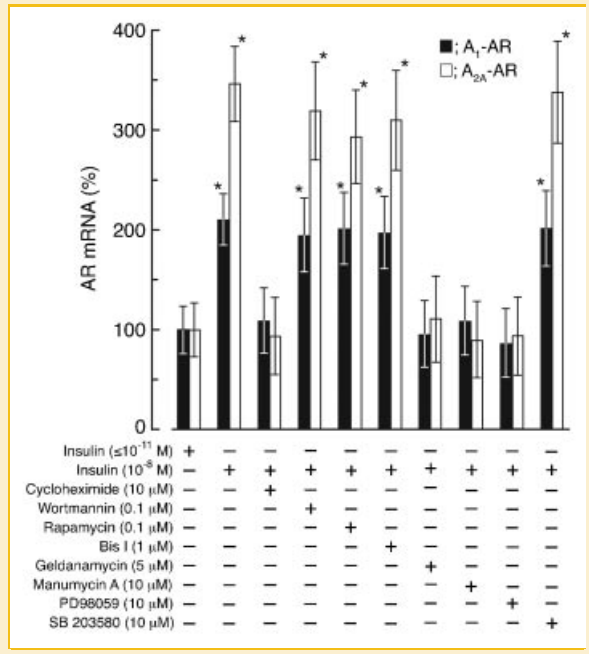

Fig. 6. The effect of translational inhibitor (cycloheximide), Ras farnesylation inhibitor (manumycin A), and PI3K (wortmannin), mTOR (rapamycin), Raf-1 (geldanamycin), PKC (Bis I), MEK (PD 98059), p38 MAPK (SB 203580), inhibitors on insulin-induced expression of A_1-AR and A_{2A}-AR mRNA in rat B lymphocytes. The cells were cultured in RPMI medium containing 10^{-11} M insulin for 24 h and then for 24 h in medium containing 10^{-8} M insulin. One hour before raising the insulin concentration to the culture medium examined inhibitors were added at indicated concentrations. Geldanamycin was included into the culture medium 24 h before addition of 10^{-8} M insulin. After 48 h incubation the cells were harvested and the A_1-AR and A_{2A}-AR mRNA were quantified as described under the Materials and Methods Section. To the control cells (no inhibitor added) appropriate volume of solvent (DMSO) was added. The data represent the mean ± SD from four experiments. $^*P < 0.05$ versus 10^{-11} M insulin.

(Fig. 7). Maximal phosphorylation of Elk-1 was evident at 8 h. Several MAP kinases are capable to phosphorylate Elk-1 including ERK kinases [Gille et al., 1995; Yang et al., 1998]. Consistent with these data we found that insulin-induced phosphorylation of Elk-1 was significantly decreased under conditions where ERK activation was blocked with the MEK inhibitor PD 98059 (Fig. 7). Moreover, blockade of ERK activation and phosphorylation with PD 98059 abolished insulin-induced expression of Fos and Jun proteins.

INSULIN-INDUCED SUPPRESSION OF A_{2B}-AR EXPRESSION IN RAT B LYMPHOCYTES DEPENDS ON ACTIVITY OF p38 MAP KINASE

Exposition of rat B lymphocytes to insulin resulted in significant decrease of the A_{2B}-AR mRNA level. This insulin action on A_{2B}-AR mRNA level was not affected by cycloheximide implying no need for protein synthesis (Fig. 8). Blockade of PI3K pathway with wortmannin or MAPK pathway with inhibitors acting on distinct steps of signaling cascade did not prevented insulin-induced suppression of A_{2B}-AR mRNA expression. The only compound capable to suppress insulin action on A_{2B}-AR expression was an inhibitor of p38 MAPK. Incubation of B cells with SB 203580 (10 μM) for 60 min before addition of insulin totally blocked the

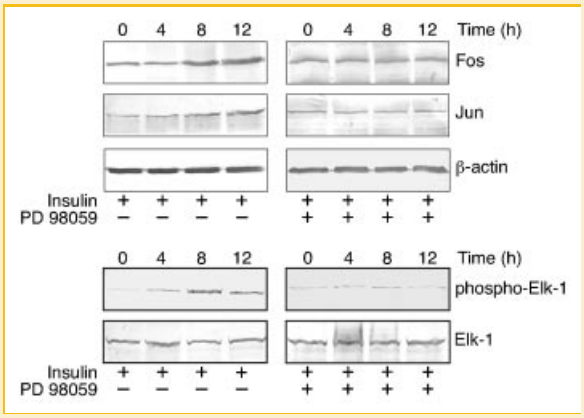

Fig. 7. The insulin effect on Fos and Jun proteins levels, and on Elk-1 phosphorylation level. Cells were cultured in the presence of 10^{-11} M insulin for 24 h. On the second day (time 0) the insulin concentration was raised to 10^{-8} M and cells were incubated with (+) or without (−) 10 μM PD 98059. PD 98059 was added to the incubation medium 1 h before changing the insulin concentration. At time indicated, cells were harvested, the protein extracts were prepared and immunoblotted with antibodies to proteins indicated on the right. Reprobing the membranes with β-actin or Elk-1 antibody controlled the equal amount of protein loading. To the control cells (no inhibitor added) appropriate volume of solvent (DMSO) was added. The presented immunoblots are representative of those obtained in at least three independent experiments.

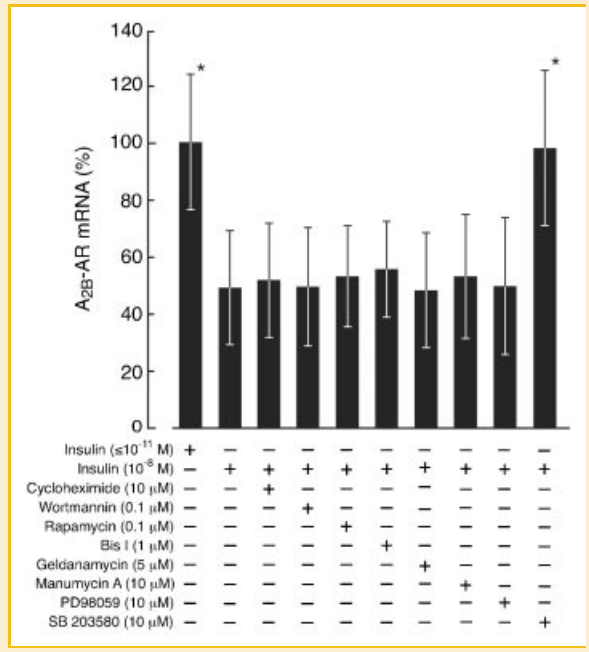

Fig. 8. The effect of translational inhibitor (cycloheximide), Ras farnesylation inhibitor (manumycin A), and PI3K (wortmannin), mTOR (rapamycin), Raf-1 (geldanamycin), PKC (Bis I), MEK (PD 98059), p38 MAPK (SB 203580), inhibitors on insulin-induced suppression of A_{2B}-AR mRNA expression in rat B lymphocytes. The cells were cultured in RPMI medium containing 10^{-11} M insulin for 24 h and then for 36 h in medium containing 10^{-8} M insulin. One hour before raising the insulin concentration to the culture medium examined inhibitors were added at indicated concentrations. Geldanamycin was included into the culture medium 24 h before addition of 10^{-8} M insulin. After 60 h incubation the cells were harvested and the A_{2B}-AR mRNA was quantified as described under the Materials and Methods Section. To the control cells (no inhibitor added) appropriate volume of solvent (DMSO) was added. The data represent the mean ± SD from four experiments. $^{*}P < 0.05$ versus 10^{-8} M insulin.

insulin action on A_{2B}-AR mRNA expression (Fig. 8). The p38 MAP kinases family is composed of four members namely α, β, γ, and δ, but only isoform p38α and p38β are inhibited by SB 203580 [Cuenda and Rousseau, 2007]. Thus, our data might suggest that insulin suppressed the expression of A_{2B}-AR in rat B lymphocytes in a p38α and/or p38β-dependent manner.

DISCUSSION

Previously reported data indicate that diabetes development results in altered expression of ARs in a variety of cell types including T lymphocytes [Grden et al., 2005, 2007; Pawelczyk et al., 2005; Sakowicz-Burkiewicz et al., 2006]. Some of these changes might be attributed to lack of insulin, which is known to regulate the expression of great number of genes. The extensiveness of insulin action on genes expression illustrates data obtained from a microarray based study demonstrating that in human skeletal muscle about 800 genes were affected during a hyperinsulinemic clamp [Rome et al., 2003]. Our previous study performed on cardiac fibroblasts showed that insulin affects the ARs expression, but the signaling pathways utilized by insulin to regulate ARs expression was not investigated [Grden et al., 2006]. Data obtained in this study document the presence of all four ARs in rat B lymphocytes, although the A_{2A}-AR protein was barely detectable in the cells incubated at low insulin concentration ($\leq 10^{-11}$ M). Moreover, we have demonstrated that insulin in a dose and time-dependent manner regulated the expression of ARs with the exception of A_3-AR which was unaffected by insulin. By conducting detailed pharmacological analysis, we determined that insulin-induced A_1-AR and A_{2B}-AR mRNA expression trough the Ras/Raf-1/MEK/ERK pathway.

MAPK pathway and PI3K pathway are the major signaling cascades downstream the insulin receptor. In the cell these two insulin-signaling branches form highly complex networks characterized by feedback loops, and crossing points [Taniguchi et al., 2006]. The cross-talk between PI3K and MAPK pathway at the level of Raf-1 [Romano et al., 2006], MEK [Bondeva et al., 1998], and ERK1/2 [Jacob et al., 2002; Keeton et al., 2003], have been reported in various cell types including T and B cells. In the present study we showed that inhibition of insulin-induced activity of PI3K with wortmannin had no effect on insulin-stimulated expression of ARs. Previously we have demonstrated that in rat T lymphocytes insulin activates p70 ribosomal S6 kinase ($p70^{S6K}$), which is a downstream element in PI3K pathway [Pawelczyk et al., 2003]. In the B cells we also observed increased phosphorylation of $p70^{S6K}$ in response to insulin. Phosphorylation and activation of $p70^{S6K}$ depends on the activity of mROR, which is sensitive to inhibition by rapamycin [Abraham and Wiederrecht, 1996]. Treatment of B cells with rapamycin suppressed the phosphorylation of $p70^{S6K}$ but do not affected insulin-induced expression of ARs. These observations

suggest that in rat B cells PI3K pathway does not play a major role in regulation of ARs expression by insulin. Several actions of insulin in the cell propagate through activation of PKC isozymes. Atypical PKCs ζ and λ are the downstream effectors of PI3K and are thought to play important roles in insulin stimulated glucose transport [Bandyopadhyay et al., 1999]. Diacylglicerol (DAG)-activated PKCs, including conventional PKCs, α, and β2 are activated by insulin independently of PI3K [Legites et al., 2002], however, there is relatively little information on the effects of DAG-dependent PKCs on insulin signaling. It had been shown previously, that activation of PKC by tetradecanoyl phorbol acetate (TPA) enhanced the A_{2A}-AR mRNA expression in human neuroblastoma cells [Peterfreud et al., 1997]. However, in our study pretreatment of B cells with a potent highly selective PKC inhibitor (Bis I) have no effect on insulin-induced expression of A_{2A}-AR mRNA and A_1-AR.

The presence of AP-1 sequences in the promoter regions of A_1-AR and A_{2A}-AR genes [Ren and Stiles, 1999; Fredholm et al., 2000] would suggest that transcription of these genes could potentially be modulated by AP-1 transcription factor. Consistent with a possible role for the AP-1 in A_1-AR and A_{2A}-AR genes transcription, we found that the expression level of Jun and Fos proteins increased on insulin addition to the incubated B cells. We observed that the increases in Fos and Jun protein levels were preceded by transient phosphorylation of Elk-1, which together with serum response factor (SRF) are the major inducers of *c-fos* gene [Nissen et al., 2001]. It had been demonstrated that Elk-1 is a direct target of ERK1/2 under both in vitro and in vivo conditions [Gille et al., 1995]. We noted that inhibition of ERK1/2 activation by the pretreatment of B cells with PD 98059 was associated with inhibition of Elk-1 phosphorylation and suppression of Fos and Jun protein expression. Therefore, our study's data point to the possible involvement of AP-1 transcription factor in insulin-induced expression of A_1-AR and A_{2A}-AR genes.

Insulin is capable to suppress and stimulate genes expression in the same cell, a phenomenon that is crucial for its role as a regulator of metabolic processes. In our study we have found that in rat B lymphocyte insulin induced the expression of A_1-AR and A_{2A}-AR, and suppressed A_{2B}-AR. Moreover, the insulin-induced suppression of A_{2B}-AR mRNA expression was neither dependent on MAPK pathway nor PI3K pathway. We observed that pretreatment of the cells with p38 MAPK inhibitor SB 203580 blocked the insulin effect on A_{2B}-AR mRNA expression. This suggests that insulin negatively regulates the A_{2B}-AR gene expression by signaling through the p38 MAPK pathway. Recently direct involvement of p38 MAPK in mechanisms that negatively regulate gene expression have been reported. In dendritic cells and macrophages the LPS-induced inhibition of MHC calss II transactivator (CIITA) gene expression was reported to be mediated by ERK and p38 MAPK. Moreover, the down-regulation of CIITA gene by ERK and p38 MAPK was associated with decreased histones acetylation at the CIITA promoter [Yao et al., 2006]. On the other hand, the ability of insulin to induce alteration of histone modification within a gene promoter was recently documented. It had been shown that insulin represses dexamethasone-induced phosphoenolpyruvate carboxy-kinase (PEPCK) gene transcription by inducing dissociation of glucocorticoid receptor, polymerase II, and several transcriptional factors from the PEPCK gene promoter. These events were associated with rapid deacetylation of lysine residues on histones H3 and H4, and demethylation of arginine-17 on histone H3 [Hall et al., 2007]. Taken together these data and our observations might suggest that insulin is able to suppress gene expression by modifying the pattern of histone methylation/acetylation and p38 MAPK would play some role in this process. However, the detailed mechanism by which insulin represses A_{2B}-AR gene remains elusive.

Nevertheless, our in vitro data indicate that insulin differentially regulate the expression of ARs in rat B cells by signaling through MAP kinase pathway. Under in vivo condition such insulin-induced mechanism of ARs expression may operate under diabetic conditions, especially in type 2 diabetes associated with insulin resistance. Metabolic insulin resistance is usually accompanied by compensatory hyperinsulinemia to maintain euglycemia. On the other hand, insulin resistance is characterized by a specific impairment of PI3K-dependent signaling cascade and increased signaling through Ras/MAPK-dependent pathway [Begum et al., 1998; Cusi et al., 2000]. Therefore, our study's data indicate that under hyperinsulinemic conditions elevation of A_1-AR and A_{2A}-AR expression, and decreased expression of A_{2B}-AR might take place in B lymphocytes. We observed that change of insulin concentration from 0.1 to 10 nM resulted in twofold increase of A_1-AR expression, whereas under such conditions the expression level of A_{2A}-AR increased threefold. Such a change in expression level of ARs might lead to differential sensitivity to adenosine. This assumption support data from our work performed on cardiac fibroblasts demonstrating that even moderate changes in expression level of ARs affected the accumulation of cAMP in response to selective ARs agonist [Grden et al., 2006].

It is generally thought that activation of A_1 and A_3 ARs stimulates immune cells function, whereas ligation of A_{2A} and A_{2B} receptors results in immunosuppression. Under given conditions the cellular response to a particular adenosine level depends on the ARs expression pattern and the adenosine concentration. Moreover, some AR can function as an anchoring protein for enzymes metabolizing adenosine. It was proposed that A_1-AR is able to bind and present adenosine deaminase to interacting cells [Gines et al., 2002]. In human B cell line, a coordinated expression of A_{2A}-AR and ecto-5′-nucleotidase was observed [Napieralski et al., 2003]. Therefore, it is possible that even moderate changes in expression level of ARs might affect the cell sensitivity to adenosine and alter the purines metabolism on the cell surface. It is likely that such changes might have significant pathophysiological implications.

Taken together, our study's data demonstrate that high insulin level differentially affect the expression of ARs in rat B lymphocytes in a fashion that might make them more sensitive to suppression by adenosine.

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REFERENCES

Abraham RT, Wiederrecht GJ. 1996. Immunopharmacology of rapamycin. Ann Rev Immunol 14:483–510.

Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR. 1995. PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. J Biol Chem 270:27489–27494.

American Diabetes Association. 2008. Diagnosis and classification of diabetes mellitus. Diabetes Care 31:S55–S60.

Antonysamy MA, Moticka EJ, Ramkur V. 1995. Adenosine acts as an endogenous modulator of IL-2-dependent proliferation of cytotoxic T lymphocytes. J Immunol 155:2813–2821.

Bandyopadhyay G, Standaert ML, Kikkawa U, Ono Y, Moscat J, Farese RV. 1999. Effects of transiently expressed atypical (z, l), conventional (a, b) and novel (b, e) protein kinase C isoforms on insulin-stimulated translocation of epitope-tagged GLUT4 glucose transporters in rat adipocytes: Specific interchangeable effects of protein kinases C-z and C-g. Biochem J 337:461–470.

Begum N, Song Y, Reinzie J, Ragolia L. 1998. Vascular smooth muscle cell growth and insulin regulation of mitogen-activated protein kinase in hypertension. Am J Physiol 275:C42–C49.

Bondeva T, Pirola L, Bulgarelli-Leva G, Rubio I, Wetzker R, Wymann MP. 1998. Bifurcation of lipid and protein kinase signals of PI3-Kg to the protein kinases PKB and MAPK. Science 282:293–296.

Cuenda A, Rousseau S. 2007. p38 MAPK-kinase pathway regulation, function and role in human diseases. Biochim Biophys Acta 1773:1358–1375.

Cuenda A, Rouse J, Doza YN, Meier R, Cohen P, Gallagher TF, Young PR, Lee JC. 1995. SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. FEBS Lett 364:229–233.

Cusi K, Maezono K, Osman A, Pendergrass M, Patti ME, Pratipanawatr T, DeFronzo RA, Khan CR, Mandarino LJ. 2000. Insulin resistance differentially affects the PI 3-kinase- and MAP kinase-mediated signaling in human muscle. J Clin Invest 105:311–320.

Davies SP, Reddy H, Caivano M, Cohen P. 2000. Specificity and mechanism of action of some commonly used protein inhibitors. Biochem J 351:95–105.

Dos Reis GA, Nobrega AF, Paes de Carvalho R. 1986. Purinergic modulation of T-lymphocyte activation: Differential susceptibility of distinct activation steps and correlation with intracellular 3′,5′-cyclic adenosine monophosphate accumulation. Cell Immunol 101:213–231.

Eibl N, Spatz M, Fisher GF, Mayr WR, Samstag A, Wolf HM, Schernthaner G, Eibl NM. 2002. Primary immune response in type-1 diabetes: Results from a controlled vaccination study. Clin Immunol 103:249–259.

Fredholm BB, Arslan G, Halldner L, Kull B, Schulte G, Wasserman W. 2000. Structure and function of adenosine receptors and their genes. Naunyn Schmiedebergs Arch Pharmacol 362:364–374.

Fredholm BB, Ijzerman AP, Jacobson KA, Klotz K-N, Linden J. 2001. International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. Pharmacol Rev 53:527–552.

Gille H, Kortenjann M, Thomae O, Moomaw C, Slaughter C, Cobb MH, Shaw PE. 1995. ERK phosphorylation potentiates Elk-1 mediated ternary complex formation and transactivation. EMBO J 14:951–962.

Gines S, Marino M, Mallol J, Canela EI, Morimoto C, Callebaut C, Hovanessian A, Casado V, Lluis C, Franco R. 2002. Regulation of epithelial and lymphocyte cell adhesion by adenosine deaminase-CD26 interaction. Biochem J 361:203–209.

Grden M, Pogorska M, Szutowicz A, Pawelczyk T. 2005. Altered expression of adenosine receptors in heart of diabetic rat. J Physiol Pharmacol 56:587–597.

Grden M, Podgorska M, Kocbuch K, Szutowicz A, Pawelczyk T. 2006. Expression of adenosine receptors in cardiac fibroblasts as a function of insulin and glucose level. Arch Biochem Biophys 455:10–17.

Grden M, Podgorska M, Szutowicz A, Pawelczyk T. 2007. Diabetes-induced alterations of adenosine receptors expression level in rat liver. Exp Mol Pathol 83:392–398.

Hall RK, Wang XL, George L, Koch SR, Granner DK. 2007. Insulin represses phosphoenolpyruvate carboxykinase gene transcription by causing the rapid disruption of an active transcription complex: A potential epigenetic effect. Mol Endocrinol 21:550–563.

Hasko G, Kuhel DG, Chen J-F, Schwarzschild MA, Deitch EA, Mabley JG, Marton A, Szabo C. 2000. Adenosine inhibits IL-12 and TNF-{alpha} production via adenosine A2a receptor-dependent and independent mechanisms. FASEB J 14:2065–2074.

Jacob A, Cooney D, Pradhan M, Coggeshall KM. 2002. Convergence of signaling patways on the activation of ERK in B cells. J Biol Chem 277:23420–23426.

Joshi N, Caputo GM, Weitekamp MR, Karchmer AW. 1999. Infections in patients with diabetes mellitus. N Engl J Med 341:1906–1912.

Karin M. 1994. Signal transduction from the cell surface to the nucleus trought the phosphorylation of transcription factors. Curr Opn Cell Biol 6:415–424.

Kaye WA, Adre MNS, Soeldner JS, Rabinowe SL, Kaldany A, Kahn CR, Bistrian B, Srikanta S, Ganda OP, Eisenbarth GS. 1986. Acquired defect in interleukin-2 production in patients with type 1 diabetes mellitus. N Engl J Med 315:920–924.

Keeton AB, Bortoff KD, Bennett WL, Franklin JE, Venable DY, Messina JL. 2003. Insulin-regulated expression of Egr-1 and Krox20: Dependence on ERK1/2 and interaction with p38 and PI3-Kinase pathways. Endocrinology 144:5402–5410.

Legites M, Plomann M, Standaert ML, Bandyopadhyay G, Sajan MP, Kanoh Y, Farese RV. 2002. Knockout of PKCa enhances insulin signaling trough PI3K. Mol Endocrinol 16:847–858.

McMahon NM, Bistrian BR. 1995. Host defences and susceptibility to infection in patients with diabetes mellitus. Infect Dis Clin North Am 9:1–9.

Nagase T, Kawata S, Tamura S, Matsuda Y, Inui Y, Yamasaki E, Ishiguro H, Ito T, Matsuzawa Y. 1996. Inhibition of cell growth of human hepatoma cell line (Hep G2) by a farnesyl protein transferase inhibitor: A preferential suppression of ras farnesylation. Int J Cancer 65:620–626.

Napieralski R, Kempkes B, Gutensohn W. 2003. Evidence for coordinated induction and repressio of ecto-5′-nucleotidase (CD37) and the A2a adenosine receptor in human B cell line. Biol Chem 384:483–487.

Nemeth ZH, Bleich D, Csoka B, Pacher P, Mabley JG, Himer L, Vizi ES, Deitch EA, Szabo C, Cronstein BN, Hasko G. 2007. Adenosine receptor activation ameliorates type 1 diabetes. FASEB J 21:2379–2388.

Nissen LJ, Gelly J-C, Hipskind RA. 2001. Induction-independent recruitment of CREB-binding protein to the c-fos serum response element trough interactions between the bromodomain and Elk-1. J Biol Chem 276:5213–5221.

O'Brien RM, Granner DK. 2004. Gene regulation. In: LeRoith D, Taylor SI, Olefsky JM, editors. Diabetes mellitus. A Fundamental and Clinical Text. Philadelphia: Lippincott-Raven. pp 382–412.

Pawelczyk T, Sakowicz M, Podgorska M, Szczepanska-Konkel M. 2003. Insulin induces expression of adenosine kinase gene in rat lymphocytes by signaling trough the mitogen-activated protein kinase pathway. Exp Cel Res 286:152–163.

Pawelczyk T, Grden M, Rzepko R, Sakowicz M, Szutowicz A. 2005. Region-specific alterations of adenosine receptors expression level in kidney of diabetic rat. Am J Pathol 167:315–325.

Peterfreud RA, Gies EK, Fink JS. 1997. Protein kinase C regulates adenoasine A2a receptor mRNA expression in SH-SY5Y cells. Eur J Pharmacol 336:71–80.

Pickup JC, Chusney GD, Thomas SM, Burt D. 2000. Plasma interleukin-6, tumor necrosis factor alpha and blood cytokine production in type 2 diabetes. Life Sci 67:291–300.

Ren H, Stiles GL. 1999. Dexamethasone stimulates human A1 adenosine receptor (A1AR) gene expression through multiple regulatory sites in promoter B. Mol Pharmacol 55:309–316.

Romano D, Pertuit M, Rasolonjanahary R, Barnier J-V, Magalon K, Enjalbert A, Gerard C. 2006. Regulation of the RAP1/RAF-1/extracellularly regulated

Kinase-1/2 cascade and prolactin release by the phosphoinositide 3-kinase/AKT pathway in pituitary cells. Endocrinology 147:6036–6045.

Rome S, Clement K, Rabasa-Lhoret R, Loizon E, Poitou C, Barsh GS, Riou J-P, Laville M, Vidal H. 2003. Microarray profiling of human skeletal muscle reveals that insulin regulates ~800 genes during a hyperinsulinemic clamp. J Biol Chem 278:18063–18068.

Sakowicz-Burkiewicz M, Szutowicz A, Pawelczyk T. 2004. Differential effect of insulin and elevated glucose level on adenosine transport in rat B lymphocytes. Int Immunol 17:145–154.

Sakowicz-Burkiewicz M, Kocbuch K, Grden M, Szutowicz A, Pawelczyk T. 2006. Diabetes-induced decrease of adenosine kinase expression impairs the proliferation potential of diabetic rat T lymphocytes. Immunology 118:402–412.

Sandberg G. 1983. Regulation of thymocyte proliferation by endogenous adenosine and adenosine deaminase. Int J Immunopharmacol 5:259–265.

Schulte TW, Blagosklonny MV, Romanova L, Mushinski JF, Monia BP, Johnston JF, Nguyen P. 1996. Destabilization of Raf-1 by geldanamycin leads to disruption of the Raf-1-MEK-mitogen-activated protein kinase signalling pathway. Mol Cel Biol 16:5839–5845.

Stancato IF, Chow YH, Hutchison KA, Perdew GH, Jowe R, Pratt WB. 1993. Raf exists in a native heterocompex with hsp90 and p50 that can be reconstituted in a cell-free system. J Biol Chem 268:21711–21716.

Taniguchi CM, Emanuelli B, Kahn CR. 2006. Critical nodes in signaling pathways: Insight into insulin action. Nat Rev Mol Cell Biol 7:85–96.

Wolberg G, Zimmerman TP, Hiemstra K, Winston M, Chi L-C. 1975. Adenosine inhibition of lymphocyte-mediated cytolysis: Possible role of cyclic adenosine monophosphate. Science 187:957–959.

Yang S-H, Yates PR, Whitmarsh AJ, Davis RJ, Sharrocks AD. 1998. The Elk-1 ETS-domain transcription factor contains a mitogen-activated protein kinase targeting motif. Mol Cell Biol 18:710–720.

Yano H, Nakanishi S, Kimura K, Hanai N, Saitoh Y, Fukui Y, Nonomura Y, Matsuda Y. 1993. Inhibition of histamine secretion by wortmannin through the blockade of phosphatidylinositol 3-kinase in RBL-2H3 cells. J Biol Chem 268:25846–25856.

Yao Y, Xu Q, Kwon M-J, Matta R, Liu Y, Hong S-C, Chang C-H. 2006. ERK and p38 MAPK signaling pathways negatively regulate CIITA gene expression in dendritic cells and macrophages. J Immunol 177:70–76.