Original article

Adenosine pretreatment attenuates angiotensin II-mediated p38 MAPK activation in a protein kinase A dependent manner

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Background: Adenosine is known as a protective and anti-inflammatory nucleoside. Angiotensin II is the main hormone of the renin-angiotensin system. It is associated with endothelial permeability, recruitment, and activation of the immune cells through induction of inflammatory mediators. Matrix metalloproteinase-9 (MMP-9) plays an important role in inflammatory processes mediated by macrophages.
Objectives: Investigate whether adenosine pretreatment modulates angiotensin II-induced MMP-9 expression and activation of signaling molecules.
Methods: Human monocytic U-937 cells were treated with either adenosine or angiotensin II alone or angiotensin II following a pretreatment with adenosine. Supernatants were analyzed for MMP-9 activity by zymography method. MMP-9 gene expression was analyzed using real-time PCR. Activation of inflammatory mediators IκB-α, NF-κB, JNK, p38 MAPK, and STAT3 were analyzed by a multi-target ELISA kit. Association of Protein kinase A (PKA) in adenosine effects was studied by pre-incubation with H89, a selective PKA inhibitor.
Results: Treatment of the cells with angiotensin II significantly increased MMP-9 production ($p < 0.05$). Adenosine pretreatment did not attenuate this angiotensin II effect. Angiotensin II treatment induced NF-κB, JNK and p38 activation. Pretreatment with adenosine prior to angiotensin II stimulation showed a 40% inhibitory effect on p38 induction ($p < 0.05$). This effect was reversed by PKA inhibition.
Conclusion: The present data confirmed that monocytic MMP-9 was a target gene for angiotensin II. Adenosine pretreatment did not inhibit MMP-9 increase in response to angiotensin II. However, it showed a potential inhibitory effect on angiotensin II inflammatory signaling.

Keywords: Adenosine, angiotensin II, matrix metalloproteinase-9, p38 MAPK, signaling

Adenosine is an endogenous immunomodulator nucleoside that exhibits anti-inflammatory and immune-suppressive properties [1]. Its extra-cellular levels rise rapidly in conditions of ischemia, hypoxia, inflammation, and trauma in which the immune system has a paramount role [2]. The effects of adenosine are mediated through its four receptors (A_1, A_{2A}, A_{2B}, and A_3). It has been reported that a variety of protective mechanisms such as modulation of cytokine production by macrophages, attenuation of free radical generation, prevention of mitochondrial damage and increasing oxygen supply/demand ratio are associated with adenosine [3, 4]. Adenosine activates cyclic-adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway through binding to its A_{2A} receptor that is known as a common inhibitory pathway of adenosine [5].

Angiotensin II is considered as the primary effector of the renin-angiotensin system. It can potentially affect all vascular cells such as endothelial cells, smooth muscle cells (SMCs), fibroblasts, monocyte/macrophages, and myocytes [6]. In pathological states, angiotensin II promotes

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hypertension, endothelial dysfunction, and vascular inflammation [7]. In the vascular wall, dysregulated function of specialized enzymes, called matrix metalloproteinases (MMPs), leads to impaired endothelial barrier function and infiltration of inflammatory cells [8]. Matrix metalloproteinase-9 (MMP-9) plays an important role in inflammatory processes mediated by macrophages [9].

It has been demonstrated that angiotensin II up-regulates MMP expression via signaling cascades that are different in cell types [10]. Angiotensin II type 1 receptor (AT_1R) stimulation activates G protein and non-G protein related signaling pathways. It further activates mitogen-activated protein kinases (MAPK), receptor and non-receptor tyrosine kinases, Janus family kinases-signal transducers and activators of transcriptions (JAK-STATs), small G proteins (Ras, Rho, Rac, etc) and NADPH oxidase [6, 11]. Among signaling molecules of angiotensin II, nuclear factor-kappa B (NF-κB), Rho kinases, Jun N-terminal kinase (JNK), p38 MAPK, STAT3 and reactive oxygen species (ROS) represent convergent points and key regulatory proteins in signaling pathways controlling the stress and inflammation responses [6, 12]. Multiple approaches such as immunosuppressive therapy and drugs against downstream targets of angiotensin II AT_1 receptor signaling have been proposed to modulate the effects of angiotensin [13].

In this study, we hypothesized that adenosine can modulate the effects and signaling mediators of angiotensin II. To test this hypothesis, we studied the effects of angiotensin II on MMP-9 expression and activity, either with or without adenosine pretreatment in a monocytic cell line model. Further, we studied the activation of key inflammatory signaling mediators in response to either angiotensin II or adenosine alone following a pretreatment with adenosine. Pre-incubation with H89, a pharmacologic protein kinase A inhibitor, was performed to elucidate PKA involvement in mediating suppressive effects of adenosine.

Materials and methods
Reagents

Adenosine, angiotensin II, lipopolysaccharide (LPS) from Escherichia coli 055:B5, gelatin, and α-tocopherol were purchased from Sigma (Saint Louis, USA). H89 dihydrochloride (protein kinase A inhibitor) was purchased from Tocris Bioscience (Bristol, UK). Cell culture reagents were purchased from Gibco (Invitrogen, USA).

Cell culture

The monocytic U-937 cell line was obtained from the cell bank of Pasteur Institute of Iran (NCBI). Cells were grown in RPMI-1640 containing 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 µg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cultures were provided with fresh media every two to three days. Cells were seeded in 12-well plates at a density of 1×10^6 cells/mL in a low serum medium (1%) and treated with LPS (100 ng/mL), adenosine (100 µM) and angiotensin II (100 nM) in combination with H89 (10 µM). Pretreatment with adenosine was performed 15 minutes prior to angiotensin II treatment. In case of PKA inhibition, cells were pre-incubated with 10 µM H89 for one hour prior to adenosine exposure.

Zymography

Assessment of MMP-9 activity in the culture media was performed by zymography method as previously described [14]. Briefly, samples of conditioned media were equally loaded on sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) containing 0.1% gelatin type B. Electrophoresis was run under non-reducing conditions. Gels are washed in 2.5 % Triton X-100 twice for 30 minutes and incubated in substrate buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, 0.01% NaN_3, pH 7.6) for 24 hours at 37°C. Gels were then stained with 1% coomassie blue R250 for one hour and were destained (45% methanol, 10% acetic acid). Gels were photographed, and the detected zymogram bands were analyzed by NIH ImageJ software based on the mean area density calculation in a grey-scale format.

Real-time polymerase chain reaction (PCR)

This technique was applied to analyze MMP-9 gene expression following adenosine and angiotensin II treatments. FAST Pure RNA extraction kit (Takara Bio, Kyoto, Japan) was employed to isolate RNA from 1×10^6 cells. Synthesis of cDNA carried out at 37°C for 30 minutes using the Primescript RT enzyme from Takara. Real-time PCR reactions performed using specific primer pairs and Taqman probes (Alpha DNA, Montreal, Canada). They are cited in RTPrimer data base with the following sequences: MMP-9 forward 5'-ACC TCG AAC TTT GAC AGC GAC-3', reverse 5'-GAG GAA TGA TCT AAG CCC AGC-3', probe FAM 5' -TGC CCG GAC CAA GGA TAC AGT TTG TT-3' TAMRA, GAPDH forward 5'-GTG AAC CAT GAG

AAG TAT GAC AAC-3', reverse 5'-CAT GAG TCC TTC CAC GAT ACC-3', and probe FAM 5'- CCT CAA GAT CAT CAG CAA TGC CTC CTG-3' TAMRA. Reactions completed using Rotor-gene 6000 thermocycler (Corbett Res, Sydney, Australia) in a total volume of $20 \mu L$, containing $2 \mu L$ cDNA, $10 \mu L$ Premix Ex-Taq (Takara Bio, Kyoto, Japan) and $0.2 \mu M$ of each primer and probe. MMP-9 gene expression was normalized against GAPDH expression. Relative quantification analysis was performed using Rotor-gene 6000 software, version 1.7 based on $\Delta\Delta Ct$ calculations.

Signaling analysis

A multi-target ELISA kit (Cell Signaling Technology, Danvers, USA) was used to assay phospho-inhibitor of kappa-B (phospho-IκB-α), NF-κB, phospho-NF-κB, phospho-JNK, phospho-p38 MAPK, and phospho-STAT3 following treatment with either adenosine or angiotensin II alone or angiotensin II following treatment with adenosine. After proper incubation times (five minutes for phospho-IκB-α, 10 minutes for NF-κB, phospho-NF-κB, and phospho-STAT3 or 15 minutes for phospho-JNK and phospho-p38), cells were washed with ice-cold PBS (phosphate buffered saline). Lysis buffer was applied and maintained on ice for five minutes followed by sonication and centrifugation. Supernatants were diluted and loaded to the corresponding ELISA wells. The assay procedure was performed according to the manufacturer's protocol. To dilute the lysates properly and to assure that equal amounts of protein were loaded in the wells, lysates were assayed for protein content using Lowry's reagents (Pierce, Rockford, USA).

Cytotoxicity assay

A cytotoxicity detection kit (Roche, Germany) was used to study cytotoxic effects of angiotensin II, adenosine, and H89 on U-937 cells. $1x10^4$ cells were seeded in 96-well plates and incubated with treating agents for 18 hours. Lactate dehydrogenase (LDH) activities in the conditioned media were assayed as indicators of cell damage. The cytotoxicity percentage was calculated by comparing the amount of released LDH following treatment to the maximum release of LDH achieved by application of lysis solution to the control cells.

Statistical analysis

The data are presented as mean ± standard error of means (SEM) of four independent determinations. Results of different groups were analyzed using ANOVA followed by Tukey multiple comparison tests. Differences with a p-value of < 0.05 were considered significant.

Results

Increased MMP-9 expression and activity by angiotensin II

To investigate the effects of adenosine and angiotensin II alone and in combination on MMP-9 production, we applied zymography and real-time PCR techniques. As shown in **Fig. 1**, Cultured U-937 cells constitutively demonstrated MMP-9 activity. Treatment of the cells with angiotensin II for 24 hours significantly increased MMP-9 activity in the supernatents (50%, $p < 0.05$). Adenosine did not show any effect on gelatinolytic activity of MMP-9. Pretreatment with adenosine did not inhibit the stimulatory effect of angiotensin II. LPS, which was applied in the experiments as a positive control, substantially induced MMP-9 activity (94%, $p < 0.01$).

MMP-9 gene expression was analyzed by real-time PCR. As shown in **Fig. 2**, incubation of cells with angiotensin II for 12 hours induced 2.6-folds increase in MMP-9 gene expression ($p < 0.05$), but no change in MMP-9 gene expression was observed following adenosine treatment. Pretreatment of cells with adenosine prior to challenging with angiotensin II did not attenuate its stimulatory effect on MMP-9.

Inhibition of angiotensin II-induced p38 MAPK activation by adenosine pretreatment

To investigate the activation of key pro-inflammatory signaling mediators after angiotensin II treatment, a commercial Path-scan ELISA kit was used. Possible modulatory effects of adenosine pretreatments on these signaling mediators was also assessed with the same ELISA kit. As shown in **Fig. 3,** basal levels of unphosphorylated NF-κB and phospho-STAT3 did not change following angiotensin II and adenosine treatments. This indicates that expression of NF-κB and activation of STAT3 could not be associated with MMP-9 up-regulation. Angiotensin II significantly induced NF-κB, JNK and p38 activation within

15 minutes (4.8, 3.8 and 3.2-folds increase, respectively, in relative to control, p <0.01). In non-stimulated conditions, phosphorylated levels of these intermediates were insignificant and adenosine treatment alone did not change their levels. Pretreatment of the cells with adenosine before angiotensin II stimulation showed a considerable inhibitory effect on p38 activation (40% reduction in phospho-p38 compare to angiotensin II stimulated state, p <0.05). The inhibition was not statistically significant for phospho-NF-κB and phospho-JNK. IκB-α is an intracellular inhibitor of NF-κB, dissociates from NF-κB following phosphorylation, leading to NF-κB activation. Angiotensin II treatment increased phospho-IκB-α level which could not be modulated by adenosine pretreatment. However, the

observed increase was not statistically significant (p <0.1).

Suppression of p38 MAPK by adenosine pretreatment

Protein kinase A is the main kinase in mediating anti-inflammatory effects of adenosine A_{2A} receptor. To investigate possible PKA contribution in the inhibitory effect of adenosine on p38, a pharmacologic inhibitor of PKA, H89, was applied in combination with adenosine and angiotensin II. H89 pre-incubation did not alter angiotensin II stimulated phospho-p38 level, but it reversed the inhibitory effect of adenosine on angiotensin II-induced phospho-p38 activation up to 74% (p <0.05) **(Fig. 4)**. H89 did not affect phospho-p38 level following one hour of incubation.
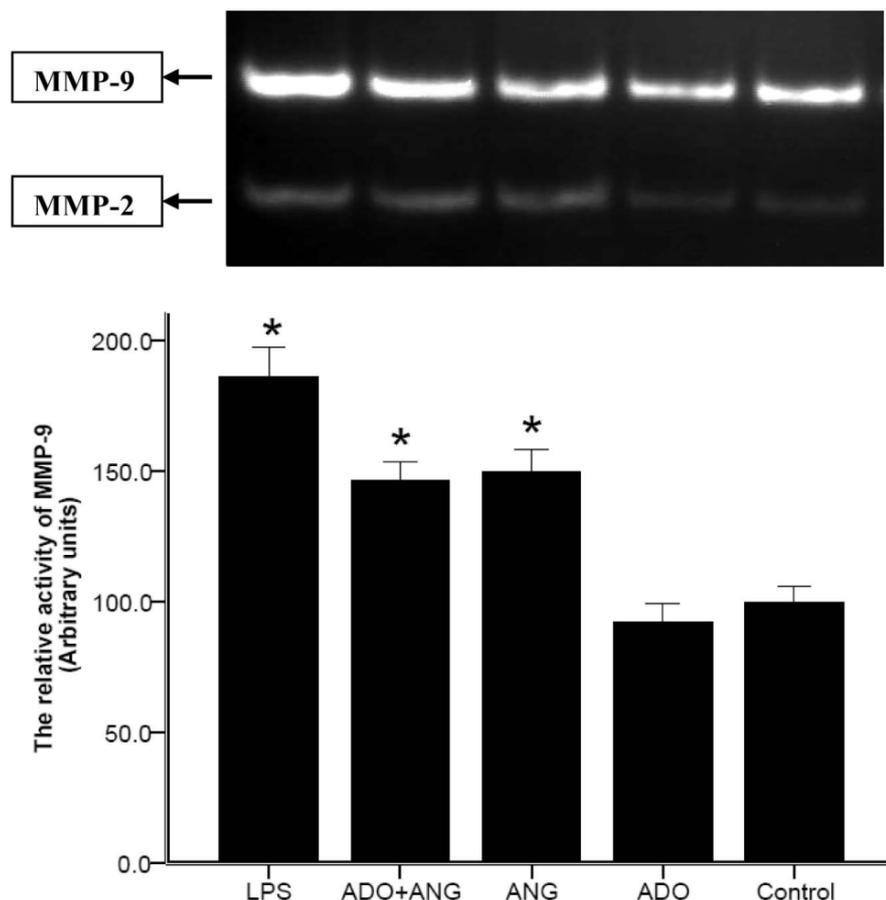

Fig. 1 Determination of MMP-9 activity in conditioned media of U-937 cells using zymography method. 1×10^6 serum starved cells were seeded in 12-well plates and treated either with LPS (100 ng/mL), adenosine (100 μM), angiotensin II (100 nM) or adenosine+angiotensin II for 24 hours. Pretreatment with adenosine was made for 15 minutes prior to angiotensin II exposure. A representative zymogram is shown (upper panel). The data are presented in relative to control as mean ± SEM (n=4) following densitometry. *p <0.05 *vs*. control. ADO = adenosine, ANG = angiotensin II, LPS = lipopolysaccharide.
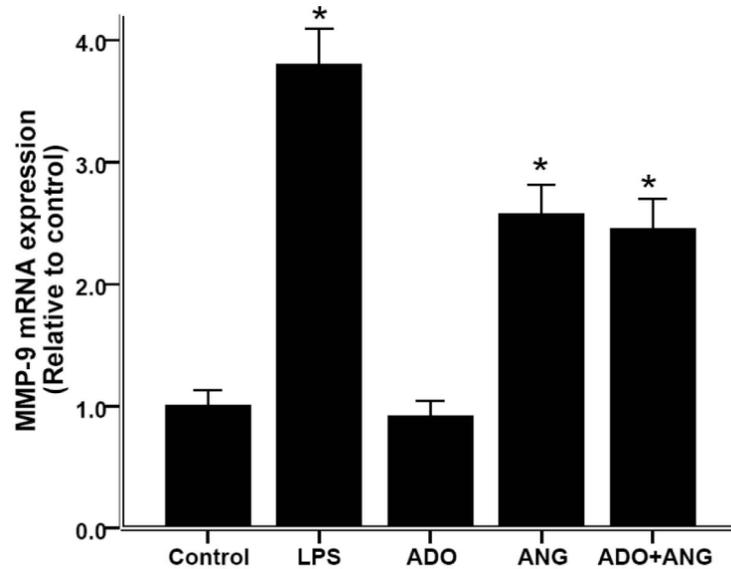
Fig. 2 Analysis of MMP-9 mRNA gene expression using real-time PCR. 1×10^6 serum starved cells were seeded in 12-well plates and incubated with LPS (100 ng/mL), adenosine (100 μM), angiotensin II (100 nM) or adenosine+angiotensin II for 12 hours. Pretreatment with adenosine was made for 15 minutes prior to angiotensin II exposure. Relative MMP-9 gene expression data are presented as mean ± SEM (n=4). *p <0.05 *vs.* control. ADO = adenosine, ANG = angiotensin II, LPS = lipopolysaccharide.
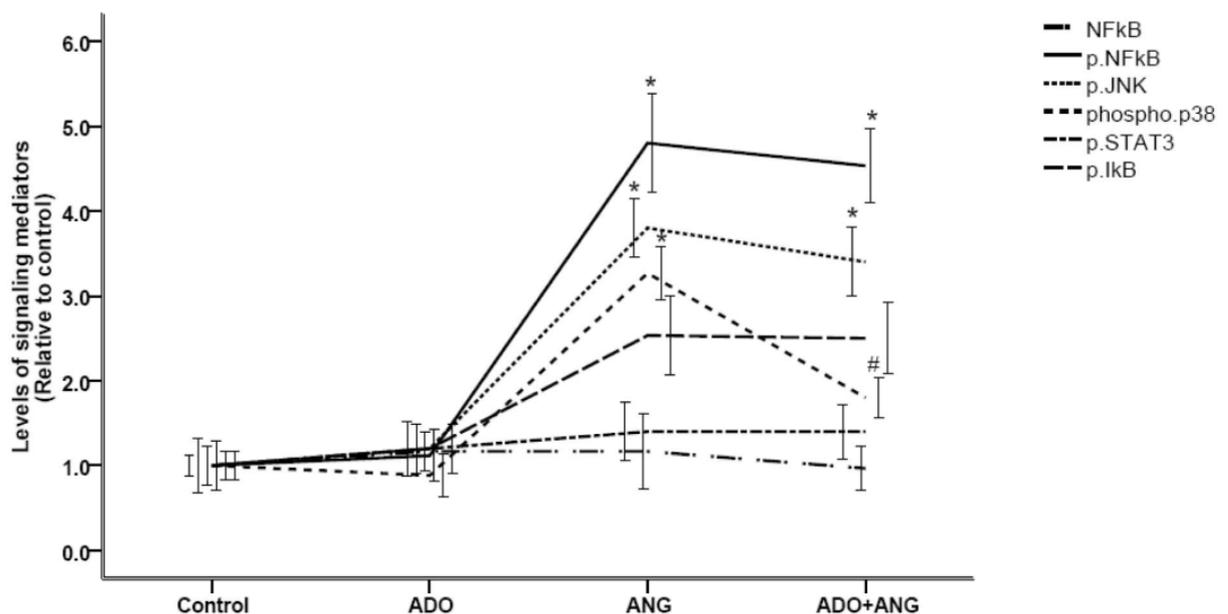

Fig. 3 Relative levels of signaling mediators of stress and inflammatory pathways following treatments. U-937 cells were cultured in a low serum medium at a density of 1×10^6 cells/mL and treated either with adenosine (100 μM), angiotensin II (100 nM) or adenosine+angiotensin II. Pretreatment with adenosine was performed for 15 minutes before challenging with angiotensin II. Following relevant incubation time (five minutes for p-IκB, 10 minutes for NF-κB, p-NF-κB and p-STAT3 and 15 minutes for p-JNK and p-p38) cells were lyzed and assayed for their protein content. Signaling mediators in cell lysates were determined using a multi-target ELISA kit. Lysates were assayed at a protein concentration of 0.5 mg/mL. Data are normalized against non-stimulated levels of analyte and represent the mean ± SEM (n=4). *p <0.01 *vs.* control, # p <0.05 *vs.* angiotensin II treated. ADO = adenosine, ANG = angiotensin II.
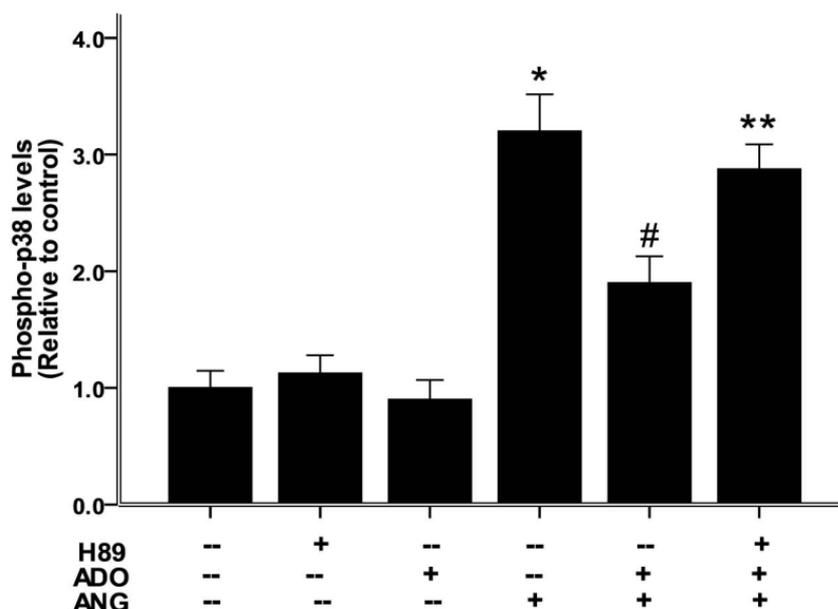
Fig. 4 The effects of pharmacologic PKA inhibition on p38 modulation by adenosine and angiotensin II. Cells were pre-incubated with 10 μM H89 for one hour followed by adenosine (100 μM, 15 minutes) and /or angiotensin II (100 nM) treatments. After 15 minutes of incubation, cells were lyzed and assayed for phospho-p38 using Path-Scan ELISA kit. Data are expressed in relative to non-stimulated control as mean ± SEM (n=4). *p <0.01 $vs.$ control, $^{\#}$p <0.05 $vs.$ angiotensin II treated, **p <0.05 $vs.$ adenosine+angiotensin II treated. ADO = adenosine, ANG = angiotensin II.

Cytotoxic effects of treatments

According to the results of LDH activity assay following treatments with adenosine, angiotensin II and H89 alone and in combination, none of them significantly elevated LDH leakage from the cells (**Table 1**). This indicates that none of them exerted cytotoxic effects at the applied concentrations.

Discussion

There are emerging concepts of regulation of angiotensin II function through receptor modification, receptor complex formation, receptor antibodies, and immunosuppressive therapy [13]. In this study, we benefited from adenosine as an immunosuppressive and anti-inflammatory metabolite to modulate angiotensin II functions. Adenosine has protective and anti-inflammatory properties. It has been shown that adenosine can modulate MMP secretion. Ernes et al. [15] showed that adenosine

inhibited MMP-9 secretion in neutrophiles via cAMP/PKA/Ca^{2+} pathway. In the study by Zhao et al. [16], hypoxia-mediated suppression of MMP-9 production was dependent on adenosine cAMP/PKA signaling pathway.

To investigate the modulatory effects of adenosine on angiotensin II signaling and MMP-9 expression, we employed U-937 cells, a human monocytic cell line. There have been reports on the application of U-937 cell line to study suppressive effects of adenosine on inflammatory cytokine production [17]. Monocytes are important target cells of angiotensin II expressing both AT$_1$ and AT$_2$ receptors. They have substantial roles in promoting vascular inflammation, foam cell formation, and MMP-9 secretion [18, 19]. Yuan et al. [20] used U-937 cells to investigate the inhibitory effect of angiotensin II type 1 receptor blockade on IL-1beta production. U-937 cells were treated with

Table 1. Cytotoxicity potentials of the drugs applied in the treatments.

Drug	ADO	ANG	ADO+ANG	H89	H89+ADO+ANG
%Cytotoxicity	5.9 ± 1.0	2.0 ± 0.9	5.4 ± 0.5	1.3 ± 0.6	5.0 ± 1.9
P-value	0.05	0.85	0.08	0.97	0.13

adenosine rather than specific agonists to activate its receptors globally. Monocyte-macrophages express all four adenosine receptor types.

In this study, we obtained harmonious data from zymography and gene expression experiments in which angiotensin II stimulation significantly augmented MMP-9 expression and activity. Adenosine pretreatment did not significantly inhibit the stimulatory effect of angiotensin II on MMP-9. Conclusions on the modulatory effects of adenosine should be made with caution, since in experiments studying anti-inflammatory and protective characteristics of adenosine, multiple variables have been shown to influence the outcome. These variables include cell type, density and type of involved receptor, different EC_{50} (concentration of agonist giving half maximal effect) values of receptor types, functionality of the intracellular signaling, specificity of the analogs employed, adenosine concentration, pre-exposure to inflammatory cytokines, and the abundance of adenosine metabolizing enzymes [21, 22, 23]. In a study by Velot et al. [24], it was shown that adenosine activated MMP-9 secretion by macrophages through their A_3 receptors. This finding is in sharp contrast to the previous observations by Ernes et al. [15] and Zhao et al. [16] who had shown adenosine inhibited MMP-9 secretion through its A_{2A} receptors.

To investigate signaling, we employed a Path-Scan ELISA kit to measure the activation of major inflammatory signaling effectors, namely IκB-α, NF-κB, JNK, p38 MAPK and STAT3. Based on earlier studies, adenosine modulates these mediators during suppression of inflammation. It has been demonstrated that adenosine can suppress activation of NF-κB in different cell types [25]. It has also been reported that adenosine A_{2A} receptor signaling regulates NADPH oxidase activity and reactive oxygen species (ROS) formation [26]. Adenosine is a negative regulator of MAP kinase signaling [27]. Adenosine inhibits cytokine receptor activation of JAK-STAT pathway via induction of suppressor of cytokine signaling-3 (SOCS-3) transcription [28]. Another mechanism of adenosine protection is through modulation of intracellular calcium release and suppression of calcium signaling [29].

Our results showed that angiotensin II treatment increased activation of NF-κB, JNK and p38. The activation of NF-κB and JNK may account for the up-regulation of MMP-9 in response to angiotensin II. Phosphorylated JNK and p38 form active transcription factor activator protein-1 (AP-1). In the promoter region of MMP-9, there are binding elements for NF-κB, AP-1, Ets-1 and STAT transcription factors [30]. Upon activation of gene expression, MMP-9 exerts its activity via degradation of matrix proteins. The lysis of matrix proteins in endothelium is considered as a prominent initiator of inflammatory processes. To exemplify, MMP-9, released by monocyte-macrophages, plays an important role in inflammatory processes such as atherosclerosis development and plaque rupture [9]. Genetic analyses have shown that polymorphisms in the MMP-9 gene are related to the presence and severity of atherosclerosis [31].

Our results showed that pretreatment with adenosine attenuated the angiotensin II-stimulated increase in phospho-p38. The attenuation was statistically significant for phospho-NF-κB and phospho-JNK. This finding is consistent with an earlier report by Fotheringham et al. [17]. They indicated that inhibition of TNF-α release by U-937 cells might be mediated via decreasing p38 activity and TNFα mRNA instability. In another study, pretreatment of HT-29 cells with adenosine resulted in reduced p38 and JNK phosphorylation followed by TNFα treatment [27]. The employed ELISA kit detects phosphorylated p65 subunit (RelA) of NF-κB complex. Phosphorylation of p65 indicates that NF-κB is activated by the classical pathway utilizing IκB phosphorylation and degradation. However, angiotensin II treatment did not significantly augment IκB phosphorylation. This observation could partly be explained by fast proteosomal degradation of phosphorylated IκB. Furthermore, alternative mechanisms could be assumed to account for NF-κB activation such as MAP kinase, Rho kinase and calcium dependent pathways [32, 33]. In our study, adenosine could not significantly inhibit angiotensin II-activated NF-κB. This can be interpreted by the finding that NF-κB inhibition by adenosine is mediated by cAMP response element binding protein (CREB). Adenosine activates CREB transcription factor in a cAMP/PKA dependent manner. Activated CREB competes with NF-κB for the co-activator, CBP, which results in decreased NF-κB transcriptional activity [34]. However, suppression of NF-κB by adenosine through cAMP/PKA and IκB modulation has been shown previously [35].

Most of anti-inflammatory effects of adenosine have been assigned to its A_{2A} receptor. The inhibitory effects of A_{2A} receptors on immune and inflammatory processes in monocytes and macrophages have been shown to occur primarily via cAMP/PKA pathway [27, 36]. This is in agreement with our finding that H89 pre-incubation reversed the inhibitory effect of adenosine on angiotensin II-induced p38. Down-regulation of p38 by adenosine treatment prior to angiotensin II, was not in accordance with down-regulation of MMP-9. It could be postulated that p38 activation was not implicated in angiotensin II-mediated MMP-9 expression.

In conclusion, our data provide further evidence to show that monocytic MMP-9 is a major target gene for angiotensin II, presumably leading to its deleterious effects. Adenosine pretreatment did not inhibit the increased MMP-9 production in response to angiotensin II. However, it showed a potential to modulate specific signaling mediators induced by angiotensin II, in which PKA has a pivotal role. Further investigations are required to elucidate the efficacy of adenosine against "angiotensin II world".

Acknowledgements

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