

Dipyridamole Inhibits Hydroxylamine Augmented Nitric Oxide (NO) Production by Activated Polymorphonuclear Neutrophils Through an Adenosine-Independent Mechanism

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Received April 1, 2003

Accepted December 18, 2003

Summary

Polymorphonuclear neutrophils (PMN) are thought to play a role in reperfusion injury and ischemia. These effects are partly mediated by toxic oxygen species (superoxide anion, hydrogen peroxide and hydroxyl radical) acting at the level of the endothelium. It was demonstrated recently that the superoxide anion reacts with nitric oxide (NO) and that interaction leads to the generation of highly toxic peroxynitrite. Several drugs were tested so far in order to affect PMN function. It was demonstrated that dipyridamole (2,6-bis-diethanolamino-4,8-dipiperidinopyrimido-(5,4-d)-pyrimidine) can influence neutrophil function by inhibiting adenosine uptake. However, this action can not fully explain all of the observed effects of dipyridamole action on PMN metabolism. The aim of our study was to evaluate the influence of dipyridamole on nitric oxide production by activated polymorphonuclear neutrophils. Incubation of PMNs with hydroxylamine (HA) and phorbol myristate acetate (PMA) generated nitrite (36.4 ± 4.2 nmol/h 2×10^6 PMN), dipyridamole at 100 μ mol/l, 50 μ mol/l and 10 μ mol/l caused a considerable drop in nitrite production (11.8 ± 1.8 , 19.7 ± 2.7 and 27.4 ± 3.2 nmol/h, respectively). Neither adenosine nor the adenosine analogue could mimic the dipyridamole effect. Moreover theophylline, an adenosine inhibitor could not reverse the dipyridamole action on PMN metabolism. We also found that dipyridamole inhibited hydrogen peroxide release from neutrophils. Catalase that scavenges hydrogen peroxide also largely abolished nitric oxide release from PMN. It is evident that dipyridamole inhibits hydroxylamine-augmented nitric oxide production by activated polymorphonuclear neutrophils through an adenosine-independent mechanism.

Key words

Nitric oxide • Neutrophils • Dipyridamole • Hydrogen peroxide • Toxic oxygen species

Introduction

The growing body of evidence suggests that infiltrating polymorphonuclear neutrophils (PMNs) may contribute to myocardial infarction and reperfusion injury (Romson *et al.* 1982, Nolte *et al.* 1991). It was also suggested that metabolism of these cells is changed in

patients with unstable angina in comparison with a stable form of the disease (Dinerman *et al.* 1990). Much of the deleterious effect of neutrophil activity is mediated by toxic oxygen derivatives (superoxide anion, hydrogen peroxide and hydroxyl radical) acting at the endothelial level (Freeman and Crapo 1982, Weiss 1989). Recent studies showed that superoxide anion (O_2^-) (produced in

large amounts by activated PMN) interact with nitric oxide (NO) to generate highly toxic peroxynitrite, thus contributing to endothelial damage (Beckman *et al.* 1990). Furthermore, nitric oxide is also generated by neutrophils (Wright *et al.* 1989, McCall *et al.* 1989) with the use of nitric oxide synthase (NOS) (Fierro *et al.* 1999) or through a NOS-independent pathway (Yan *et al.* 1994). Detection of nitric oxide release from neutrophils is quite difficult, because NO reacts with O_2^- , thus escaping disclosure. Moreover, large amounts of heme-enzymes in neutrophils bind NO, preventing its detection. Markert *et al.* (1994) pointed out that inhibition of heme-containing enzymes by hydroxylamine led to a massive release of NO from neutrophils activated by phorbol myristate acetate (PMA). The NO generation seems to depend on O_2^- production since superoxide dismutase (SOD) largely abolished this process (Markert *et al.* 1994).

Increased NO production was reported in patients with hypotension during hemodialysis (Yokokawa *et al.* 1995). Also Ocha *et al.* (1992) showed that circulating nitric oxide correlates with extreme hemodynamic changes in patients subjected to tumor immunotherapy. On the contrary, NO donors reduce myocardial necrosis and neutrophils accumulation in a model of canine myocardial infarction and reperfusion (Lefler *et al.* 1993).

Dipyridamole (2,6-bis-diethanolamino-4,8-dipiperidinopyrimido-(5,4-d)-pyrimidine) has been used in several clinical and experimental studies due to its action on platelets, endothelial cells, macrophages and neutrophils (McElroy and Philp 1975, Crutchley *et al.* 1980, Colli and Tremoli 1991). It was shown that this drug can inhibit platelet aggregation, induce coronary vasodilation, inhibit leukotriene B_4 as well as O_2^- generation by PMN (Colli and Tremoli 1991, Suzuki *et al.* 1992). Here we present evidence that dipyridamole can also inhibit NO production by activated neutrophils (most probably by NOS-independent pathway).

Methods

Reagents

Phorbol myristate acetate (PMA), cytochrome C type VI, superoxide dismutase (SOD), horseradish peroxidase (HRP), hydroxylamine (HA), dimethyl sulfoxide (DMSO), naphthylethylenediaminedihydrochloride, sulfanilamide, sodium nitrite, fmet-leu-phe (FMLP), calcium ionophore (CaI), 5'-deoxy-5'-(ethylamino)-5'-oxy-adenosine (NECA) and adenosine were purchased from Sigma-Aldrich Chemie GmbH. CaI, FMLP, PMA were dissolved in DMSO at 2×10^{-2} M, 10^{-2} M, 3×10^{-2} M respectively and stored in aliquots at -20 °C. NECA was dissolved in DMSO and methanol (1:1), all dilutions were made in MHS before use. The concentration of DMSO and methanol used did not interfere with neutrophil stimulation nor with the drug effect and did not produce PMN stimulation. The composition of cell buffers used were as follows: modified Hanks (MHS without calcium and magnesium)- K_2HPO_4 1.668 g, NaCl 8.5 g, glucose 2.0 g per liter, pH 7.2, phosphate buffered saline (PBS) KH_2PO_4 0.144 g, NaCl 9.0 g, Na_2PO_4 0.795 g per liter.

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Blood collection and separation of polymorphonuclear neutrophils

Peripheral venous blood was collected from healthy volunteers in heparin (10 U/ml) for isolation of PMN. Blood was layered on density gradient medium (Gradisol G, Polfa) and centrifuged at 500 $\times g$ for 20 min. PMNs were collected and washed in PBS. The obtained population contained ~96 % of neutrophils. Cell viability was estimated by trypan blue exclusion and was ~97 %. After the last washing cells were suspended in a modified Hanks solution (MHS) containing Ca^{2+} and Mg^{2+} .

Nitric oxide production by polymorphonuclear neutrophils

The reaction of NO with oxygen in the aqueous solution leads to the formation of nitrite detected by the Griess reagent (Markert *et al.* 1994). PMN (2×10^7 cells/ml) were suspended in MHS supplemented with Ca^{2+} and Mg^{2+} . Hundred μl of the neutrophil suspension were incubated in final volume of 1 ml in polypropylene tubes at 37 °C for 60 min. Various stimuli such as hydroxylamine (HA, 5 mmol/l), PMA (1 $\mu g/ml$), FMLP (1 $\mu mol/l$), CaI (2 $\mu mol/l$) were added to the cell suspension. In order to evaluate the influence of dipyridamole (Behringer Ingelheim, Germany) on nitrite production by PMN, the drug was added to the reaction mixture at increasing concentrations (0.1-100 $\mu mol/l$). After incubation, cells were centrifuged (1200 $\times g$, 10 min) and the supernatant was collected and mixed 1:1 with Griess reagent (one part of 0.1 % naphthylethylenediamine dihydrochloride in distilled H_2O plus one part of 1 % sulfanilamide in 5 % concentrated H_3PO_4 , the two parts being mixed together within 2 h). After 10 min of incubation the absorbancy was estimated at

546 nm. Nitrite production was compared with standard curve generated with known amounts of NaNO_2 . Results are expressed as $\text{nmol nitrite}/2 \times 10^6 \text{ cells/h}$.

Hydrogen peroxide production by polymorphonuclear neutrophils

H_2O_2 generation was assayed by the method of Pick and Mizell (1981). Briefly, 0.25 ml PMN suspension in MHS with Ca^{2+} and Mg^{2+} was added to the tubes (final vol. 1 ml) containing 0.5 ml phenol red solution [4.64 ml HBSS, 0.09 ml phenol red (28 mmol/l), 0.07 ml HRP (12 mg/ml)]. Cells were stimulated by the addition of PMA (1 $\mu\text{g}/\text{ml}$), FMLP (1 $\mu\text{mol}/\text{l}$) or CaI (2 $\mu\text{mol}/\text{l}$) for 30 min at 37 °C. The reaction was stopped by adding 1 N NaOH. PMN were centrifuged (1200 $\times g$, 10 min), and absorbancy in a cell-free supernatant was assayed at 600 nm. The obtained extinction was compared with a standard curve generated with known amounts of H_2O_2 . The results are expressed as $\text{nmol H}_2\text{O}_2/1.25 \times 10^6 \text{ PMN}/30 \text{ min}$.

Statistical analysis

Results are expressed as mean \pm S.E.M. Data for control and treated cells were compared by using two-tailed t-test for paired observation or one-way analysis of variance (ANOVA). Bonferroni's correction for multiple comparisons was used to determine the level of significance. $P < 0.05$ value was considered significant.

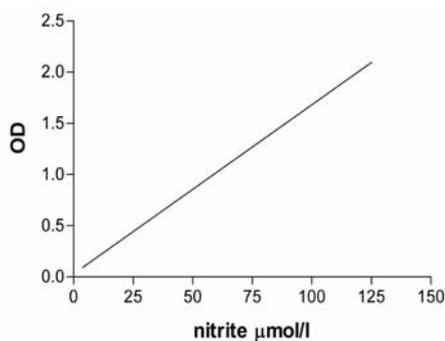


Fig. 1. Standard curve generated with known amounts of nitrite ($r=0.90$, $p < 0.001$).

Results

Effect of dipyridamole on the stimulated nitrite production from human PMN

It was previously shown that PMN incubated with hydroxylamine and stimulated with PMA generated large amounts of nitric oxide which could be

subsequently detected as nitrite (Markert *et al.* 1994). That process was mediated by O_2^- generation. It is evident that superoxide anion production by PMN is inhibited by dipyridamole (Colli and Tremoli 1991). Therefore we investigated whether this drug can also affect nitric oxide production by activated PMNs. Supernatants obtained from the suspension of stimulated cells were assayed for the presence of nitrite. Results were compared with a standard curve obtained with a known amount of nitrite (Fig. 1). As demonstrated in Figure 2A, stimulation of neutrophils with HA (5 mmol/l) and PMA (1 $\mu\text{g}/\text{ml}$) leads to massive production of nitrite. Increased NO generation was effectively inhibited by dipyridamole (100 $\mu\text{mol}/\text{l}$). Figure 2B shows that the effect of dipyridamole was dose-dependent. Incubation of PMNs with HA and PMA caused the generation of nitrite ($36.4 \pm 4.2 \text{ nmol/h } 2 \times 10^6 \text{ PMN}$). Dipyridamole at 100 $\mu\text{mol}/\text{l}$, 50 $\mu\text{mol}/\text{l}$ and 10 $\mu\text{mol}/\text{l}$ caused a considerable drop in nitrite production (11.8 ± 1.8 , 19.7 ± 2.7 and $27.4 \pm 3.2 \text{ nmol/h}$, respectively).

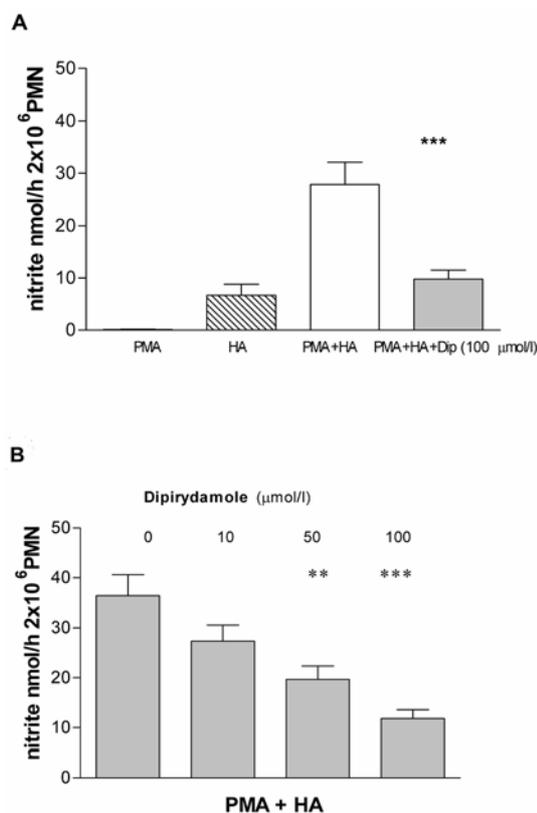


Fig. 2. Neutrophils (2×10^6 cells) were incubated with HA and stimulated with PMA (8 separate experiments). Additional samples were incubated with increasing concentrations of dipyridamole. Results were expressed as mean \pm S.E.M. (from nine separate experiments. In part A of the figure *** indicate $p < 0.001$, when compared with data for PMA + HA (two-tailed Student t test). In part B of the figure *** indicate $p < 0.001$, ** $p < 0.01$, when compared with sample without dipyridamole (ANOVA with Bonferroni multiple comparison test).

Colli and Tremoli (1991), demonstrated that dipyridamole inhibits O_2^- generation by PMN stimulated with FMLP and CaI but not with PMA. We found that neutrophils stimulated with CaI and HA produced significant amounts of nitrite (26.7 ± 3.9 nmol/ 2×10^6 cells/h), while activation of PMN with FMLP in addition to hydroxylamine is not produce additive effect (data not shown). The increased production of nitrite caused by HA and calcium ionophore was also inhibited by dipyridamole (to the value of 4.18 ± 3.6 nmol/ 2×10^6 cells/h).

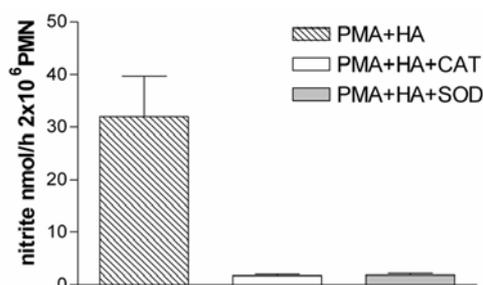


Fig. 3. Neutrophils (2×10^6 cells) were incubated with HA and stimulated with PMA. Additionally, some samples were incubated with catalase (CAT, 200 U/ml) or superoxide dismutase (SOD, 200 U/ml). Results were expressed as mean \pm S.E.M. (4 separate experiments).

Effect of oxygen metabolite scavengers on nitrite production by human PMN

Markert *et al.* (1994) demonstrated that superoxide dismutase inhibited nitrite production by cells activated with HA and PMA. It was therefore concluded that the superoxide anion is involved in NO production by PMN. It was also noted that exogenously added hydrogen peroxide enhanced nitrite generation by PMN. Colli and Tremoli (1991) showed that neutrophils stimulated with PMA produced O_2^- that cannot be inhibited by dipyridamole. We also observed that dipyridamole could not diminish superoxide generation by PMN activated with PMA while O_2^- production augmented by CaI or FMLP was significantly diminished (data not shown). Moreover, H_2O_2 production by neutrophils stimulated with PMA, was significantly diminished by dipyridamole (Table 1). Therefore, we used catalase that scavenges hydrogen peroxide to assess the contribution of H_2O_2 to nitrite generation by PMN. It was found (Fig. 3) that both SOD and catalase are equally effective in abolishing nitrite generation by activated neutrophils ($p < 0.001$).

Adenosine and adenosine agonist did not affect nitrite generation by PMN

It was previously shown that neutrophils possess adenosine receptors. When occupied, A_2 receptors inhibit stimulated superoxide anion and hydrogen peroxide production (Cronstein *et al.* 1985, 1987). It was also proved that one of the mechanisms of dipyridamole action is the inhibition of cellular uptake and adenosine metabolism. This mechanism is responsible for inhibition of O_2^- generation by neutrophils. In order to determine whether adenosine may mediate some of the dipyridamole influence on nitrite production by PMN, we incubated neutrophils with increased concentrations of adenosine (up to 20 μ mol/l). Figure 4A shows that adenosine did not inhibit nitrite production by HA and PMA activated PMNs (24.2 ± 6.4 vs. 20.0 ± 5.5 nmol/ 2×10^6 PMN/h, $n=5$ separate experiments). Furthermore, NECA (1 μ mol/l), a selective A_2 receptor agonist also failed to inhibit NO generation by neutrophils (24.2 ± 6.4 vs. 21.2 ± 2.0 nmol/ 2×10^6 PMN/h, Fig. 4B). The fact that both adenosine or NECA were unable to diminish activated generation of nitrite by PMN seems to indicate that an adenosine-independent mechanism is involved in dipyridamole-mediated NO inhibition.

Table 1. The influence of dipyridamole on stimulated hydrogen peroxide production (nmol/ 1.25×10^6 PMN/30 min) by polymorphonuclear neutrophils.

	-	Dipyridamole (μ mol/l)		
		100	10	0.1
PMA	43.6 ± 7.8	$4.0 \pm 1.0^{**}$	26.7 ± 9.2	44.8 ± 7.2
CaI	18.5 ± 2.8	$2.1 \pm 0.4^{**}$	10.1 ± 3.4	15.2 ± 4.2
FMLP	6.8 ± 1.3	$2.0 \pm 0.3^*$	$1.1 \pm 0.3^{**}$	4.8 ± 1.5

Neutrophils were incubated with increasing concentration of dipyridamole and stimulated with hydroxylamine (5 mmol/l) and activators PMA (1 μ g/ml), CaI (2 μ mol/l) and FMLP (1 μ mol/l). Results are expressed as mean \pm S.E.M. of 4 separate experiments, * denotes $p < 0.05$, ** $p < 0.01$ (comparisons were carried out by ANOVA with Bonferroni's multiple comparisons).

Theophylline did not reverse the effect of dipyridamole on stimulated PMN nitrite production

It was hypothesized that the effect of dipyridamole on polymorphonuclear nitric oxide metabolism is mediated by the inhibition of adenosine uptake by the cells and increase of adenosine concentration in the extracellular milieu. In order to confirm this hypothesis we tried to reverse the dipyridamole effect by incubating PMN in the presence

of theophylline (an adenosine antagonist). We have found that theophylline itself did not affect activated nitrite production (data not shown). Furthermore, the inhibition of hydroxylamine-PMA stimulated production in the presence of dipyridamole was not reversed by theophylline (36.2 ± 5.3 nmol/ 2×10^6 PMN/h for PMA and HA stimulation, 11.6 ± 2.8 nmol/ 2×10^6 PMN/h for PMA+HA + dipyridamole 100 μ mol/l vs. 11.2 ± 3.2 nmol/ 2×10^6 PMN/h for PMA+HA + dipyridamole 100 μ mol/l + theophylline 100 μ mol/l) (Fig. 4B).

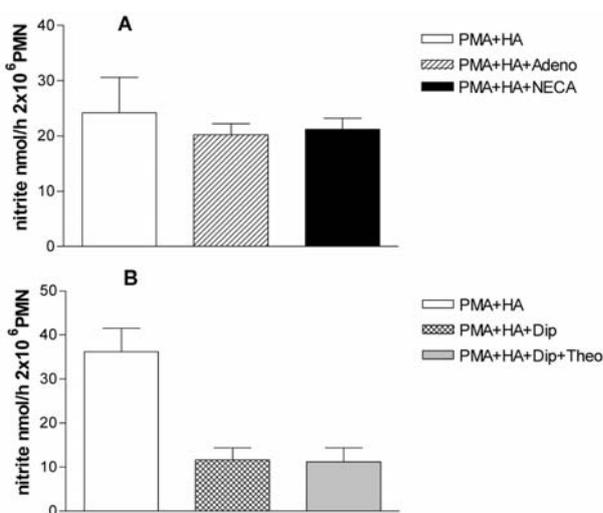


Fig. 4. Neutrophils 2×10^6 cells were incubated with HA and stimulated with PMA. Additionally, some samples were incubated with dipyridamole (Dip 100 μ mol/l), adenosine (Adeno, 20 μ mol/l), theophylline (Theo, 100 μ mol/l) or NECA (1 μ mol/l). Results were expressed as mean \pm S.E.M. (2-4 separate experiments)

Discussion

In recent years much attention has been focused on the role of polymorphonuclear neutrophils in the pathogenesis of coronary artery disease (CAD). There are reports indicating that PMNs are involved in various forms of CAD. These cells participate in ischemia and reperfusion injury of the myocardium. This effect is partly mediated by cytotoxic properties of oxygen metabolites – superoxide anion, hydroxyl radicals, hypochlorous acid and hydrogen peroxide produced by activated neutrophils.

Despite the large body of evidence concerning the role of PMN in CAD pathological mechanisms by which these cells participate in the progression or complication of coronary artery disease are not well understood. Particularly, better knowledge is necessary

before designing a rational approach to treatment strategies aiming to modulate the PMN functional status. Recently, Pietersma *et al.* (1995) showed that late lumen loss after coronary angioplasty is associated with activation status of circulating neutrophils. Surprisingly, activated PMNs seem to prevent luminal restenosis. The protective mechanism by which activated neutrophils prevent late lumen loss is not well established. Authors speculate that facilitation of tissue repair, generation of 6-keto-prostaglandin- E_1 (that inhibits aggregation of platelets) and providing 13-hydroxy-octadecadienoic acid to inhibit platelet adhesion may be responsible for this effect.

Nitric oxide produced by PMN is also thought to play an important role in tissue injury. This molecule can also influence PMN adherence to the endothelium. Recently, Neilly *et al.* (1995) have demonstrated that plasma nitrate concentration (stable-end product of NO oxidation) which reflects endogenous NO production, largely depends on the amount of neutrophils in peripheral blood. Moreover, these authors showed that non-neutropenic patients with suspected septicemia and hypotension have usually a high concentration of nitric oxide in the plasma. In contrast, neutropenic patients with septicemia but without hypotension had low levels of plasma NO. These data strongly suggest that PMNs are an important source of nitric oxide, thus modulation of nitric oxide released by PMNs may be of clinical relevance.

A number of studies that were designed to test the effect of various substances on PMN metabolism *in vitro* and *in vivo* rarely address this issue. Here we demonstrated that dipyridamole, a drug that affects PMN oxygen metabolism, significantly decreased nitric oxide production by these cells. The effect was dose-dependent and observed after a short period of incubation (about 2 min, data not shown). Dipyridamole inhibits both superoxide anion production and hydrogen peroxide generation by activated PMN. However, production of superoxide by cells activated with PMA is not affected by dipyridamole (Colli and Tremoli 1991). In our study, nitrite production by neutrophils stimulated with PMA and hydroxylamine was inhibited by dipyridamole. Thus, we concluded that this effect may be mediated in part by the diminished H_2O_2 generation by activated neutrophils. Furthermore, catalase that scavenges hydrogen peroxide significantly impaired nitrite generation by stimulated PMN. Since the action of dipyridamole on neutrophils was partly mediated by inhibition of adenosine uptake

(Colli and Tremoli 1991), we decided to further explore this important mechanism. We showed that exogenously added adenosine did not mimic the effect of dipyridamole. Furthermore, theophylline, a competitive antagonist at the adenosine receptor, did not reverse dipyridamole action on nitrite production by stimulated neutrophils. Thus, we conclude that inhibition of NO production by dipyridamole is adenosine-independent process. Colli and Tremoli (1991) showed that dipyridamole inhibits dose-dependently the synthesis of leukotriene B₄ and C₄ by stimulated neutrophils. Furthermore, this inhibition was not mediated by adenosine. Imai *et al.* (1993) demonstrated that nitric oxide production by macrophages is regulated by arachidonic acid metabolism. These authors reported that nordihydroquaiaretic acid, a preferential inhibitor of lipoxygenase pathway, caused considerable reduction of NO₂⁻ accumulation. Although production of NO in macrophages is mediated by inducible nitric oxide synthase, it is tempting to speculate that inhibition of leukotriene production by dipyridamole may influence NO release in activated neutrophils. It is also possible that antioxidant properties of dipyridamole (Pick and Mizell 1981) may also contribute to diminished synthesis of nitric oxide by PMNs.

Nitric oxide can react with superoxide anion to form the powerful oxidant, peroxynitrite anion (Beckman *et al.* 1990). It seems that inhibition of this reaction may be beneficial. On the other hand, reduction of nitric oxide production may enhance PMN adhesion to endothelial cells resulting in augmented tissue destruction. In addition, choosing the moment of such inhibition may be crucial for the final outcome, e.g. drug administration before, during or after reperfusion. Moreover, selective inhibition of PMN oxygen metabolites may be necessary to prevent some unwanted effects without antagonizing a

favorable response. It seems that some of these processes may be responsible for the controversy concerning the involvement of nitric oxide in reperfusion injury. The vasodilatory effect of NO and inhibitory effect on PMN adhesion to endothelium were most probably responsible for cardioprotective effects observed in some experiments (Lefer *et al.* 1993). On the contrary, it was demonstrated that overproduction of NO during reperfusion is deleterious (Yamabe *et al.* 1992). Recently, Woolfson *et al.* (1995) showed that inhibition of nitric oxide synthesis reduced infarct size by an adenosine-dependent mechanism. Adenosine is a strong inhibitor of PMN respiratory burst and diminished neutrophil adhesion to endothelium (Cronstein *et al.* 1992). It is produced in large amounts during ischemia and may serve as an endogenous modulator of PMN function (Cronstein *et al.* 1985). However, here we show that adenosine could not reduce NO production by activated PMN. Interestingly, recently more stress is put on local delivery of drug to the site of its action. Singh *et al.* (1994) showed that dipyridamole inhibited smooth muscle cell proliferation *in vitro* and locally delivered inhibited cell replication in arteries and intimal thickening after balloon injury.

Despite numerous studies, the beneficial effect of inhibiting PMN function in clinical setting of myocardial ischemia is still largely illusive, partly due to a poorly understood mechanism of the action of drugs aiming to affect PMN metabolism and function. Most of these drugs do not act selectively but rather exert a pleiotropic action affecting PMNs oxygen metabolism, leukotrienes generation, chemotactic activity and proteolytic enzyme release. It is thus difficult to predict the net effect of any given drug. We believe that the information provided here will be of some help in a better understanding the effect of dipyridamole action in various research paradigms.

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Reprint requests

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