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# Adenosine $A_{2B}$ receptor mediates an increase on VEGF-A production in rat kidney glomeruli

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**Abstract**

Up-regulation of the glomerular expression and the activity of vascular endothelial growth factor-A (VEGF) have been identified as an early pathogenic event for the progression of diabetic nephropathy. Currently, however the mediators are not yet clearly recognized. In this study we identified all four adenosine receptor (AR) subtypes, i.e. $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$ in isolated rat kidney glomeruli. We localized the expression of $A_{2B}$AR in podocytes, the primary VEGF producing cells. The ex vivo treatment of kidney glomeruli with adenosine or a general AR agonist NECA, increases VEGF protein content. In addition, NECA treatment elicits VEGF release. These effects were blocked by the $A_{2B}$AR selective antagonist MRS1754 supplementation. Furthermore, we showed that $A_{2B}$AR activation was necessary to promote a higher expression of VEGF in kidney glomeruli upon exposure to high D-glucose concentration, a pathogenic condition like those observed in diabetic nephropathy.


*Keywords:* VEGF-A; Adenosine receptor; Glomerulus; High D-glucose

The vascular endothelial growth factor-A (VEGF-A or VEGF) is a homodimeric glycoprotein of 45 kDa with potent effects on vascular permeability and angiogenesis [1]. It is also important for normal nephrogenesis to occur [2], while the adult kidney maintains an unusual constitutive expression of VEGF mainly restringed to glomerular visceral epithelial cells (podocytes), renal tubule cells and collecting ducts [3]. Relevant glomerular functions are dependent on VEGF activity [4]. Among the proposed roles are the regulations of blood flow through the capillary tuft and to maintain the filtration activity [5]. Autocrine VEGF production could play a role in the homeostasis and podocytes survival [6] and in the expression of the filtration barrier protein nephrin [7]. It has also been implied in mesangial cell survival and differentiation [8].

The increase in glomerular VEGF expression and activity observed at early stages of the human diabetic nephropathy and in experimental models of diabetes is the consequence of the pathological role of this growth factor [3]. In addition, the renal VEGF over production by transgenesis demonstrated that it directly causes the glomerular hypertrophy that is associated with proteinuria [9].

The most potent stimuli for VEGF production is hypoxia [10] and also hyperglycemia [11]. Particularly, it has been demonstrated that in a mouse podocytes cell line the expression of VEGF increases under exposition to high D-glucose concentrations [12,13]. At present however, it is not clear how glomerular VEGF production is up-regulated in response to diabetes or high glucose concentration.

The nucleoside adenosine regulates essential renal functions by means of local modification of its extracellular

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bioavailability to activate members of the $P_1$ family of purinoceptors that contain $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$ adenosine receptor (AR) subtypes [14]. However, very little is known about adenosine receptor expression and function in glomerular cells types.

## Materials and methods

*Glomeruli isolation.* Renal cortex of male rats (Sprague–Dawley) weighing 200–250 g was sieved through 212 μm, 150 μm, 106 μm and 75 μm meshes. The material collected with the narrowest sieve corresponded to glomeruli [15].

*Experimental treatment conditions.* Purified glomeruli (10,000 per well) were incubated in 2 ml of HAM-F10 medium (5 mM D-glucose) (Invitrogen, USA) supplemented with 10 μmol/L adenosine, 1 μmol/L NECA (non selective $P_1$ receptors agonist), 50 nmol/L MRS1754 ($A_{2B}$AR antagonist), 100 nmol/L CGS21680 ($A_{2A}$AR agonist), 10 nmol/L ZM241385 ($A_{2A}$AR antagonist), 30 nmol/L CPA ($A_1$AR agonist), 30 nmol/L DPCPX ($A_1$AR antagonist) or D-glucose 25 mM at standard conditions (37 °C and 5% $CO_2$) for 6 h. Following incubation, the glomeruli were collected by centrifugation and supernatants were stored at −70 °C. Total protein extracts were obtained from glomeruli resuspended in 150 μl RIPA buffer containing protease inhibitors (1 mM PMSF, 2 μM aprotinin, 1 μg/μl leupeptin and pepstatin). The concentrations of adenosine receptors modulators were derived from Fredholm et al. [16].

*Western blots.* Total proteins extracts (100 μg) fractionated under none reducing conditions by polyacrylamide gel (10%) electrophoresis were transferred to nitrocellulose membranes and probed with monoclonal anti-VEGF antibody (1:1000) (C-1, Santa Cruz Biotechnology, USA). Membranes were washed in Tris buffer saline 0.1% Tween, and incubated (1 h) in TBST/0.1% BSA containing HRP-conjugated goat anti-mouse IgG antibody. Immunodetections were revealed by enhanced chemiluminescence and quantitated by densitometry. Following the stripping procedure the membranes were probed with a monoclonal anti-β actin antibody (1:5000) (Sigma–Aldrich, USA) and revealed as described above [17].

*Reverse transcription.* Total RNA was isolated from glomeruli using the Trizol® Reagent (Invitrogen, USA) [18]. RNA quality and integrity were assured by gel visualization and spectrophotometric analysis ($OD_{260/280}$), quantified at 260 nm and precipitated to obtain 2 μg/μl. Aliquots of 1 μg of total RNA were reversed transcribed into cDNA using oligo ($dT_{18}$) plus random hexamers (10-mers) and MMLV reverse transcriptase (Invitrogen, USA) [19].

*Polymerase chain reaction.* PCR were performed in a total volume of 20 μl containing 1 μl of cDNA (dilution 1:10), 1× PCR buffer, 1.5 mM $Mg^{+2}$, 0.4 mM dNTP's, 2U Taq DNA polymerase (Invitrogen, USA) and 0.5 μM of gene-specific oligonucleotide primers. Samples were incubated for 5 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 56 °C, 30 s at 72 °C and a final extension of 5 min at 72 °C. RTPCR products were sequenced in both directions by Taq dideoxyterminator cycle sequencing with the automated ABI Prism 3730 DNA sequencer (Applied Biosystems/Hitachi). Rat specifics oligonucleotide primers were: $A_1$AR 5′-CTCCATTCTGGCTCT GCTCG-3′ and 5′-ACACTGCCGTTGGCTCTCCA-3′, $A_{2A}$AR 5′-C CA TCTTTAGCCTCTTGGCT-3′ and 5′-AATCCGTAGGTAGATGGC CA-3′, $A_{2B}$AR 5′-TTCTGCACGGACTTTCACAG-3′ and 5′-AAGG AGTCAGTCCAATGCCA-3′, $A_3$AR 5′-TGGAGGTCCAGATGCACT TC-3' and 5′-CGAAACGGAAGTGGCATGAG-3′, β actin 5′-GATGA CCCAGATCATGTTTG-3′ and 5′-CAGGAGGAGCAATGATCTTG-3′.

*Immunohistochemistry.* Rat kidney tissues were fixed in formalin, paraffin embedded and 5 μm sections were mounted on xylanized slides. For immunodetection the slides were sequentially deparaffined and rehydrated, incubated with 10 mM sodium citrate (pH 6.0) for 30 min, hydrogen peroxide (70% methanol, 3% perhydrol) for 5 min, and blocked with PBS 1× containing 1% bovine serum albumin, 0.3% Triton X-100 and 5% fat free milk for 30 min at room temperature. The slides were incubated with polyclonal anti-$A_{2B}$AR (1:1000) antibody (R-20, Santa Cruz Biotechnology, USA) in blocking solution over night at 4 °C. Then, the slides were washed three times with PBS 1× for 5 min and the immunosignals revealed using the LSAB+System-HRP system (Dako-Cytomation, USA) [18].

*Enzyme-linked immunosorbent assay (ELISA).* The amounts of VEGF secreted by glomeruli were measured by a quantitative solid-phase ELISA enzyme immunoassay designed to recognize rat $VEGF_{164}$ (R&D Systems, USA). The sensitivity of the assay was 8.4 pg/ml.

*Statistical analysis.* Values are means ± SEM, where $n$ indicates number of animals. Statistical analyses were carried out on raw data using the Peritz $F$ multiple means comparison test. Student's $t$-test was applied for unpaired data.

## Results

### *Adenosine receptor subtypes in rat kidney glomeruli*

We obtained reproducible glomeruli preparations with a high degree of purity with less than 5% of renal tubules contamination. Glomeruli mostly lack of bowman capsule thus, allowing the ex vivo exposure of the glomerular cells to incubation media, as desired.

While in isolated rat kidney glomeruli we identified the expression of all four AR genes family, only $A_1$AR and $A_{2B}$AR transcripts were present in cultured podocytes (Fig. 1A). Using immunohistochemistry in rat kidney sections we recognized the expression of $A_{2B}$AR mainly in glomerular podocytes localized toward bowman's space and wrapping the capillary tuft (Fig. 1B).

### *Effect of the adenosine receptors activation on VEGF-A production*

The VEGF production in adult rat kidney glomeruli has been mainly recognized in podocytes therefore, we evaluated the effect of activating the AR present in this cell type on VEGF expression. First we established that the activation of adenosine receptors using the endogen ligand adenosine (10 μM) after 6 h of stimulation increased the active VEGF dimeric 45 kDa protein content over basal levels in 66 ± 16.8% ($P < 0.01$). At 15 h the VEGF content decreased nearly to the level observed at time 0 (Fig. 2A). Furthermore, we exposed the glomeruli to the general AR agonist NECA. Similarly, NECA (1 μM) induced an increase of the VEGF expression (36 ± 7.1%, $P < 0.01$) (Fig. 2B).

The increase of glomerular VEGF induced by NECA was blocked by MRS1754, an $A_{2B}$AR subtype antagonist (Fig. 3A). On the other hand, neither addition of $A_1$AR subtype agonist (CPA) nor $A_{2A}$AR agonist (CGS21680) were able to increase VEGF content in glomeruli (Fig. 3A).

Furthermore, we looked for a correlation between the increased expression of VEGF upon $A_{2B}$AR subtype activation and VEGF release. The general $P_1$ agonist NECA (1 μM) increased the VEGF content in the incubation medium more than 4-fold over the constitutive amount released from rat kidney glomeruli (23 ± 0.7 versus 119 ± 0.7 ng/ml, $P < 0.01$). This augmented VEGF