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CELLULAR MECHANISMS OF THE MACROPHAGE DEACTIVATING EFFECT OF ADENOSINE

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Introduction

Adenosine is an endogenous purine nucleoside which following its release from cells or after being formed extracellularly travels to the cell membrane of surrounding cells where it binds specific cell surface structures that recognize it, termed adenosine receptors. The concept of adenosine as an extracellular signaling molecule was established following the seminal study of Szentgyörgyi and his colleague demonstrating that adenosine extracted from heart muscle, has pronounced biological effects, including heart block and arterial dilatation.

Adenosine exerts its effects via basically two different mechanisms. Firstly, adenosine decreases the energy demand of the tissue via a direct inhibitory effect on parenchimal cell function, exemplified by the negative inotropic effect of adenosine on the heart muscle or the attenuation of neuronal firing and neurotransmission in the central nervous system. Secondly, adenosine indirectly protects the tissue by providing a more favorable environment for parenchimal cells, for which the best example is that adenosine augments nutrient availability by inducing vasodilation.

Immune organs subjected to metabolic stress represented by hypoxia or ischemia release adenosine originating from the immune cells themselves but not nerve terminals. Finally, specific inflammatory stimuli, such as bacterial products are also capable of triggering adenosine release from immune cells. Adenosine

receptors have been subdivided according to molecular, biochemical, and pharmacological evidence into four subtypes. These are A_1, A_{2a}, A_{2b}, and A_3 receptors. Adenosine affects almost all macrophage functions and these effects occur via occupancy of specific adenosine receptors.

Aims of the study

1. To investigate whether adenosine receptor stimulation exerts an inhibitory effect on TNF-α production by LPS-stimulated RAW 264.7 macrophages.

2. To examine whether adenosine has any effect on intracellular TNF-α levels in immune-stimulated RAW 264.7 macrophages.

3. To determine whether IL-10 production can be modulated by adenosine treatment in LPS-stimulated RAW 264.7 macrophages.

4. To investigate whether adenosine can modify the NF-κB transcription factor system in LPS-treated RAW 264.7 macrophages.

5. To examine the effect of adenosine receptor stimulation on the activation of the CREB transcription factor pathway in RAW 264.7 macrophages.

6. To study whether gene expression can be up- or down-regulated by adenosine treatment in LPS-stimulated RAW 264.7 macrophages

Methods

TNF-α and IL-10 determination from supernatants and TNF-α from cell extracts

NF-κB and CREB electromobility shift assay (EMSA) and supershift

Transient transfection and luciferase activity

Western blot analysis of inhibitory κB (IκB) and p65

Western blot analysis of phospho-CREB, phospho-p38, and phospho-p42/44

RT-PCR and Affymetrix gene chip analysis

Results

Adenosine receptor agonists decrease TNF-α production and intracellular TNF-α levels in LPS-stimulated RAW 264.7 macrophages. First, we examined whether adenosine receptor stimulation decreased the production of the NF-κB-regulated cytokine TNF-α by macrophages. Stimulation of cells with LPS for 4 hours induced the release of TNF-α into the medium. Adenosine (10-100 μM) pretreatment of cells 30 min before the LPS challenge reduced the release of TNF-α, which occurred in a concentration-dependent fashion. The adenosine receptor agonists CPA, CCPA, CGS-21680, NECA, and IB-MECA, all mimicked the effect of adenosine in suppressing the production of TNF-α by LPS-stimulated RAW 264.7 cells. None of these purinergic agents had any effect on cell viability,

as determined using the MTT assay. These data obtained using LPS-stimulated RAW 264.7 cells confirm the previous observations of studies using other macrophage systems that adenosine receptor stimulation attenuates the production of TNF-α. Next, we asked the question, whether adenosine acted by decreasing the accumulation of intracellular TNF-α or if it affected the release of this cytokine. The results of this experiment showed that treatment of the cells with LPS induced the appearance of intracellular TNF-α, which was suppressed by adenosine pretreatment. These results indicate that adenosine does not interfere with the release process of TNF-α.

Lack of effect of adenosine on NF-κB activation in RAW 264. 7 macrophages. Because NF-κB is an important regulator of TNF-α production by macrophages, we next tested the possibility that adenosine decreased activation of the NF-κB transcription factor system. Using nuclear extracts from LPS-treated RAW 264.7 cells, we observed an increase in NF-κB binding, when compared to LPS-untreated cells. Supershift studies confirmed the observation by previous reports that the DNA binding complex induced by LPS contained both p65 and p50. However, neither adenosine nor adenosine receptor agonists affected this induction of NF-κB DNA binding. Further, adenosine did not prevent either the LPS-induced accumulation of p65 in the nucleus or LPS-elicited IκB degradation.

Adenosine does not affect LPS-induced NF-κB-dependent transcriptional activity. The possibility still existed that adenosine could prevent NF-κB transcriptional activity without interfering with NF-κB DNA binding. To test this hypothesis, we transiently transfected cells with a NF-κB-luciferase reporter construct. Then, the transfectants were pretreated with adenosine or its vehicle for 30 min, which was followed by stimulation with LPS for 6 hours. The effect of adenosine on NF-κB-dependent gene transcription was assessed using the luciferase assay. Similar to results of the DNA binding experiments, adenosine failed to suppress LPS-stimulated NF-κB-dependent gene transcription. Finally, adenosine alone failed to affect NF-κB-dependent gene transcription.

Adenosine upregulates IL-10 production by LPS-stimulated RAW 264.7 macrophages. IL-10 was initially described as a T helper (Th)2 product that inhibited the secretion of cytokines by Th1 T cell clones. Recently, it has been demonstrated that monocytes and macrophages also produce IL-10 and that macrophages appear to be a major source of circulating IL-10 response to LPS. IL-10 inhibits the synthesis of various cytokines (TNF-α, IL-1, IFN-γ, IL-6 and granulocyte-macrophage CSF) secreted by monocytes/macrophages in response to activation by LPS. Since our previous results show a clear anti-inflamatory effect of adenosine receptor stimulation, we examined whether treatment with adenosine increases the production of the anti-inflammatory cytokine IL-10 by LPS-

stimulated macrophages. Stimulation of RAW 264.7 macrophages with LPS (10 μg/ml) for 5 hours resulted in an increase in IL-10 production. Exposure of the cells to 100 μM adenosine for 5 hours upregulated this LPS-induced IL-10 response.

Adenosine augments CREB-dependent transcriptional activity. Since IL-10 production can be increased following CREB activation, we next studied whether extracellular adenosine affected transcriptional activity of a CREB-firefly-luciferase construct transfected into RAW 264.7 macrophages. To control transfection efficacy, cells were cotransfected with pCMV-*Renilla*-luciferase vector. Exposure of the cells to adenosine (10-100 μM) increased the firefly/*Renilla* ratio as compared to vehicle-treated cells, which indicates that adenosine augments CREB-dependent transcriptional activity. In the next set of experiments, we determined whether this increase in CREB- driven transcriptional activity following adenosine treatment was secondary to increased CREB DNA binding. To assess CREB DNA binding, cells were treated with adenosine (100 μM) for 0, 30, and 90 minutes and nuclear extracts were prepared and subjected to EMSA using a CREB consensus oligonucleotide. The intensity of the only major CREB DNA binding complex (CREB-1) that was observed at the 0 minute time point did not change 30 or 90 min after adenosine administration. Furthermore, the composition of this complex was not altered by adenosine treatment, because it

contained CREB-1 but not ATF-1 at all of the time points. That is because a CREB-1 antibody but not an ATF-1 antibody caused a complete supershift of this complex at all time points examined.

Adenosine induces CREB phosphorylation. Since adenosine did not alter CREB DNA binding, the next possibility was that theadenosine-induced increment in CREB transcriptional activity was a consequence of increased CREB phosphorylation on Ser133. The reason for this proposition is that Ser133 phosphorylation of CREB is one of the predominant mechanisms by which CREB-driven transcriptional activity is stimulated. Adenosine (100 μM) administration to RAW cells for 30 minutes elicited an increase in CREB phosphorylation, indicating that adenosine enhances CREB-driven transcriptional activity via CREB phosphorylation. Extracellular adenosine has been reported to activate both p38 and p42/44. In addition, both of these mitogen-activated protein (MAP) kinases have been shown to be intermediaries of CREB activation following extracellular stimuli. Therefore, we tested the possibility that either p38 or p42/44 was involved in mediating the stimulatory effect of adenosine on CREB transcriptional activity. First, we examined whether adenosine triggered activation of p38 and p42/44 in RAW macrophages using Western blotting utilizing antibodies against the active, double- phosphorylated form of p38 and p42/44. We found that adenosine (100 μM) increased the activation of p38. Furthermore, adenosine enhanced activation

of p42/44 with a more pronounced effect on p44. We next investigated whether MAP kinase inhibition decreased adenosine-stimulated CREB transcriptional activity. Treatment of RAW cells with the selective p38 pathway inhibitor SB203580 produced a blunting of the CREB transcriptional response to adenosine, because adenosine increased CREB transcriptional activity by 172% in vehicle-treated cells, whereas the adenosine-induced increase in CREB-driven transcriptional activity amounted to only 79% in SB203580-treated cells. On the other hand, the selective p42/44 pathway inhibitor PD98059 failed to affect CREB transcriptional activity, because adenosine increased CREB transcriptional activity to the same extent in both vehicle-treated and PD98059-treated cells.

Microarray analysis of gene expression in RAW 264.7 cells treated with adenosine and/or LPS. Stimulation with LPS induced a ≥ 2-fold induction of 98 genes after 3 hours whereas 32 genes were repressed ≥ 2-fold by LPS at this time point. However none of the LPS-induced induced genes, including the NF-κB-regulated ones, such as TNF-α, IκBα, and IL-1 receptor antagonist were altered by at least 1.5 fold by adenosine. In addition, none of the LPS-repressed genes were changed (at least 1.5 fold) by adenosine treatment. Adenosine (no LPS) treatment did not affect gene expression as compared to treatment with vehicle (no LPS). Interestingly, while the A_{2a} receptor mRNA was not expressed in either LPS-

untreated or LPS-treated cells, the mRNA for A_{2b} receptor was not present in LPS non-stimulated cells, but became detectable in LPS-stimulated cells.

RT-PCR analysis of TNF-α and A2b receptor gene expression. RT-PCR analysis confirmed that TNF-α mRNA was induced by LPS but was not affected by adenosine pretreatment. Furthermore, the A_{2b} receptor was upregulated in response to LPS, but was unchanged in adenosine-pretreated cells. Finally, it was confirmed using RT-PCR that the A2a receptor was not expressed in RAW cells.

Discussion

We examined the possibility that the modulatory effects of extracellular adenosine and adenosine receptor agonists on cytokine production observed in macrophages were mediated by interference with activation of the NF-κB and/or CREB transcription factor systems. One of the major finding of our study is that despite the fact that adenosine receptor stimulation decreased both extracellular and intracellular concentrations of TNF-α, a prototype NF-κB-regulated proinflammatory cytokine, adenosine did not interfere with NF-κB activation. There are three lines of evidence to support this proposition. First, adenosine as well as a series of adenosine receptor agonists failed to decrease DNA binding of NF-κB. Secondly, adenosine was unable to decrease NF-κB-driven promoter activity of a luciferase construct. Finally, global analysis of gene expression using

cDNA microarray demonstrated that while LPS induced expression of a number of NF-κB regulated genes, adenosine failed to alter this response.

While these results argue against a role of NF-κB and even a transcriptional effect of adenosine in macrophages, there are several caveats that need to be discussed. First, gene expression was assessed only at the 3-hour time point, whereas it is possible that adenosine may affect gene expression at other time points. Secondly, although adenosine itself had no effect on the expression of cytokine mRNAs in the current study using RAW 264.7 macrophages, we found that the selective A_3 adenosine receptor agonist IB-MECA decreased MIP-1α mRNA levels in the same cell type in an earlier study. Since adenosine itself is a relatively week agonist at A_3 receptors it is possible that selective A_3 receptor stimulation can decrease the levels of cytokine mRNAs.

The mechanism of action for the macrophage deactivating effect of adenosine is incompletely understood. A recent study by Sajjadi et al. demonstrated that adenosine decreased TNF-α mRNA steady state levels in an LPS-stimulated human monocytic cell line, which results are contradictory to our findings in LPS-stimulated mouse macrophages showing a failure of adenosine to inhibit TNF-α mRNA accumulation. Nevertheless, this reduction in TNF-α mRNA steady state levels following adenosine receptor stimulation in human macrophages was not associated with a decrease in NF-κB activation. On the other hand, it

appears that under certain conditions, adenosine can decrease NF-κB activation. For example, adenosine suppressed NF-κB activation in both myeloid and lymphocytic, as well as epithelial cells, when TNF-α but not when LPS was used to stimulate the cells. Clearly, further studies will be necessary to dissect the signaling pathways whereby adenosine exerts its anti-inflammatory effects. It is also important to point out that at this point it is unclear, which receptors mediated the suppressive effect of adenosine on TNF-α production in the current study. While the general view is that the A_{2a} receptor may be the most important one in regulating cytokine production and macrophage activation, it is clear that this was not the case here. That is because the microarray analysis found no A_{2a} receptor mRNA expression in the RAW cells. In addition, in our previous study, the selective A_{2a} receptor CGS-21680 was much less potent (EC 50 in the low micromolar range) in suppressing MIP-1α production by RAW cells than would have been expected. On the other hand, in a study utilizing primary peritoneal macrophages, we found that the potency of CGS-21680 in decreasing cytokine production was much more consistent with an effect on A_{2a} receptors (EC 50 in the nanomolar range). A further support for the role of A_{2a} receptors in peritoneal cells came from the observation that CGS-21680 lost its efficacy in cells taken from A_{2a} receptor knockout mice. Nevertheless, adenosine itself, although to a lesser extent, was still capable of decreasing cytokine production by peritoneal cells from A_{2a}

knockout mice, suggesting that both A_{2a} and other receptors are involved in the anti-inflammatory effects of adenosine. Since RAW 264.7 cell do not appear to express A_{2a} receptors, this cell line may be a powerful tool to study the A_{2a} receptor-independent effects of adenosine on macrophage function.

In addition to the NF-κB transcription system we also investigated the possible involvement and function of CREB transcriptional pathway. Our study provides evidence for the first time that adenosine activates the CREB transcription factor system in macrophages. Thus, macrophages can be added to the growing list of cell types, including intestinal epithelial cells endothelial cells, smooth muscle cells, neurons, and skeletal muscle cells that exhibit an increment in CREB activation following adenosine receptor occupancy. This adenosine-induced activation of CREB transcriptional activity in macrophages follows the conventional route of CREB activation in that it is regulated primarily by Ser133 phosphorylation of CREB without major changes in CREB DNA binding. Another major novel finding of the current study is that adenosine stimulates the phosphorylation of both p38 and p42/44 in macrophages. In addition, p38 activation but not p42/44 activation contributes to the adenosine-elicited increase in CREB transcriptional activity, because blockade of the p38 pathway decreases adenosine-induced CREB activation.

These results raise the interesting question of which genes are the targets of adenosine-induced CREB activation in macrophages. In a recent study using cDNA microarray analysis of approximately 12,000 genes, we found that adenosine exposure of RAW macrophages failed to induce expression of any gene when measured 3.5 hours after adenosine treatment. Furthermore, while the prototypical macrophage activating agent lipopolysaccharide stimulated expression of a few hundred genes, adenosine co-treatment of the cells did not alter the expression of these LPS-induced genes. One obvious explanation for our failure to detect any effect of adenosine on gene expression despite a substantial stimulation of CREB activation is that mRNA levels of CREB-induced genes may have subsided by 3.5 hours after adenosine treatment. This possibility is underlined by the general observation that the CREB transcriptional response to extracellular stimuli is very rapid and mRNA levels of CREB-regulated genes fade by 3-4 hours post-stimulation. A further possibility is that CREB activation itself is not sufficient to activate gene transcription in these cells. Rather, CREB activation may play a regulatory role in gene expression stimulated by some other pathway. It has to be emphasized at this point that it is possible that the expression IL-10 was upregulated following adenosine administration, however, IL-10 did not show up on the gene chip. This lack of expression of IL-10 mRNA on the gene chip is not completely unexpected, because IL-10 mRNA is notoriously difficult to detect due

to the low copy number of mRNA for this cytokine. Another possibility, supported by previous data is that IL-10 mRNA has a delayed kinetics following LPS stimulation of macrophages and the 3 hour time point may have been too early for the generation of sufficient IL-10 mRNA levels. Further studies will be required to exactly pinpoint the mechanisms whereby adenosine enhances IL-10 production by LPS-stimulated macrophages.

Summary

While it is likely that adenosine receptor ligation targets a common major intracellular pathway to exert a general anti-inflammatory effect in macrophages, the nature of this intracellular target remains unclear. The possibility that NF-κB, a central transcription factor mediating most of the proinflammatory effects of LPS, could be such a target was dismissed, since not only adenosine failed to decrease LPS-mediated NF-κB activation but it also had no effect on cytokine transcript levels as assessed by cDNA array analysis. Thus, our data seem to support the proposition that many of the regulatory actions of adenosine on cytokine production are post-transcriptional.

On the other hand, our results demonstrating that adenosine induces CREB activation, uncovers a major intracellular regulatory mechanism, which may explain some of the changes in macrophage phenotype following adenosine

exposure. Furthermore, since both p38 and p42/44 are central factors in conveying signals from the extracellular space to regulate cell function, activation of these two MAPKs by adenosine may represent an important mechanism contributing to macrophage deactivation under conditions of ischemia, inflammation, and sepsis. Future studies will be required to examine how these various pathways initiated by extracellular adenosine regulate macrophage function.

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