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Adenosine mediates transforming growth factor-beta 1 release in kidney glomeruli of diabetic rats

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ABSTRACT

Up regulation of the transforming growth factor-beta 1 (TGF-β1) axis has been recognized as a pathogenic event for progression of glomerulosclerosis in diabetic nephropathy. We demonstrate that glomeruli isolated from diabetic rats accumulate up to sixfold more extracellular adenosine than normal rats. Both decreased nucleoside uptake activity by the equilibrative nucleoside transporter 1 and increased AMP hydrolysis contribute to raise extracellular adenosine. Ex vivo assays indicate that activation of the low affinity adenosine A_{2B} receptor subtype (A_{2B}AR) mediates TGF-β1 release from glomeruli of diabetic rats, a pathogenic event that could support progression of glomerulopathy when the bioavailability of adenosine is increased.

1. Introduction

Diabetic nephropathy (DN) is the leading cause of end-stage renal failure in the world. Approximately 40% of patients with type 1 and 2 diabetes will develop DN [1]. The major clinical features of human DN include albuminuria, progressive reduction of GFR and hypertension, and increased risk for cardiovascular diseases [1]. The DN pathogenesis is associated with glomerular angiogenesis and hyperfiltration. In addition, thickening of the glomerular basement membrane, the expansion of mesangial cells, glomerulosclerosis and tubulointerstitial fibrosis are observed in patients with DN [2].

Transforming growth factor-beta 1 (TGF-β1) and its receptors are up-regulated in both experimental and human diabetic nephropathy [2]. Enhanced expression of TGF-β1 receptors, TGF-β1 bioactivity, and responsiveness to exogenous TGF-β1 has been noted to occur in response to high glucose in glomerular cells [3–6]. Blocking of TGF-β1 signaling or expression prevent and/or reverse the hypertrophic and profibrotic effects of the diabetic state [2]. Currently, new pharmacological targets that allow blocking the TGF-β1 mediated glomerulosclerosis are the focus of much research.

The nucleoside adenosine regulates essential renal functions by means of local modification of its extracellular bioavailability to activate members of the P_1 purinoceptor family enclosing A_1, A_{2A}, A_{2B} and A_3 adenosine receptor (AR) subtypes [7]. All subtypes have been detected in the glomerulus, but their physiological roles have been scarcely studied [8–10].

Extracellular adenosine availability in body compartments is regulated by the capability of cells to uptake adenosine to be metabolized, accumulating extracellularly when uptake is decreased [11–16]. The uptake of adenosine by mammalian cells is mediated by the activities of the equilibrative (ENTs, Na^+-independent) and the concentrative (CNTs, Na^+-dependent) nucleoside transporters [17]. In addition, the activity of ecto enzymes able to metabolize purine nucleotides, particularly the ecto 5′-nucleotidase (CD73) that hydrolyze AMP [18], is also a relevant contributor to produce the ligand for adenosine receptors.

Actually the mechanisms controlling extracellular adenosine bioavailability in the glomerulus are poorly understood. It has been shown that a source of adenosine is provided by the metabolism of ATP released from glomerular cells [19]. In this study, we evaluate the effect of experimental diabetes on the activity of the nucleoside

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transporters and the ecto 5′-nucleotidase in the rat glomerulus. Further the probably pathogenic role of adenosine receptors to support the activation of the TGF-β1 axis, which mediates glomerulosclerosis, in response to changes in adenosine availability.

2. Materials and methods

2.1. Animals and tissue samples

Diabetes was induced in male rats (Sprague–Dawley) weighing 250 g by single intravenous injection of streptozotocin (65 mg/kg) dissolved in citrate buffer pH 4.5. Control rats were injected with vehicle. Blood glucose was measured weekly. Diabetic group were animals presenting blood glucose levels higher than 450 mg/dl. Three weeks post-induction, the kidney were perfused with PBS 1X and recollected. Glomeruli were isolated using a differential sieving method [10].

2.2. Adenosine quantification

Total glomeruli freshly purified from individual rat were incubated in 1 ml of tyrode's buffer at 37 °C and 5% CO_2 for 1 h. Incubation medium and glomeruli were separated by centrifugation at 2000×*g* for 5 min. The adenosine content in supernatants was quantified using derivatization with chloroacetaldehyde and HPLC with fluorometric detection.

Reverse transcription and Real Time Polymerase chain reactions. Total RNA were isolated from purified glomeruli using the Trizol® Reagent (Invitrogen). Aliquots of 1 μg of total RNA were reversed transcribed using oligo (dT_{18}) and MMLV Reverse Transcriptase (Invitrogen) [10]. Amplifications were performed using a LightCycler™ rapid thermal cycler (Roche Diagnostics). Reactions (10 μl) included 1 μl of template cDNA (dilution 1:10), 0.2 μM primers and components provided by Lightcycler® Fast Start DNA Master SYBR®Green (Roche). Templates for standard curves were prepared as described [10]. Reactions were conducted for 5 min at 95 °C, followed by 40 cycles including denaturation for 10 s at 95 °C, annealing for 10 s at 60 °C and extension for 30 s at 72 °C. Rat gene-specific primers (sense, antisense) were: A_1AR 5′-CTCCATTCTGGCTCTGCTCG-3′, 5′-ACACTGCCGTTGGCTCTCCA-3′; A_{2B}AR 5′-TTCTGCACGGACTTTCACAG-3′, 5′-AAGGAGTCAGTCCAATGCCA-3′; CD73 5′-GATAACGGTGTGGAAGGACT-3′, 5′-CTGCA ACG-CAGTGACTTCAT-3′; ENT1 5′-TCTGCTTTCATCTGGAGGAC-3′, 5′-GAAGATGAGCCAGACAGCCT-3′; ENT2 5′-ATAGGACTGCGGACATCATG-3′, 5′-TTGAAGGTGTCTGTGGGACT-3′; β-actin 5′-GATGACCCAGATCATGTTTG-3′, 5′-CAGGAGGAGCAATGATCTTG-3′.

2.3. Transport assay

Overall uptake rates were measured in isolated glomeruli by the rapid filtration technique [20]. Freshly purified glomeruli were resuspended in choline buffer (in mM: 5.4 KCl, 1.8 $CaCl_2$, 1.2 $MgSO_4$, 10 Hepes, 137 choline chloride, pH 7.4). Uptake was initiated by diluting the glomeruli suspension 5-fold in choline buffer supplemented with [5,6-^{3}H]uridine (1 μM, 1 μCi/ml) and incubated at 22 °C for 1 min. Incubation was terminated by the addition of 1 ml of ice-cold STOP solution (137 mM NaCl and 10 mM Hepes, pH 7.4), filtered and washed twice with 3 ml of STOP solution. Particular uptake rates for equilibrative nucleoside transporters 1 or 2 (ENT1, ENT2) were assigned to transport activities inhibited by 1 μM NBTI or 2 mM hypoxanthine, respectively [21]. Total nucleoside uptakes were also measured in transport medium containing sodium chloride. Sodium-dependent uptakes rates were calculated from the differences between sodium and choline uridine uptakes.

2.4. AMP hydrolysis

Glomerular ecto 5′-nucleotidase activity (CD73) was revealed by histochemical lead phosphate method. Frozen sections (12 μm) were obtained from rat kidneys perfused with 0.15 M NaCl. The procedure to fix the sections and assay AMP hydrolyzing activity was similar as described [18]. Preincubation in presence of 5 mM levamizole were carry out to block alkaline phosphatase activity. To assure specific CD73 ecto 5′-nucleotidase activity, the AOPCP inhibitor (Sigma) was used as control.

2.5. TGF-β1 release

Purified glomeruli (200 000 per well) were incubated in 2 ml of HAM-F10 medium (5 mM D-glucose) supplemented with 1 μmol/l NECA non-selective P_1 receptor family agonist, 50 nmol/l MRS1754 A_{2B}AR antagonist, 30 nmol/l CPA A_1AR agonist, 30 nmol/l DPCPX A_1AR antagonist, 100 nM CGS21680 A_{2A}AR agonist, 10 nM ZM241385 A_{2A}AR antagonist, 1 μM 2-CI-IBMECA A_3AR agonist and 10 μM MRS1220 A_3AR antagonist at 37 °C and 5% CO_2 for 12 h. Binding affinities were described [22]. Following incubations, glomeruli were collected by centrifugation and the supernatants were stored at −70 °C. Aliquots of supernatants from ex vivo treated glomeruli were used to quantify active TGF-β1 using the ELISA Mouse/Rat/Porcine/Canine TGF-β1 Immunoassay Quantikine® system (R&D Systems). TGF-β1 values were normalized to glomeruli total protein contents in individual assays.

2.6. Immunohistochemistry

Rat kidney tissues were fixed in formalin, paraffin embedded and 5 μm sections were mounted on sylanized slides. Immunodetection was performed as described [10]. The primary polyclonal anti-A_{2B}AR, -A_1AR and primary monoclonal anti-TGF-β1 antibodies were obtained from Santa Cruz Biotechnology. The immunosignals were revealed using the LSAB+ System–HRP system (DakoCytomation).

2.7. Statistical analysis

Values are means ± S.E.M., where *n* indicates number of animals. ANOVA was applied for unpaired data and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Short term diabetes modifies adenosine bioavailability in rat glomeruli

Purified glomeruli by differential sieving method were mostly lacking of bowmann's capsule. Quantification of adenosine indicates that diabetic glomeruli were able to accumulate more than sixfold extracellular adenosine compared to levels in the supernatants of normal rat glomeruli. Values were 37.4 ± 1.79 nM in diabetic vs 6.0 ± 0.36 nM in normal rat glomeruli (values are means ± S.E.M. from individuals determinations normalized to 1 μg of total glomerular proteins, $n = 5$, $P < 0.001$). In order to identify the mediators involved in modifying the outer adenosine accumulation in diabetic rats, we quantified the effect of diabetes on the mRNA levels for nucleosides transporters ENT1 and ENT2 and their activities. Neither ENT1 nor ENT2 transcript contents were significantly modified (Fig. 1A); however, uptake rates were decreased in short term diabetes. Overall uridine uptake in sodium-containing medium was significantly ($P < 0.05$) reduced by 30.8% in glomeruli from diabetic rats. This reduction was mostly, if not

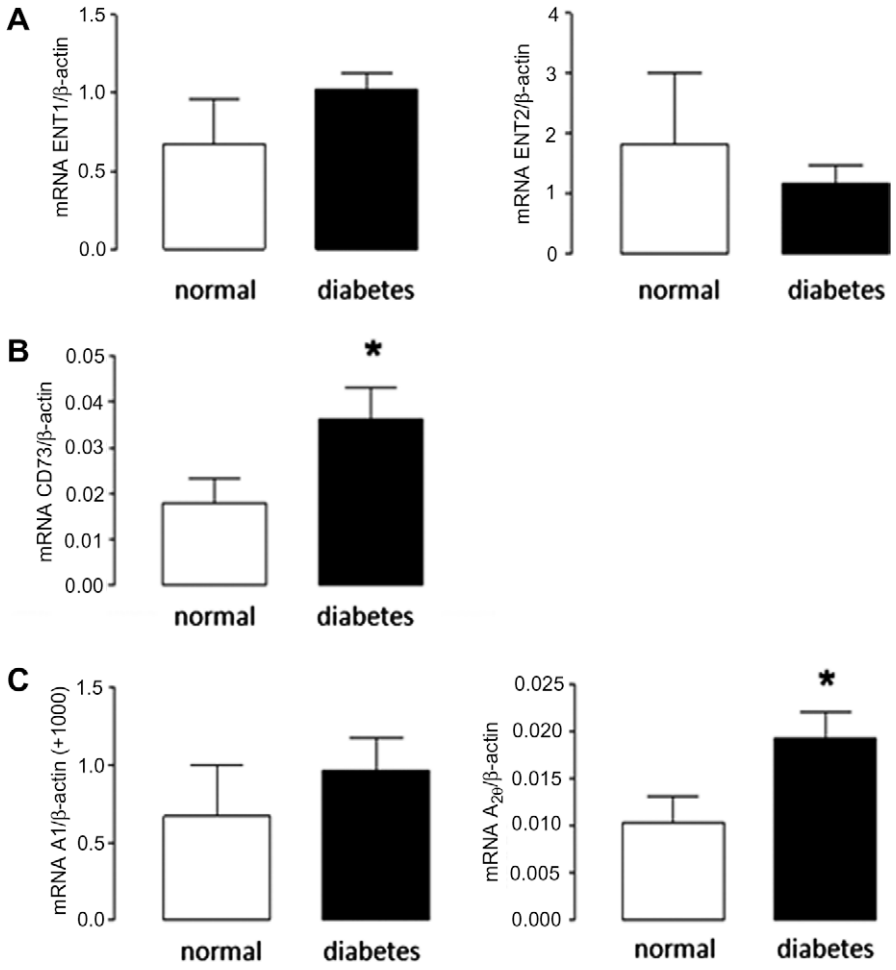

Fig. 1. mRNA content of genes implicated in extracellular adenosine accumulation and adenosine receptors. Quantification of transcript amounts of (A) ENT1 and ENT2, (B) the ecto 5′-nucleotidase (CD73) and (C) adenosine A_1 and A_{2B} receptor subtypes genes, were performed by real time PCR in total RNA from glomeruli of normal (□) and diabetic (■) rats ($n = 5$). The graphs depict the means ± S.D. of the ratios between numbers of target gene/β actin copies. * indicate significant statistical difference ($P < 0.05$).

exclusively, accounted for by the sodium-independent component of uridine transport, which was decreased by 33.8% by diabetes ($P < 0.05$) (Table 1). The evaluation of individual uridine uptake rates for ENT1 and ENT2, showed a significant decrease ($P < 0.05$) in the activity of ENT1 in diabetic rats glomeruli (Table 1).

Previous reports showed that the hydrolysis of AMP by the ecto 5′-nucleotidase (CD73) is a limiting step in the rat glomerulus [19]. We quantified an increase in the mRNA contents of CD73 in glomeruli from diabetic rats (Fig. 1B). Hystochemical analysis revealed that evident 5′-nucleotidase activity occurs at luminal membranes of proximal tubules while very less activity was observed in the glomerulus of normal rats (Fig. 2). Comparison between normal and pathological rat renal slides indicates an increase in the 5′-nucleotidase activity in the glomerulus of diabetic animals (Fig. 2B), but no differences were apparent in tubule cells.

3.2. Adenosine A_{2B} receptor mediates TGF-β1 release from diabetic glomeruli

Following 3 weeks of diabetes induction, the rat glomeruli showed an increased expression of TGF-β1 located mainly to mesangial cells as previously reported [23,24], which contrasts with normal glomeruli where the immunostaining was undetected and it was only evident in certain tubular epithelial cells (Fig. 4). In addition, extracellular matrix deposition at this stage was a remarkable feature occurring at the onset of glomerusclerosis (Fig. 4). The up regulation of the TGF-β1 receptors and the responsiveness of glomerular cells have been previously reported in the diabetic condition, thus suggesting that the ligand availability is an essential pathogenic event. In glomeruli isolated from normal rats the treatment with the general P_1 agonist NECA (1 μM) does

Table 1
Effect of diabetes on the activity of nucleoside transporters in ex vivo glomeruli.

Uridine uptake	Normal (pmol Urd/mg prot/min)	Diabetes (pmol Urd/mg prot/min)
Sodium buffer	1.418 ± 0.083	0.982 ± 0.081*
Choline buffer	1.100 ± 0.096	0.728 ± 0.052*
Sodium-dependent	0.318 ± 0.053	0.255 ± 0.076
ENT1 mediated	0.410 ± 0.044	0.243 ± 0.049*
ENT2 mediated	0.188 ± 0.071	0.055 ± 0.033

The values represent the means ± S.E.M. of triplicate experiments in glomeruli isolated from normal ($n = 4$) and diabetic ($n = 6$) rats.
* Significant difference, $P < 0.05$.

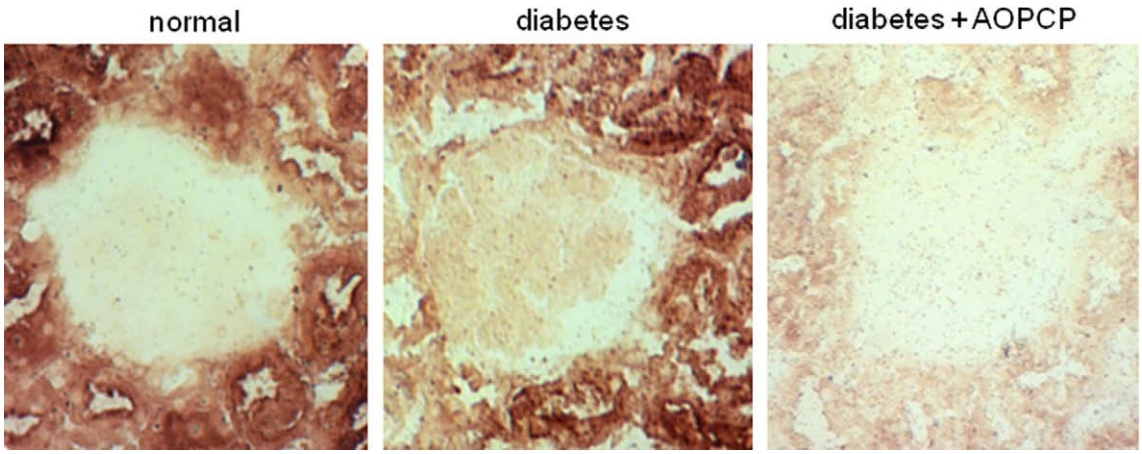

Fig. 2. CD73-mediated AMP hydrolysis in rat glomerulus. Histochemical staining of lead orthophosphate derived from AMP hydrolysis in kidney sections from normal and 21 days diabetic rats. The specificity of hydrolysis was verified using the selective CD73 inhibitor AOPCP. Original magnification 400×.

not mediate a significant change in the release of the TGF-β1 (Fig. 3). In contrast, the treatment of glomeruli from diabetic rats with the general adenosine receptor agonist elicits a 2.8-fold increase in the release of the TGF-β1. This effect can be blocked by a selective adenosine A_{2B} receptor antagonist, MRS1754 (Fig. 3). Due to the fact that selective adenosine A_{2B} receptor agonists are not available we tested selective agonists for all other receptors, and not significant increase in the growth factor release was observed (Fig. 3).

3.3. Effect of diabetes mellitus on adenosine receptors expression

Due to the probably pathogenic role of adenosine A_{2B} receptor and the changes in the ligand bioavailability, we studied the effect of diabetes on its expression. Short term diabetes was correlated with a 1.7-fold higher A_{2B}AR transcripts content ($P < 0.05$) (Fig. 1C). Immunohistochemical analysis located the protein mainly in glomerular podocytes of normal rats (Fig. 4), showing a similar pattern of expression with the podocyte marker VEGF (data not shown), whilst a broader protein distribution was observed in glomeruli of diabetic rats, including mesangium as occurs with TGF-β1 (Fig. 4). On the contrary, no significant changes were quantified for A_1AR mRNA content (Fig. 1C) and decreased protein abundance in glomerular podocytes was observed when comparing pathological to control rat kidney glomeruli (Fig. 4).

4. Discussion

Previous studies have measured basal rate of ATP release from isolated rat glomeruli being approximately 0.30 pmol/min/1000 glomeruli [19]. Interestingly, exogenous ATP was rapidly degraded by the glomeruli suspension. Adenosine was generated following ATP hydrolysis, although the AMP concentration was higher than its other hydrolysis products. Therefore, under normal conditions, hydrolysis of ATP to adenosine occurs at low rate [19], being AMP hydrolyzing activity a limiting step. Our finding shows that diabetic milieu increases AMP nucleotidase activity and sixfold the accumulation of adenosine with respect to controls, as quantified in our experimental conditions. This is consistent with observations showing that in the diabetic rat kidney, adenosine levels

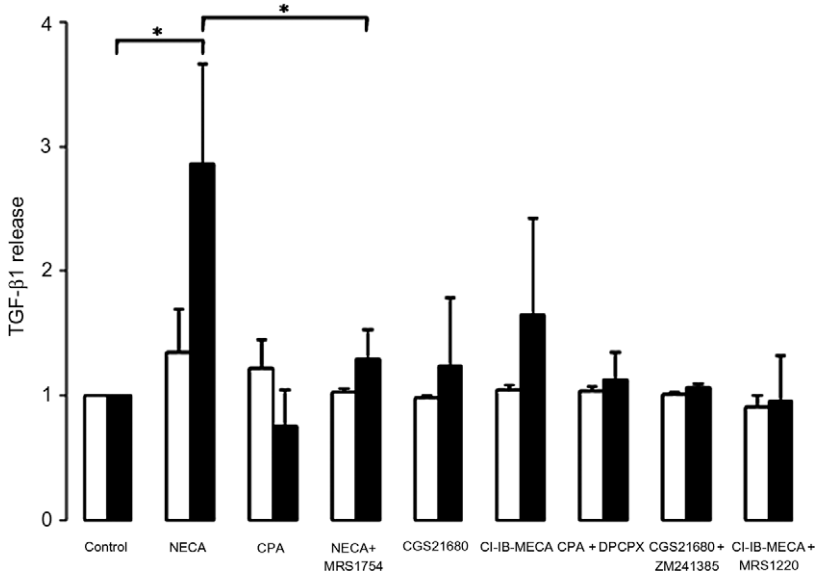

Fig. 3. Role of the adenosine receptors on TGF-β1 release in kidney glomeruli. Glomeruli isolated from normal (□) or diabetic rats (■) were exposed to pharmacological modulators of adenosine receptors (see Section 2). Active form of TGF-β1 released to incubation medium was quantified by ELISA. The amount of growth factor released from control glomeruli was normalized to 1. The graph depicts the means ± S.E.M. * shows statistical differences ($P < 0.05$), $n = 5$.

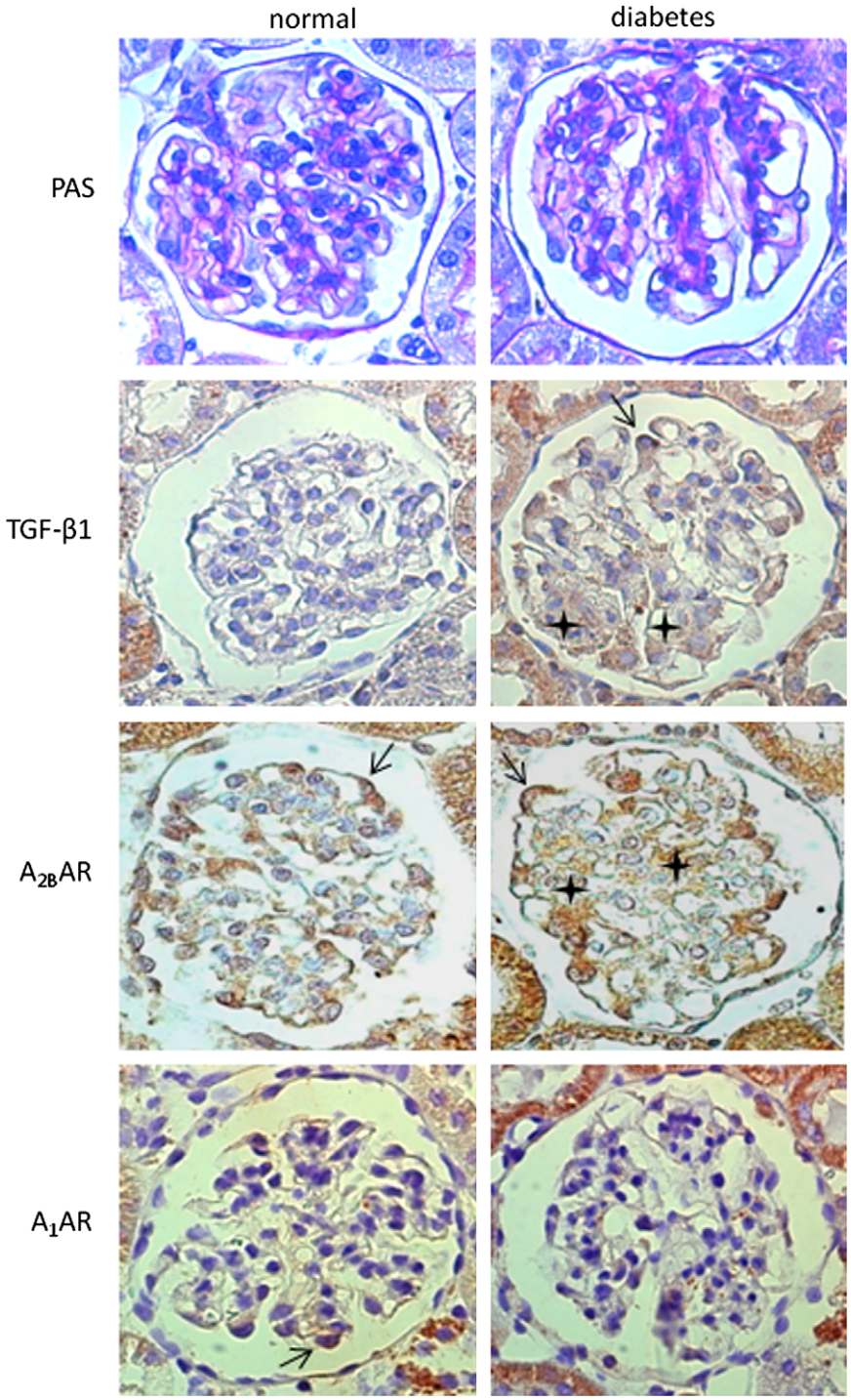

Fig. 4. Effect of diabetes on adenosine receptors expression in the rat glomerulus. Periodic acid-Schiff (PAS) staining and immunolocalization of TGF-β1, adenosine A_{2B} receptor (A_{2B}AR) and adenosine A_1 receptor (A_1AR) subtypes were performed in kidney sections of rats following 3 weeks of diabetes. Immunosignals in podocytes (arrows) and mesangial areas (stars) are denoted in the glomerulus. Original magnification 400×.

were not altered in the artery but were significantly increased in the renal vein plasma [25]. In addition, it was demonstrated that the ecto 5′-nucleotidase activity was increased in medulla and cortex membrane fractions from diabetic rats [9], therefore the enzyme activity or its expression may be modulated.

A variety of physiological models have shown that pharmacological inhibition of the ENT activities results in an increased extracellular adenosine concentration, triggering a specific biological effect via P_1 receptor activation [11–16]. The expression and activity of ENTs is also altered by the diabetic syndrome, glucose and insulin [21,26]. Experiments performed in rats revealed that on day 10 following streptozotocin administration, the mRNA level of rENT1 was slightly (10%) lowered whilst the level of rENT2 mRNA was reduced by 40% in total kidney [27]. We have not found differences in ENT1 or ENT2 mRNA contents in glomeruli, but a marked reduction of ENT1 activity was found in diabetes. In addition, ENT3 mRNA expression was detected in the glomerulus but its related activity, assayed to pH 5.5 and inhibited by

dideoxycytidine, was negligible in control or diabetic glomeruli, probably confirming its intracellular distribution, as reported earlier [28] (data not shown). We conclude that, in addition to an increase in CD73 activity, a lower uptake activity mediated by equilibrative nucleoside transporters (mostly by ENT1) brings about extracellular adenosine accumulation in the diabetic state. The possibility that ENT2 and, to a minor extent, concentrative nucleoside transporters are also contributing to this accumulation cannot be ruled out.

Experimental models correlated adenosine elevations and adenosine receptors activities with development of fibrosis [29–31]. The involvement of receptor A_{2B} in TGF-β1 release attributes a pathogenic role to adenosine in diabetic glomeruli at a stage where diffuse expansion of mesangial matrix occurs. This response to adenosine appears to be an attribute of diabetic glomeruli, thus no effects have been observed in response to adenosine or selective A_1 and A_{2A} agonists on collagen and fibronectin expression in cultured mesangial cells [32]. Probably, the expression of this receptor subtype in mesangial cells contributes to mediate its effects on TGF-β1 release in diabetes. In addition, we reported earlier that A_{2B}AR mediates overproduction of the vascular endothelial growth factor (VEGF) in glomeruli exposed to high glucose concentrations [10], another remarkable feature of diabetic glomerulopathy. Further, increased A_{2B} receptor protein was observed when glomeruli where exposed to high glucose concentration [10]. It has been established that induction of the receptor's expression appears to be mediated through transcriptional up regulation in the promoter of the A_{2B}AR gene via the hypoxia inducible factor-1 alpha (HIF1α) [33]. Interestingly, the use of cDNA microarrays demonstrated that in hyperglycemia HIF-1α was up-regulated in the glomerulus in parallel with an alteration of genes related to oxidative stress and glucose and lipid metabolism [34].

Due to differential glomerular expression patterns of adenosine receptors and ligand availability determined in the early diabetic stage, a condition that may favor signaling by the low affinity adenosine A_{2B} receptors, it is possible to conclude that pharmacological intervention of adenosine signaling could be a new alternative to block some of the events triggering diabetic glomerulopathy.

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