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Short communication

Reduction of postischemic leukocyte-endothelium interaction by adenosine via A_2 receptor

Dirk Nolte[1], Anna Lorenzen[2], Hans-Anton Lehr[1], Franz-Josef Zimmer[2], Karl-Norbert Klotz[2], and Konrad Messmer[1]

[1] Institute for Surgical Research, University of Munich, Klinikum Großhadern, Marchioninistrasse 15, W-8000 Munich, Federal Republic of Germany
[2] Institute of Pharmacology, University of Heidelberg, W-6900 Heidelberg, Federal Republic of Germany

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Summary. The adhesion of leukocytes to the endothelium of postcapillary venules hallmarks a key event in ischemia-reperfusion injury. Adenosine has been shown to protect from postischemic reperfusion injury, presumably through inhibition of postischemic leukocyte-endothelial interaction. This study was performed to investigate in vivo by which receptors the effect of adenosine on postischemic leukocyte-endothelium interaction is mediated.

The hamster dorsal skinfold model and fluorescence microscopy were used for intravital investigation of red cell velocity, vessel diameter, and leukocyte-endothelium interaction in postcapillary venules of a thin striated skin muscle. Leukocytes were stained in vivo with acridine orange (0.5 mg kg^{-1} min^{-1} i.v.). Parameters were assessed prior to induction of 4 h ischemia to the muscle tissue and 0.5 h, 2 h, and 24 h after reperfusion. Adenosine, the adenosine A_1-selective agonist 2-chloro-N^6-cyclopentyladenosine (CCPA), the A_2-selective agonist CGS 21,680, the non-selective adenosine receptor antagonist xanthine amine congener (XAC), and the adenosine uptake blocker S-(p-nitrobenzyl)-6-thioinosine (NBTI) were infused via jugular vein starting 15 min prior to release of ischemia until 0.5 h after reperfusion.

Adenosine and CGS 21,680 significantly reduced postischemic leukocyte-endothelium interaction 0.5 h after reperfusion ($p < 0.01$), while no inhibitory effect was observed with CCPA. Coadministration of XAC blocked the inhibitory effects of adenosine. Infusion of NBTI alone effectively decreased postischemic leukocyte-endothelium interaction.

These findings indicate that adenosine reduces postischemic leukocyte-endothelium interaction via A_2 receptor and suggest a protective role of endogenous adenosine during ischemia-reperfusion.

Key words: Adenosine receptors – Ischemia/reperfusion – Leukocyte/endothelium interaction – Microcirculation

Introduction

Ischemia and ensuing reperfusion are associated with the activation of leukocytes which first roll and then become stuck to the microvascular endothelium via upregulation of specific adhesion receptors. Through the release of cytotoxic enzymes and formation of oxygen free radicals, sticking leukocytes contribute to postischemic reperfusion injury, as characterized by enhanced microvascular leakage (Lehr et al. 1991; Menger et al. 1988; Nolte et al. 1991 a), and decrease of nutritional blood flow due to endothelial swelling (Mazzoni et al. 1989). In keeping with this concept, therapeutic inhibition of postischemic leukocyte-endothelium interaction by monoclonal antibodies directed against leukocyte adhesion receptors (Arfors et al. 1987) or by leukocyte depletion using anti-neutrophil serum (Hernandez et al. 1987) effectively reduces the extent of reperfusion injury.

Adenosine has been shown to reduce postischemic reperfusion injury in different organ systems (Grisham et al. 1989; Dux et al. 1990). These effects of adenosine have been ascribed to its vasodilatory action (Berne 1980) as well as to its potential to decrease leukocyte activation (Cronstein et al. 1986), presumably via activation of the cyclic AMP pathway (Iannone et al. 1987; Omann et al. 1987).

Most of the physiological effects of adenosine are mediated through membrane bound receptors, the A_1 (R_i) subtype and the A_2 (R_a) subtype (Londos et al. 1980), both of which are expressed on human leukocyte membranes (Marone et al. 1985). There is evidence that activation of the adenosine A_2 receptor is followed by decreased leukocyte responsiveness to stimulation as as-

Correspondence to D. Nolte at the above address

Abbreviations. AC, adhesion coefficient; CAMAS, computer-assisted microcirculation analysis system; CCPA, 2-chloro-N^6-cyclopentyladenosine; CGS21,680, 2-[p-(2-carboxyethyl)-phenethylamino]-5'-N-ethylcarboxamido-adenosine; DMSO, dimethyl sulfoxide; NBTI, S-(p-nitrobenzyl)-6-thioinosine; RBCV, red blood cell velocity; WBCV, white blood cell velocity; XAC, xanthine amine congener

sessed by superoxide radical formation (Cronstein et al. 1985), release of H_2O_2, myeloperoxidase and cytotoxic enzymes (Riches et al. 1985; Iannone et al. 1987), adhesion of stimulated leukocytes to cultured endothelial cells and leukocyte-induced endothelial damage (Cronstein et al. 1986).

Using the dorsal skinfold chamber model for intravital microscopy in awake hamsters, we have recently demonstrated that adenosine reduces postischemic leukocyte-endothelium interaction in striated muscle tissue in vivo (Nolte et al. 1991b). Although numerous in vitro data suggest the involvement of an A_2 receptor-mediated mechanism for this inhibitory action of adenosine on postischemic leukocyte-endothelial interaction, to date no study has been performed to investigate the role of adenosine receptor subtypes under postischemic conditions in vivo.

Materials and methods

Materials. Adenosine, acridine orange, and S-(p-nitrobenzyl)-6-thioinosine (NBTI) were purchased from Sigma Chemical Co., Deisenhofen, FRG. Pentobarbital-Natrium was from Sanofi/CEVA, Hannover, FRG. 2-chloro-6-aminocyclopentyladenosine (CCPA) and xanthine amine congener (XAC) were kindly provided by Prof. Dr. U. Schwabe, Institute of Pharmacology, University of Heidelberg, FRG. 2-[p-(2-carboxyethyl)-phenethylamino]-5'-N-ethylcarboxamido-adenosine (CGS 21,680) was a gift from Dr. M. F. Jarvis, Summit, N.J., USA.

Animal model. Experiments were carried out in the dorsal skinfold chamber model of the Syrian golden hamster (60–80 g, 6–8 weeks of age). This preparation allows intravital microscopic investigation of the microcirculation in striated muscle and skin in conscious animals. Dorsal skinfold chambers and permanent venous and arterial catheters were implanted in 36 animals as described previously in detail (Endrich et al. 1980). Under pentobarbital anesthesia (50 mg kg^{-1} body weight i.p.), animals were surgically fitted with 2 symmetric titanium frames positioned on the dorsal skinfold, in such a fashion that they sandwiched the extended skinfold. A circular 15 mm diameter area of one of the layers was completely removed and the remaining layer, containing striated muscle and subcutaneous tissue, was covered with a removable cover glass incorporated into one of the frames. Fine polyethylene catheters were passed subcutaneously from the dorsal to the ventral side of the neck and placed into jugular vein and carotid artery. A recovery period of 48 h was allowed between the chamber implantation and the microscopic investigations in order to eliminate side effects of anesthesia and surgical trauma on the microcirculation.

Ischemia for 4 h was induced to the striated muscle contained within the observation window by means of an adjustable screw clamp equipped with a transparent silicone pad (Sack et al. 1987; Menger et al. 1988). The tissue in the chamber was observed by transillumination while the pad was gently pressed form the posterior side onto the tissue until blood supply to the tissue was completely stopped.

Intravital fluorescence microscopy. Four to six postcapillary venules (20–60 µm in diameter) were investigated in each animal. Vessel segments of 200 µm length were examined at predetermined intervals using a stepping motor-driven platform that allows the computerized relocalization of previously defined vessel segments. Vessel diameters and leukocyte-endothelium interaction were examined by intravital fluorescence microscopy (magnification 560×). Intravital microscopic pictures were recorded on videotape and evaluated offline, using the computer-assisted microcirculation analysis system (CAMAS, Zeintl et al. 1989).

For visualization of leukocytes, 0.1 ml of the fluorescent marker acridine orange was infused intravenously during the microscopic investigations (0.5 mg kg^{-1} min^{-1}). Each leukocyte was assigned an adhesion coefficient (AC), calculated from the velocity of each leukocyte (WBCV) and the velocity of the surrounding red cell column (RBCV) as AC = (RBCV-WBCV)/RBCV. This ratio differentiates free-flowing leukocytes ($0 < AC < 0.8$), rolling leukocytes ($0.8 \leq AC < 1.0$) and sticking leukocytes (AC = 1.0). As shown in a validation experiment, this approach yields reproducible results (Zeintl et al. 1989). Sticking leukocytes were expressed as number of cells per mm^2 of endothelial surface that did not move or detach from the endothelial lining within the entire observation period of 1 minute, calculated from diameter and length (200 µm) of the vessel segment under investigation. Rolling leukocytes were expressed as percentage of nonadherent leukocytes (= free-flowing + rolling leukocytes) passing through the observed vessel segment within one minute. RBCV measurements were performed with a photometric analyzer and the dual slit technique (Velocity tracker Model 102B, I.P.M., San Diego, Calif.; USA).

Experimental protocol. Animals were randomly assigned to control or test groups. Baseline values for leukocyte-endothelium interaction, RBCV, and vessel diameters were assessed prior to induction of 4 h ischemia. The same parameters were measured in the identical vessel segments 0.5 h, 2 h, and 24 h after reperfusion. During the experiment the ambient temperature of the experimental system and infusions were maintained at room temperature.

The following drugs were infused intravenously at an infusion rate of 0.18 ml h^{-1} starting 15 min prior to release of ischemia and continued until 0.5 h after reperfusion: a) 10 mmol l^{-1} adenosine, b) 0.1 µmol l^{-1} CCPA, c) 10 µmol l^{-1} CGS 21,680, d) 10 mmol l^{-1} adenosine +0.7 µmol l^{-1} XAC and e) 0.24 µmol l^{-1} NBTI. Control animals received equivalent volumes of isotonic saline. In each experimental group ($n = 6$ animals), blood pressure and heart rate were continuously monitored via an indwelling catheter in the carotid artery. The described drug concentrations were chosen following the criterion that blood pressure and heart rate were not affected in order to rule out alterations of the macrohemodynamic flow conditions. In order to rule out side effects of DMSO (Hameroff et al. 1983), which has been used as solvent for CCPA and NBTI, six animals were infused with DMSO doses equivalent to the content in the NBTI solution. No effects of this solvent on macrocirculatory parameters (blood pressure and heart rate) or leukocyte-endothelium interaction were observed as compared to saline-treated control animals.

Statistic. Statistical tests were performed only on experiments where measurements at all time points of investigation were complete. For lack of parametric distribution, Wilcoxon test was used to test for significant differences between the groups (SAS® Version 5, 1989, SAS Institute Cary, N.C., USA). Differences were considered significant at $p < 0.05$ and depicted in the figures. Values in figures and tables are given as mean ± SD of six animals investigated per experimental group.

Results

During performance of the experiments the macrohemodynamic parameters blood pressure and heart rate as well as the microhemodynamic parameters vessel diameter and red cell velocity were not affected by the administration of the drugs as compared to the saline-treated controls (data not shown).

Effects of adenosine, CCPA, CGS 21,680, XAC, and NBTI on postischemic leukocyte-endothelium interaction

A 4 h ischemia and ensuing reperfusion elicited a drastic enhancement of both postischemic leukocyte sticking to and rolling of leukocytes along the endothelial lining of postcapillary venules in striated skin muscle of the hamster. The application of adenosine (10 mmol l^{-1}) during the early reperfusion period significantly attenuated this increase of the number of sticking leukocytes (Fig. 1). Infusion of the adenosine A_1-selective agonist CCPA had

no inhibitory effect on postischemic leukocyte sticking. In contrast, 24 h after reperfusion an approximately three-fold increase of the number of sticking leukocytes was observed as compared to controls while infusion of the A_2-selective agonist CGS 21,680 resulted in a potent reduction of postischemic leukocyte sticking as found under treatment with adenosine (Fig. 1). Simultaneous infusion of adenosine with XAC resulted in complete abrogation of the adenosine-induced effects on postischemic leukocyte sticking. Infusion of NBTI alone effectively reduced leukocyte sticking until 0.5 h after reperfusion.

Postischemic leukocyte rolling was significantly reduced by adenosine, CGS 21,680, and NBTI after 0.5 h reperfusion as compared to the saline-treated controls while no inhibitory effects were observed when animals were treated with CCPA or a simultaneous infusion of adenosine+XAC (Fig. 1).

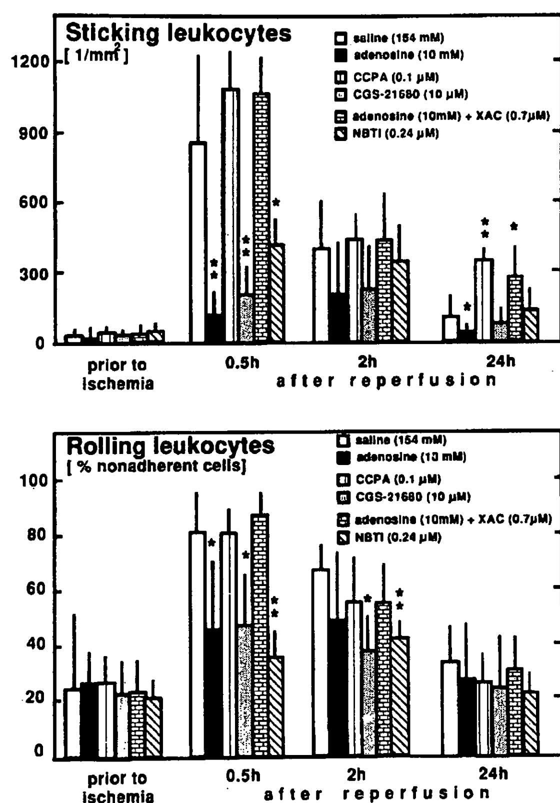

Fig. 1. Effects of adenosine, CCPA, CGS 21,680, XAC, and NBTI on postischemic leukocyte sticking and rolling in striated muscle of the hamster. A 4 h ischemia was induced to a thin striated skin muscle of the hamster. Leukocyte-endothelium interaction was assessed in 4–6 postcapillary venules (20–60 µm diameter) before ischemia and at predetermined intervals after reperfusion using CAMAS. Sticking leukocytes (AC = 1) were defined as cells that did not move or detach from the endothelial lining within the entire observation time of 1 min and given as cells per mm² endothelial surface. Rolling leukocytes were defined as cells with $0.8 \leq AC < 1$ and given in percentage of nonadherent leukocytes passing the vessel segment. Drugs were infused 15 min prior to release of ischemia until 0.5 h after reperfusion at 0.18 ml h^{-1}. Control animals received equivalent volumes of isotonic saline. Values are means±SD of 6 animals investigated per experimental group. * $p < 0.05$, ** $p < 0.01$ vs. saline, Wilcoxon test

Discussion

Studies from our laboratory suggest that the beneficial effects of adenosine on ischemia-reperfusion injury are due to its inhibitory action on postischemic leukocyte-endothelium interaction (Nolte et al. 1991 b). The demonstration in this manuscript that the effects of adenosine can be mimicked by infusion of the adenosine A_2-selective agonist CGS 21 680 (Jarvis et al. 1989) but not by the A_1-selective agonist CCPA (Lohse et al. 1988), and can be blocked by the non-selective antagonist XAC, suggests that the effects of adenosine are mediated via the adenosine A_2 receptor.

The abrogation of the adenosine effects by XAC indicates the involvement of these extracellular receptors. Activation of the A_2 receptor results in stimulation of adenylate cyclase (Londos et al. 1980). This results in an increase of intracellular cyclic AMP concentration which has been shown to counteract leukocyte activation (Omann et al. 1987), presumably through the inhibition of the leukocyte-activating phospholipase C pathway (Teitelbaum 1990). On the other hand, the lack in our experiments of inhibitory effects on leukocyte-endothelium interaction by treatment with the A_1-selective agonist CCPA, along with the finding of an enhanced leukocyte sticking 24 h after reperfusion, points to a facilitation of postischemic leukocyte activation via the A_1 receptor. We therefore suggest a dual action of adenosine on postischemic leukocyte-endothelium interaction with A_1 receptor stimulation resulting in enhancement, while A_2 receptor activation resulting in reduction of postischemic leukocyte-endothelium interaction. Furthermore, the finding that the uptake inhibitor NBTI was also effective to decrease leukocyte-endothelium interaction, suggests a protective role for endogenous adenosine during ischemia-reperfusion.

Before leukocytes can emigrate towards the inflammatory focus, they have to marginate in order to start rolling which is achieved by non-firm interaction with the microvascular endothelium, mediated through expression of specific adhesion receptors of the selectin family (Lawrence and Springer 1991). For the emigration process, firm adhesion of leukocytes to the microvascular endothelium is required and considered as the second step after leukocyte rolling (Von Andrian et al. 1991). Therefore, our results may be interpreted as an action of adenosine on postischemic leukocyte rolling alone and/or leukocyte sticking.

There is evidence that changes of the intracellular cyclic AMP concentration by adenosine affect the expression of membrane-embedded receptors such as CD4, CD8, and Fc_γ receptors on T-lymphocytes, accounting for its immunoregulatory function (Birch and Polmar 1986). Likewise, it has recently been reported that the rise of the intracellular cyclic AMP concentration by 2-chloro-adenosine inhibits motility and adhesion receptor clustering of human umbilical vein endothelial cells (Lampugnani et al. 1990). Therefore, it is intriguing to assume that in response to the adenosine-induced transmembrane signal the expression of adhesion receptors either on leukocyte and/or endothelium is affected. Shear

force-dependent alterations of leukocyte-endothelium interaction by infusion of the drugs can be ruled out since neither changes of blood pressure and heart rate nor changes of the microhemodynamic parameters vessel diameter and red cell velocity have been observed under the given drug doses.

This study provides first in vivo evidence that an A_2 receptor-dependent mechanism mediates the effects of adenosine on postischemic leukocyte-endothelium interaction. The finding that inhibiton of adenosine uptake alone has the potential to reduce leukocyte rolling and sticking points to a pathophysiologic role of endogenous adenosine in this event. However, it still remains to be investigated whether the effects of adenosine are secondary to the modulation of the release of inflammatory mediators which are known to affect leukocyte-endothelium interaction, or a direct effect on the expression of adhesion receptors on leukocyte and/or endothelium.

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