Adenosine Inhibits Exocytotic Release of Endogenous Noradrenaline in Rat Heart: A Protective Mechanism in Early Myocardial Ischemia

Gert Richardt, Wolfgang Waas, Roger Kranzhofer, Eckart Mayer, and Albert Schömig

The effects of exogenous and endogenous adenosine on exocytotic noradrenaline release were studied in rat hearts perfused in situ. Exocytotic release of endogenous noradrenaline (determined by high pressure liquid chromatography) was induced by electrical stimulation of the left cervicothoracic ganglion. Exogenous adenosine significantly reduced noradrenaline overflow from the heart. This suppression of noradrenaline overflow was not influenced by desipramine, indicating a mechanism independent from noradrenaline reuptake. The A1 subtype specific agonists cyclohexyladenosine and R-phenylisopropyladenosine had inhibitory effects at lower concentrations than adenosine and S-phenylisopropyladenosine, suggesting the relevance of presynaptic inhibitory adenosine receptors of the A1 subtype. Short ischemic periods of 3 minutes resulted in a marked coronary venous overflow of adenosine during reperfusion. This was accompanied by an inhibition of noradrenaline release evoked by nerve stimulation during ischemia. The adenosine antagonists theophylline and 8-phenyltheophylline prevented this suppression of noradrenaline release. Blockade of oxidative phosphorylation by cyanide in combination with glucose-free perfusion induced an increased formation of endogenous adenosine and suppression of stimulation-evoked noradrenaline overflow. Again, in the presence of the adenosine antagonists theophylline or 8-phenylthcophylline, this suppression was abolished. These results indicate that adenosine is a potent inhibitor of exocytotic noradrenaline release in the heart with relevance during conditions of increased endogenous adenosine formation such as myocardial ischemia. (Circulation Research 1987;61:117-123)

Clinical and experimental observations suggest deleterious effects of catecholamines in myocardial ischemia.1,2 High local noradrenaline levels may be achieved by either local metabolic non-exocytotic release3-5 or exocytotic noradrenaline release due to activation of cardiac sympathetic nerves.6 The nonexocytotic release does not occur before 10 minutes of total ischemia6 and therefore might not be involved in the very early disturbances caused by ischemia. The exocytotic release, however, is thought to be stimulated in early myocardial ischemia because fear and pain increase central sympathetic activity7 and autonomic reflexes are activated due to impaired hemodynamics and stimulation of myocardial chemoreceptors.8

However, noradrenaline release evoked by stimulation of cardiac sympathetic nerves was found to be suppressed just after short periods of global ischemia in the rat heart.9 These results suggest an endogenous suppression of exocytotic noradrenaline release occurring during the first minutes of myocardial ischemia. The mechanisms of this phenomenon, which protects the ischemic myocardium from excess sympathetic activation, is still not understood.

Adenosine is a plausible candidate for modulating noradrenaline release during myocardial ischemia because it is known to accumulate in the ischemic myocardium to concentrations in the micromolar range10-11 and because purine nucleosides interfere with sympathetic neurotransmission.12,13

To test the hypothesis that adenosine is the endogenous inhibitor of exocytotic noradrenaline release during early ischemia, two experimental approaches were used. In the rat heart retrogradely perfused in situ, first the effects of exogenous adenosine and various adenosine agonists on the release of endogenous noradrenaline evoked by electrical nerve stimulation were investigated; second, the effect of increased endogenous adenosine formation on noradrenaline release was studied in myocardial ischemia and in an experimental model of energy deficiency with unchanged coronary flow (cyanide intoxication in the absence of glucose).

Materials and Methods

Male Wistar rats (180-250 g) (Ivanovas, Kislegg, FRG) were anesthetized with thiobutabarbitral (50 mg/kg ip). The thorax was opened and a cannula inserted and tied into the ascending aorta for retrograde coronary perfusion14 in situ. Each heart was perfused at constant flow of 4 ml/min using a peristaltic pump. Throughout the experiment, the perfusion started with a modified Krebs-Henseleit solution (KHS) with a composition of (in mM) NaCl 125.0, NaHCO3 16.9, NaHPO4 0.2, KCl 4.0, CaCl2 1.85, MgCl2 1.0, glu-
The temperature of the perfusate at the point of entry was 37.5° C. A polyethylene cannula was placed in the right atrium for collection of coronary venous effluent. Preparations with a perfusate gassed with oxygen, and the pH was adjusted to 7.4 by CO₂. The buffer was equilibrated period of 30 minutes, 2 subsequent stimulations (S1 and S2) with intervals of at least 10 minutes were performed. The first stimulation (S1) was used as individual control. The stimulation periods were 60 seconds when exogenous adenosine or adenosine agonists were administered and 30 seconds in ischemia and cyanide experiments. The noradrenaline release evoked by electrical nerve stimulation as described has been characterized previously to be exocytotic. At the end of the experiments, the hearts were excised and weighed (mean weight = 0.79 g).

The following agents were used in the experiments: adenosine (Sigma, Munich, FRG), cyclohexyladenosine (CHA) (Sigma), desipramine (DMI) (Ciba-Geigy, Basle, Switzerland), both stereoisomers of phenylisopropyladenosine (R-Pia and S-Pia) (Boehringer, Mannheim, FRG), 8-phenyltheophylline (Sigma), cyanide (Merck, Darmstadt, FRG), and theophylline (Sigma). Addition of the agents started 1 minute after the first stimulation, except desipramine, which was added 10 minutes before the first stimulation.

Ischemia was induced by interruption of perfusion flow. The hearts were covered in a warmed chamber and the myocardial temperature, monitored by a fine temperature-sensitive probe, maintained between 36 and 38° C. The effluente was collected 1 minute before, during, and after the stimulation period or during the first 3 minutes of reperfusion in case of global ischemia. The samples for estimation of endogenous noradrenaline and endogenous purines were put on ice, stabilized either by the addition of sodium EDTA to a final concentration of 10 mM for noradrenaline measurements or by perchloric acid to a final concentration of 0.3 M for purine measurements. The samples were stored at −80° C until assayed. The results of noradrenaline overflow were given as cumulative overflow during stimulation and poststimulation sampling periods or as S2/S1 ratio of the 2 subsequent stimulations.

**Assay of Noradrenaline**

Noradrenaline was measured using a high pressure liquid chromatography (HPLC) method as described by Schömig et al. Briefly, the pH of the samples was adjusted to 8.5 by the addition of NH₄Cl-NH₄OH buffer (2 M, pH 8.5) containing 0.5% sodium EDTA and 0.2% diphenylborate. Catecholamines were extracted into 5 ml organic phase consisting of 99% n-heptane and 1% octanol in the presence of 0.25% tetracycliammonium-bromide. Back extraction of catecholamines into aqueous phase was performed by shaking the organic phase with 0.15 ml 0.2 M phosphoric acid. One-tenth milliliter of the aqueous phase was injected into the HPLC system. Separation was performed using a 5-μM C18 Latek (Heidelberg, FRG) reversed phase column with a flow of 0.8 ml/min. The solvent was 0.2 M phosphate buffer (pH 3.0) containing sodium EDTA (40 μM) and ocystsulfate (25 μM). Quantitative analysis was performed by electrochemical detection (Bioanalytical Systems model LC 4B, West Lafayette, Ind.) at a voltage of 0.6 V. The retention time of noradrenaline was 4.5 minutes. None of the drugs used in the experiments interfered with extraction, separation, or detection of noradrenaline. The recovery of noradrenaline was 98%, the limit of detection was 0.1 nM, and the coefficient of variation was 5.9%.

**Assay of Purines**

Adenosine, inosine, and hypoxanthine were assayed by an HPLC method according to Agarwal et al. One ml of the sample was neutralized by ion exchange (AG-1X2, acetate form, Biorad, Munich, FRG) and lyophilized. The samples were redissolved in 0.2 ml water, and 0.1 ml was injected into the HPLC system. The nucleosides and bases were separated using a μBonpak C18-column with a Waters guard-PACK pre-column module. The elution was performed by a linear gradient (20 minutes) of 50 mM potassium phosphate buffer, pH 6.0 (gradient 100 to 70%) against 80% methanol at a flow rate of 0.8 ml/min. The absorbance of the eluate was monitored at 254 nm using an UV detector. The limit of detection was 20 nM. Recovery rates for this procedure were 90% for adenosine, 88% for hypoxanthine, and 94% for inosine. The coefficient of variation was less than 10% for the 3 compounds.

**Statistical Methods**

Results are expressed as mean ± SEM. The noradrenaline overflow of the 2 subsequent stimulations in each experiment group was tested for statistical differences by Student's t tests for paired data. A p value of less than 0.05 was considered significant.

**Results**

**Effect of Exogenous Adenosine and Adenosine Analogues on Noradrenaline Overflow Evoked by Nerve Stimulation**

Control experiments (n = 5) proved good correspondence of 2 subsequent stimulations with identical composition of the perfusate (Figure 1). Adenosine (10 μM) added before the second stimulation reduced the noradrenaline overflow from 202 ± 2.5 pmol/g to 11.6 ± 2.4 pmol/g (n = 6, p < 0.01) (Figure 1). During blockade of neuronal reuptake of noradrenaline (uptake, by desipramine (0.3 μM), noradrenaline overflow was markedly increased without a significant difference between 2 subsequent stimulations (n = 5). Inhibition of noradrenaline overflow by adenosine was still effective after the blockade of uptake, (S1 = 50.1 ± 1.7, S2 = 19.3 ± 3.8, n = 5, p < 0.01) (Figure 1)
Adenosine and Noradrenaline

In another series of experiments, the effects of metabolically stable adenosine agonists were studied to characterize the adenosine receptor involved in the modulation of noradrenaline release. 0.1 μM CHA suppressed noradrenaline overflow (S1 = 20.4 ± 1.5, S2 = 8.3 ± 1.8, n = 6, p < 0.01) and 10 μM CHA nearly abolished the release of noradrenaline (S1 = 19.8 ± 2.9, S2 = 2.3 ± 1.4, n = 6, p < 0.01) (Figure 2). The inhibition of noradrenaline release induced by the stereoisomers of PIA (1 μM) significantly differed in favor of R-PIA, which is more selective for A1 receptors (R-PIA: S1 = 23.6 ± 4.7, S2 = 3.4 ± 0.9, n = 5, p < 0.01; S-PIA: S1 = 28.0 ± 9.7, S2 = 15.2 ± 2.6, n = 5, p < 0.05) (Figure 2).

Effect of Ischemia on the Overflow of Endogenous Adenosine and Stimulation-Induced Release of Noradrenaline

Total ischemia for a period of 1 minute did not significantly increase the adenosine release during reperfusion (70.8 ± 17.7 nM, n = 7; control = 69.2 ± 11.8 nM, n = 14). Inosine and hypoxanthine concentrations did not exceed basal levels either (Figure 3). Stimulation during the last 30 seconds of this ischemic period (S2 = 21.6 ± 8, n = 6) revealed nearly the same amount of noradrenaline as collected during control stimulation (S1 = 27.1 ± 3.3) (Figure 4). To prevent noradrenaline reuptake, which is enhanced under stop flow conditions, all ischemia experiments were performed in the presence of desipramine (0.3 μM).

If total ischemia was extended to 3 (n = 6) or 10 minutes (n = 7), purine levels in reperfusate progressively increased up to concentrations in the micromolar range. The increase of inosine (the deaminated metabolite of adenosine) was more pronounced indicating a high local degradation of adenosine under stop flow conditions. Stimulation during the last 30 seconds of a 3-minute (S1 = 22.9 ± 3.2, S2 = 11.1 ± 1.9, n = 8, p < 0.01) or a 10-minute (S1 = 41.5 ± 6.5, S2 = 16.6 ± 1.1, n = 8, p < 0.01) ischemic period resulted in a significantly suppressed noradrenaline release. Pretreatment with the adenosine antagonists theophylline 100 μM (S1 = 25.1 ± 4.0, S2 = 21.2 ± 5.1, n = 8, NS) and 8-phenyltheophylline 10 μM (S1 = 31.2 ± 3.5, S2 = 32.7 ± 5.9, n = 6, NS) abolished this suppression of stimulation-evoked noradrenaline release in the 3-minute ischemia experiments. The same pretreatment, however, was not effective if ischemia was prolonged to 10 minutes and only minor

![Figure 1](http://circres.ahajournals.org/)

**Figure 1** *Effect of adenosine (10 μM) on stimulation-induced overflow of endogenous noradrenaline in rat heart.* Left columns: Overflow of noradrenaline induced by 2 subsequent electrical stimulations (S1, S2) without (control, n = 5) and with administration of adenosine (10 μM, n = 6) 10 minutes before second stimulation. Right columns: Identical experiments after blockade of uptake by 0.3 μM desipramine (DMI, control, n = 5, adenosine, n = 5). DMI was present from 10 minutes before first stimulation throughout experiments. Mean ± SEM, **p < 0.01.

![Figure 2](http://circres.ahajournals.org/)

**Figure 2** *Effects of adenosine agonists cyclohexyladenosine (CHA 0.1 μM, n = 6, CHA 10 μM, n = 6), R- and S-phenylisopropyladenosine (R-PIA, n = 5, S-PIA, n = 5) on stimulation-induced noradrenaline overflow in rat heart.* Agonists were added 10 minutes before the second (S2) of both stimulations. Mean ± SEM, *p < 0.05, **p < 0.01.
amounts of noradrenaline could be found in the venous effluent during reperfusion (Figure 4)

**Effect of Energy Deficiency on the Overflow of Endogenous Adenosine and Noradrenaline**

As noradrenaline release might be influenced by changes in the composition of the interstitial fluid occurring during ischemia, such as acidosis and high potassium concentrations, another experimental model was used that leads to the formation of endogenous adenosine despite unchanged flow, thus avoiding major extracellular accumulation of potassium and protons. Release of endogenous adenosine was caused by perfusion of the hearts without substrate in the presence of 1 mM cyanide, which blocks oxidative phosphorylation (n = 8). Cyanide instead of protonophores such as dinitrophenol was used to block oxidative phosphorylation because cyanide does not interfere directly with noradrenaline transport and accumulation in the storage vesicles.5 The concentration of adenosine and its metabolites, inosine and hypoxanthine, in perfusate increased from the first minute after cyanide administration and reached levels in the micromolar range (Figure 5). The release of the degradation products followed the time course of the adenosine release with short delay. The presence of glucose in the perfusate (n = 5) prevented an increase of adenosine and its metabolites (Figure 5). In this experimental model with extracellular accumulation of endogenous adenosine in the presence of unchanged coronary flow, stimulation-induced noradrenaline overflow was tested 3 minutes after inhibition of energy production. Under these conditions, noradrenaline overflow was significantly suppressed (S1 = 11.4 ± 1.8, S2 = 3.1 ± 0.9, n = 10, p < 0.01) (Figure 6). The adenosine antagonist theophylline 100 μM prevented this reduction of stimulation-induced noradrenaline release in the presence of cyanide and the absence of glucose (S1 = 17.3 ± 2.8, S2 = 14.7 ± 2.8, n = 7, NS). Similar results were found with the more specific adenosine A1 receptor antagonist 8-phenyltheophylline 10 μM (S1 = 10.8 ± 2.8, S2 = 10.2 ± 3.3, n = 6, NS) (Figure 6). In the presence of glucose and cyanide, a significant suppression of noradrenaline overflow was not found (S1 = 11.9 ± 4.3, S2 = 8.5 ± 2.8, n = 8, NS) (not shown in the figures).

**Effect of Various Periods of Total Ischemia on Adenosine, Inosine, and Hypoxanthine Concentrations in Coronary Venous Effluent During First Minute of Reperfusion**

Controls, n = 14; 1-minute ischemia, n = 7, 3-minute ischemia, n = 6, 10-minute ischemia, n = 7 Mean ± SEM.
Discussion

The results document a potent inhibition by adenosine of exocytotic noradrenaline release in the rat heart. The data demonstrate that this effect is due to presynaptic suppression via the adenosine A_2 (Ri)-receptor and that adenosine-mediated suppression of exocytotic noradrenaline release gains pathophysiologic relevance under conditions of enhanced endogenous adenosine formation, such as myocardial ischemia.

Effect of Exogenous Adenosine and Adenosine Analogues on Stimulation-Induced Noradrenaline Overflow

The inhibition of stimulation-evoked noradrenaline release by exogenous adenosine is consistent with data published previously that suggest suppression of neurotransmission by adenosine in various peripheral tissues, such as isolated blood vessels, vas deferens, and kidney. Also, adenosine was found to inhibit sympathetic transmission in hearts from rabbit and guinea pig. A methodical problem in most of the aforementioned studies is the use of electrical field stimulation, which in contrast with nerve stimulation may have side effects such as nonexocytotic catecholamine release due to application of high voltages. Furthermore, no author, except Hedqvist and Fredholm, determined the release of endogenous noradrenaline; instead all authors measured the overflow of tritium from tissues prelabelled with ^3H-noradrenaline. This approach does not allow a distinction between noradrenaline and its metabolites. Moreover, in experiments with exogenous adenosine administration, there are general difficulties in assessing quantitatively the efficacy of adenosine in blocking the transmitter release because of unknown adenosine concentration gradients between perfusate and interstitial space. This is due to an endothelial sink and rapid metabolization of adenosine.

Metabolically stable adenosine agonists were used to characterize the adenosine receptor involved in presynaptic modulation of noradrenaline release. Adenosine antagonists available so far have less subtype specificity and therefore have not been used for receptor characterization. The adenosine analogues CHA and the stereoisomers of PIA are the most...
suitable ligands available for the identification of adenosine-mediated responses. The order of adenosine agonistic potency, CHA > R-PIA > S-PIA, characterizes an A₁-mediated response. Therefore, our experiments using these compounds suggest a presynaptic A₁-receptor-mediated inhibition of norepinephrine release in the rat heart. From experiments using brain preparations, evidence has accumulated that activation of the A₁ (R₁)-receptor interferes with the entry of calcium into the neuron, thus depressing excitation-secretion coupling. The role of adenylate cyclase in mediating presynaptic adenosine effects is controversial

**Effect of Energy Deficiency and Ischemia on Adenosine Formation and Stimulation-Induced Norepinephrine Release**

Our results demonstrate endogenous adenosine formation under conditions of energy deficiency. The major source of endogenous adenosine in the ischemic or anoxic myocardium is the ATP pool of the myocytes. Ischemia or blockade of oxidative phosphorylation by cyanide in combination with exhaustion of glycolysis by glucose-free perfusion is followed by degradation of ATP, which finally results in a pronounced release of adenosine from the myocyte. In our experiments, adenosine levels in the perfusate reach a concentration range sufficient for inhibition of stimulation-induced norepinephrine release. A parallel increase of the adenosine metabolites indicates even higher interstitial levels of adenosine because adenosine is metabolized to inosine and hypoxanthine during its passage from the interstitial space to the coronary sinus. These metabolites have no effect on norepinephrine release.

A causal relation between adenosine formation and suppression of norepinephrine release is suggested by the observation that adenosine antagonists restore the norepinephrine overflow evoked by nerve stimulation during ischemia. The use of theophylline to block adenosine receptors implicates the problem that this compound inhibits phosphodiesterase with an inhibitory constant of 100–300 μM. Therefore, the adenosine antagonist 8-phenyltheophylline was used, which is accepted to be adenosine-specific in the concentration used (10 μM). Neither methylxanthine had an effect on adrenergic transmission in normoxic experiments using a stimulation duration of 30 seconds. However, Hedqvist et al observed an enhanced stimulation-induced norepinephrine release in the presence of theophylline under normoxic conditions in the kidney. This discrepancy may be due to longer stimulation periods applied by these authors, since we also could demonstrate that blockade of adenosine receptors increases stimulation-induced norepinephrine release from the heart during longer stimulation periods. Such stimulations were accompanied by increased formation of adenosine. It may be assumed, therefore, that at longer stimulation periods, the effect of theophylline reflects antagonism to endogenous adenosine.

After 10 minutes of ischemia, the adenosine antagonists did not abolish the suppression of norepinephrine release in our experiments. Under these conditions, extremely high interstitial concentrations of adenosine might occupy the receptors, even in the presence of competitive adenosine antagonists. The complete ineffectiveness of theophylline and 8-phenyltheophylline, however, makes it difficult to explain the resistantly suppressed norepinephrine release by this assumption. The most plausible reason for the breakdown of exocytotic norepinephrine release is the progressive ATP-depletion of the sympathetic nerve cell during the course of ischemia since exocytosis depends on the presence of high energy phosphates.

**Mechanisms Preventing Myocardial Catecholamine Excess During Early Ischemia**

In acute myocardial infarction, the impulse rate of cardiac sympathetic efferents is assumed to be enhanced. In the normally perfused myocardium, this enhanced activity results in an increased norepinephrine release and an intensified adrenergic stimulation of the muscle cells. In the ischemic area, however, several mechanisms have been described to protect the myocardium from being flooded with catecholamines in early myocardial ischemia (phase 1).

1. The elimination of released norepinephrine by uptake prevents an excessive extracellular accumulation of the neurotransmitter. This mechanism, energetically dependent on an intact sodium gradient across the plasmalemma of the sympathetic neuron, is operative up to at least 10 minutes of total ischemia.

2. With longer-lasting ischemia and progressive ATP-depletion of the nerve cell, exocytotic release of norepinephrine has to collapse because exocytosis depends on the presence of high energy phosphates within the neuron.

During the further course of ischemia, however, the myocardium is no longer protected from excessive adrenergic stimulation because nonexocytotic mechanisms of norepinephrine release progressively gain significance (phase 2) and lead to rapid extracellular accumulation of norepinephrine reaching micromolar concentrations after 20 minutes of ischemia. In this phase of ischemia, adenosine has lost its release modulating effect since this nonexocytotic release is independent of nerve stimulation and is not controlled by presynaptic receptors. Moreover, the protective role of...
uptake, has turned to the opposite because, during phase 2 of ischemia, noradrenaline is released via the uptake, mechanism, which has changed its transport direction and carries noradrenaline from the cytoplasm of the neuron to the extracellular space where it can play a detrimental role.

Acknowledgment

We would like to thank Dr Martin Lohse for helpful discussion and Michaela Oestinger and Peter Stefan for excellent technical assistance.

References


Key Words: adenosine, adenosine A1 receptor, exocytotic noradrenaline release, presynaptic modulation of noradrenaline release, myocardial ischemia, perfused rat heart.
Adenosine inhibits exocytotic release of endogenous noradrenaline in rat heart: a protective mechanism in early myocardial ischemia.
G Richardt, W Waas, R Kranzhöfer, E Mayer and A Schömig

Circ Res. 1987;61:117-123
doi: 10.1161/01.RES.61.1.117

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1987 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/61/1/117

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/