The Effect of Dipyridamole on Vascular Cell-Derived Reactive Oxygen Species

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Received May 24, 2005; accepted July 19, 2005

ABSTRACT
Platelet and vascular stimulation leads to release of reactive oxygen species (ROS) that are known to influence vascular reactivity and thrombosis. Dipyridamole is a vasodilator and platelet inhibitor that has previously been shown to have direct antioxidant properties. The antioxidant effects of dipyridamole on vascular cell-derived ROS are not known; therefore, dipyridamole was incubated with endothelial cells and platelets and cellular redox status and release of endogenous ROS were assessed. Dipyridamole decreased intracellular basal ROS generation from endothelial cells as measured by DCFDA (2',7'-dichlorodihydrofluorescein diacetate) oxidation. Incubation of endothelial cells with dipyridamole also attenuated t-butyldihydroperoxide-induced oxidative stress. Using a redox-sensitive fluorescent dye, dipyridamole improved cellular activity after treatment with t-butyldihydroperoxide. Incubation with dipyridamole did not alter platelet release of nitric oxide or hydrogen peroxide but significantly attenuated peroxide release. Using flow cytometry and confocal microscopy, dipyridamole decreased platelet ROS generation. Dipyridamole also suppressed platelet-soluble CD40 ligand release. In summary, at therapeutically relevant concentrations, dipyridamole suppresses the formation of ROS in platelets and endothelial cells and improves cellular redox status. These data suggest that the redox-dependent properties of dipyridamole have a direct effect on vascular cells.

Dipyridamole is a platelet inhibitor that is primarily recognized as an antithrombotic agent. In addition, through the generation of adenosine, dipyridamole evokes vasodilation and, through the combination of these antiplatelet and vasodilator functions, probably improves tissue perfusion. The European Stroke Prevention Study 2 involving over 6600 patients with transient ischemic attacks or stroke demonstrated that treatment with dipyridamole was as effective as low-dose aspirin in the reduction of stroke risk (Diener et al., 1996), and combination therapy with dipyridamole and aspirin was more than twice as effective as aspirin alone. Because dipyridamole is a weak direct platelet inhibitor, such clinical observations suggest that dipyridamole may have additional beneficial vascular effects. Dipyridamole has been reported to have antioxidant properties (Iuliano et al., 1989), but the direct effect on vascular cells is not known. Dipyridamole is a highly efficient chain-breaking antioxidant with fluorescence that is quantitatively quenched upon reaction with peroxy radicals (Iuliano et al., 2000). Serving as oxygen-derived free radical scavenger, dipyridamole has been shown to prevent membrane and mitochondrial lipid peroxidation as well as oxidative modification of low-density lipoprotein (Selley et al., 1994; Iuliano et al., 1996). When used as a superoxide scavenger, dipyridamole prevented pyrogallol-induced stimulation of platelets (De la Cruz et al., 1992). The clinical relevance of these antioxidant effects is suggested by the attenuation of cerebral oxidative stress associated with human carotid endarterectomy occurring with pretreatment with dipyridamole (Kusmic et al., 2000a).

It is unknown whether antioxidant properties of dipyridamole directly alter endogenous release of reactive oxygen and nitrogen species in the vasculature. Because vessel patency, tone, and thrombosis may all be affected by endogenous formation of vascular ROS, the redox-dependent prop-

ABBREVIATIONS: DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; CMDCFDA, 5- and 6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester; DPPH, 1,1-diphenyl-2-picrylhydrazyl; TRAP, thrombin receptor-activating peptide; sCD40L, soluble CD40 ligand; PMA, phorbol 12-myristate 13-acetate; DMEM, Dulbecco’s modified Eagle’s medium; FRAP, ferric-reducing antioxidant power; DMSO, dimethyl sulfoxide; BAEC, bovine aortic endothelial cells; NO, nitric oxide; ROS, reactive oxygen species; DHR, dihydrorhodamine; eNOS, endothelial NO synthase.
properties of dipyridamole were determined in both platelets and endothelial cells.

Materials and Methods

DCFDA, CMDCFDA, sodium pyruvate, and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma-Aldrich (St. Louis, MO). Dipyridamole [2,6-bis(diethanolamino)-4,8-dipiperidino-pyrimido(5,4-d) pyrimidine] was obtained from Boehringer Ingleheim USA (Ridgefield, CT). Alamar Blue dye was purchased and is a proprietary product from BioSource International (Camarillo, CA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, and glutamine were obtained from Invitrogen (Carlsbad, CA). Ferrozine [3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4 triazine] was obtained from Bio Vectra (Abingdon, MD). DPPH was purchased from Cayman Chemicals (Ann Arbor, MI).

Direct Antioxidant Properties of Dipyridamole. The antioxidant activity of dipyridamole was determined by two methods: 1) by measuring its effect on the scavenging capacity of free radical DPPH and 2) by the ferric-reducing antioxidant power (FRAP) assay (Aaby et al., 2004; Shon et al., 2004; Tavridou and Manolopoulos, 2004; Xu et al., 2004; Firuzi et al., 2005). DPPH is a stable free radical, and its reduction has been used for the determination of efficacy of antioxidant compounds (Aaby et al., 2004; Alvarez-Gonzalez et al., 2004; Gandhi and Nair, 2004; Ligeret et al., 2004; Shon et al., 2004; Tavridou and Manolopoulos, 2004; Xu et al., 2004). The reduction of stable free radical DPPH was determined as described previously (Ligeret et al., 2004) by adding DPPH to increasing concentrations of dipyridamole and by measuring the decrease in absorption at 515 nm over time.

The measurement of Fe3+ reduction using the FRAP assay (Aaby et al., 2004; Xu et al., 2004; Firuzi et al., 2005) determined the antioxidant property of dipyridamole by monitoring ferrous ferrozine complex formation after conversion of Fe3+ to Fe2+. As described previously (Ligeret et al., 2004). In brief, using ferrozine (100 μM final concentration) and ferric chloride (100 μM final concentration), the reaction was initiated by the addition of increasing concentrations of dipyridamole and the absorbance of the Fe2+/ferrozine complex formation was monitored spectrophotometrically at 568 nm as a function of time. Control samples were run in parallel with DMSO and/or dipyridamole without Fe3Cl4 or ferrozine.

Platelet Isolation and Endothelial Cell Culture. Blood from healthy human volunteers on no medications or vitamin supplements was drawn in a syringe containing 10% sodium citrate. The healthy human volunteers on no medications or vitamin supplements was drawn in a syringe containing 10% sodium citrate. The study protocol, and all of the patients gave their written informed consent to participate.

Platelet-rich plasma was prepared after centrifugation of blood (150g). Washed platelets were prepared as described previously (Freedman et al., 1996a,b). Platelet pellets were resuspended in HEPES buffer, pH 7.4, for subsequent analysis. Platelet counts were determined in a Coulter Counter (model ZM; Beckman Coulter, Inc. (Fullerton, CA). Bovine aortic endothelial cells (BAEC) were cultured in DMEM containing 10% fetal bovine serum, 2.5 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin as described previously (Freedman et al., 1995).

Measurement of Reactive Oxygen Species. Confluent endothelial cells washed with fresh DMEM were incubated with DCFM premixed with DCFDA (10 μM) with and without dipyridamole and incubated for 2 h, and fluorescence readings were taken immediately after adding fresh DMEM. Fluorescence was recorded in a microplate reader (Molecular Devices, Sunnyvale, CA) over time after excitation at 485 nm and emission at 535 nm. To normalize fluorescence, cellular proteins were measured using the Bio-Rad D, protein assay kit (Bio-Rad, Hercules, CA).

Influence of dipyridamole on stimulation-induced generation of reactive oxygen species from endothelial cells was also verified by confocal microscopy. Confluent BAECs were incubated for 45 min at 37°C in Krebs buffer in the presence of 10 μM CMDCFDA along with DMSO (vehicle control) or 10 μM dipyridamole. Cells were subsequently washed and then treated with 1 U/ml thrombin, and images were captured using two-photon confocal microscopy and processed by using NIH image software.

Treatment of Endothelial Cells with t-Butylhydroperoxide and Evaluation of Redox State by Using Alamar Blue. Confluent BAECs were washed once with DMSO and incubated with and without dipyridamole. Increasing concentrations of t-butylhydroperoxide were added and incubated for 2 h, and readings were recorded over time. To study the resulting redox state as originating from cellular growth, cells were incubated with redox-sensitive water-soluble nontoxic dye Alamar Blue (Ahmed et al., 1994; Collins and Franzblau, 1997; Franzblau, 2000). After incubation with t-butylhydroperoxide, cells were washed with fresh DMEM and Alamar Blue in DMEM (10% Alamar Blue final concentration) was added. Readings were taken (550-nm excitation/570-nm emission) in a fluorescent microplate reader. Cells were normalized by protein content as described above using the Bio-Rad protein assay kit. Potential endothelial cell toxicity due to t-butylhydroperoxide was assessed using a lactate dehydrogenase assay (Lautraite et al., 2003).

Measurement of Platelet Nitric Oxide Production and Aggregation. We developed a NO-selective microelectrode (Inter Medical Co., Ltd., Nagoya, Japan) for use in a standard platelet aggregometer (Payton Associates, Buffalo, NY) in order to monitor platelet NO production and aggregation simultaneously, as described previously (Freedman et al., 1996a,b). Platelet NO production was rigorously quantified as the integrated area under the curve of the signal detected by the microelectrode after platelet activation with 20 μM TRAP. As compared with measuring peak height, this approach allows enhanced quantification and comparison of relative signal. TRAP aggregation-induced platelet NO generation was determined after standard calibration with spermine nonoate (Cayman Chemicals).

Measurement of Platelet-Superoxide Production. Lucigenin-derived chemiluminescence was used to estimate aggregation-dependent superoxide production from stimulated platelets using a lumiggregometer (whole blood lumiaggregometer, model 500-CA; Chronolog Corp., Havertown, PA) as detailed previously (Freedman and Keeay, 1999). Washed platelets (4 × 10^5 platelets/μL, preincubated with 250 μM lucigenin (bis-N-methylacridinium nitrate) in HEPES buffer for 3 min, were placed into the lumiaggregometer (aggregometer with luminescence detection). After stabilization of the signal, 0.1 μM PMA-stimulated superoxide production and aggregation were simultaneously measured while stirring at 1000 rpm at 37°C. Washed human platelets were treated with increasing concentrations of dipyridamole, stimulated with PMA, and compared with vehicle control (DMSO).

Platelet Generation of Hydrogen Peroxide. In situ generation of stimulation-induced platelet hydrogen peroxide was carried out in the presence of dipyridamole using a sensitive hydrogen peroxide detection kit (Amplex Red; Invitrogen). Reactions were carried out in HEPES buffer with 20 μM dipyridamole. Amplex Red reagent was added, readings were taken at 560-nm excitation/590-nm emission, and fluorescence was monitored as a function of time after the addition of 0.1 μM PMA. In separate experiments, catalase was used to verify assay specificity.

Assessment of ROS by DCFDA and Platelet Flow Cytometry and Confocal Microscopy. Freshly isolated washed platelets from healthy volunteers were incubated with the fluorescent probe DCFDA for 5 min in the presence of vehicle control or varying concentrations of dipyridamole. After TRAP stimulation for 3 min,
the samples were immediately analyzed in a fluorescence-activated cell sorter (DakoCytomation California Inc., Carpinteria, CA). Platelet-specific events (10–50,000) were collected for each set of data points. The activation-induced platelet fluorescence induction was calculated using appropriate gating of the platelet population.

Confocal images were captured in a two-photon confocal microscopy. In brief, freshly isolated platelets in HEPES buffer were incubated in the presence of redox-sensitive probe dihydorhodamine (DHR, 10 μM) for 10 min in the presence and absence of 20 μM dipyridamole. Confocal images were captured at different time points before and after TRAP (20 μM) stimulation. Images were processed after laser background subtraction using NIH image software.

**Platelet-Soluble CD40 Ligand Release.** Washed human platelets (2 × 10⁹/ml) were stimulated with thrombin (0.2 U/ml) for 2 h in the presence of varying concentration of dipyridamole at room temperature. Platelet supernatant was collected by centrifugation. The released sCD40L was measured by enzyme-linked immunosorbent assay (Bender Med Systems, San Bruno, CA).

**Statistical Analysis.** Differences between groups were determined using an unpaired Student’s *t* test. The effects of interventions were analyzed using a Student's paired *t* test. A statistically significant difference was assumed with a value of *P* < 0.05. All of the data are expressed as the mean ± S.E.M.

**Results**

**The Direct Antioxidant Properties of Dipyridamole.** Previous studies have suggested the direct antioxidant effects of dipyridamole. To confirm these properties and establish the relevance of our platelet and endothelial cell findings, the direct antioxidant effects of dipyridamole were studied by two methods: 1) the effect on the scavenging capacity of free radical DPPH and 2) by the FRAP assay (Aaby et al., 2004; Shon et al., 2004; Tavridou and Manolopoulos, 2004; Xu et al., 2004; Firuzi et al., 2005). DPPH, a stable free radical, is used for the determination of efficacy of antioxidant compounds (Aaby et al., 2004; Alvarez-Gonzalez et al., 2004; Gandhi and Nair, 2004; Ligeret et al., 2004; Shon et al., 2004; Tavridou and Manolopoulos, 2004; Xu et al., 2004). The reduction of DPPH was determined after the addition of increasing concentrations of dipyridamole, and the findings indicate a modest direct antioxidant effect (Fig. 1). Using the FRAP assay (Aaby et al., 2004; Xu et al., 2004; Firuzi et al., 2005), the antioxidant property of dipyridamole, as indicated by monitoring ferrous ferrozine complex formation, demonstrates a dose-dependent antioxidant effect for dipyridamole over time (Fig. 1B). The FRAP assay directly determines the reducing capacity of a compound (Firuzi et al., 2005). A good correlation has been observed between the FRAP assay and electrochemical results, confirming the reliability of this assay as a method for the evaluation of the antioxidant activity of compounds.

**The Effect of Dipyridamole on Endothelial Cellular Redox State and Generation of Reactive Oxygen Species.** To determine whether the antioxidant effects of dipyridamole alter endothelial cell release of reactive oxygen species, BAECs were incubated with increasing concentrations of dipyridamole and DCFDA fluorescence was determined. DCFDA is a fluorescence-based probe that has been recently developed to detect intracellular production of ROS. DCFDA diffuses passively into cells and is trapped inside generating DCFH after deacetylation by intracellular esterases. It is subsequently oxidized to a fluorescent product in presence of intracellular ROS. Oxidation of DCFDA is conveniently monitored for the determination of intracellular oxidative stress (Halliwell and Whiteman, 2004). As seen in Fig. 2A, there is a significant dose-dependent reduction of basal DCFDA fluorescence as a result of dipyridamole incubation. These effects were seen immediately after washing out the dipyridamole and persisted as well as, increasing at 1, 2, and 3 h after washing (data not shown). Representative confocal images are shown in Fig. 2B.

t-Butylhydroperoxide is used to generate oxidative stress in various biological systems (Lautraite et al., 2003). As shown in Fig. 3A, the addition of t-butylhydroperoxide leads to enhanced oxidative stress and this effect is attenuated after incubation with dipyridamole. Dipyridamole also improves endothelial metabolic activity after exposure to t-butylhydroperoxide-induced oxidative stress, as shown by the redox-sensitive dye Alamar Blue (Fig. 3B). Alamar Blue is a redox-sensitive dye, a reduction of which reflects metabolic activity of the cells (Ahmed et al., 1994; Collins and Franzblau, 1997; Franzblau, 2000). To confirm that the t-butylhydroperoxide treatment did not cause cellular toxicity, endothelial cells that had been incu-
bated with t-butylhydroperoxide were tested for the release of lactate dehydrogenase. No significant toxicity was observed at all of the concentrations during the periods of incubation (data not shown).

The Effect of Dipyridamole on Activation-Induced Platelet Generation of Reactive Oxygen and Nitrogen Species. Incubation with dipyridamole alters endothelial cell redox status and ROS generation. To determine whether these effects are specific for the endothelial cells or are also relevant in platelets, the release of platelet reactive oxygen and nitrogen species was determined after incubation with dipyridamole. Platelet-superoxide release was measured using a lumiaggregometer after PMA-induced stimulation. PMA is used as the agonist, because it has the most marked effect on platelet-superoxide release (Freedman and Keaney, 1999). Incubation of platelets with 20 μM dipyridamole led to a marked suppression of platelet release of superoxide (Fig. 4A). This effect was noted over a range of dipyridamole concentrations (2–100 μM; data not shown).

To determine whether dipyridamole alters the release of other endogenous reactive species, platelet levels of NO and hydrogen peroxide were also measured. There was no significant effect of dipyridamole on platelet release of NO as measured by microelectrode (Fig. 4B) or hydrogen peroxide (data not shown) as measured by the Amplex Red assay over a range of concentrations. Platelet generation of ROS after stimulation as measured by DCFDA oxidation by flow cytometry was studied. The generation of ROS was significantly attenuated as a function of increasing dipyridamole concentration (Fig. 5A). Washed platelets were also incubated in the presence of redox-sensitive probe DHR (10 μM) for 10 min in the presence and absence of 20 μM dipyridamole. Confocal images (Fig. 5B) were captured at different time points before and after TRAP (20 μM) stimulation (displayed images were taken 10 min after TRAP stimulation). The presence of dipyridamole attenuated TRAP activation-induced platelet fluorescence induction (Fig. 5B).

To determine whether the change in redox status of the
platelet by dipyridamole has an effect on the platelet-inflammatory response, platelet release of sCD40L was determined. Platelets were stimulated with thrombin for 2 hr in the presence of increasing concentrations of dipyridamole. Platelet supernatant was collected, and the released sCD40L was measured by enzyme-linked immunosorbent assay. As seen in Fig. 6, incubation with dipyridamole leads to a modest but significant suppression of sCD40L release from activated platelets.

**Discussion**

Platelet and vascular stimulation leads to the release of ROS that are known to induce vasoconstriction and platelet activation and stimulate the atherothrombotic processes. Dipyridamole is a vasodilator and platelet inhibitor that has previously been shown to have direct antioxidant properties; however, its effects on vascular cells are unknown. Therefore, we studied the effect of dipyridamole on platelet- and endothelial cell-derived release of ROS. Consistent with previous studies (Iuliano et al., 1989), the direct antioxidant properties of dipyridamole were confirmed. In addition, at a therapeutically relevant concentration (3.5 μM) (Aktas et al., 2003), dipyridamole suppressed stimulation-dependent endothelial ROS formation and platelet release of soluble CD40 ligand. Previous studies using higher concentration of dipyridamole (~20 μM, peak concentration) reached in blood after chronic intake of the habitual dose diminished platelet-neutrophil interaction (De la Cruz et al., 2000) and also attenuated neutrophil-superoxide generation (Vargas et al., 2003). Our present study, with a dose range of 0.5 to 20 μM dipyridamole, displayed a wide range of antioxidant/anti-inflammatory properties both in platelets and endothelial cells.
These data suggest that the antioxidant effect of dipyridamole is related to its partition in the lipid phase of the mitochondrial membrane and not to a specific interaction with membrane proteins. This protection may be due either to a direct inhibition of the propagation steps or a scavenger effect on the radical species that would trigger the peroxidative process (Nepomuceno et al., 1999). Our findings that specifically showed a suppression of superoxide (Fig. 4) would support the previous suggestions as well as our own findings (Fig. 1, A and B) that dipyridamole has a direct antioxidant effect.

Oxidant stress can lead to endothelial dysfunction, and in turn, this contributes to the genesis of the atherothrombotic plaque. Therefore, it is plausible that dipyridamole promotes vascular protection and improves endothelial function through its antioxidant actions. Dipyridamole had been shown to enhance inhibition of platelet function by amplifying the effect of exogenous NO donors, suggesting enhancement of the NO/cGMP pathway (Aktas et al., 2003). Although dipyridamole did not alter endogenous release of platelet-derived NO (Fig. 4B), the findings of our study are consistent with these previous observations (Aktas et al., 2003) because the dipyridamole-dependent enhancement of exogenous NO could be mediated through the suppression of platelet-superoxide release (Fig. 4A), thus limiting bioavailable NO. However, further in vitro and in vivo studies are warranted to characterize the role of dipyridamole in influencing endothelial NO and superoxide generation.

Although the effects on endothelial cells and platelets have not been investigated, dipyridamole was previously shown to scavenge ROS secreted by activated neutrophils (Vargas et al., 2003). Dipyridamole has also been shown to protect erythrocyte membranes from oxidation (Kusmic et al., 2000b) and neuronal cells from chemically mediated oxidative damage (Blake, 2004). In addition, dipyridamole was recently shown to prevent damage in a liver ischemia-reperfusion model (Taniguchi et al., 2004). These redox-specific effects may extend to other cellular antioxidant/anti-inflammatory interactions that are known to be important in acute and chronic cardiovascular disease. Flow cytometry data generated in the AGATE (Aggrenox versus Aspirin Therapy Evaluation) study (Serebruany et al., 2004) indicates that subjects taking Aggrenox have a depression (beyond aspirin alone) of receptors important in cell-cell interactions. Our results show that low concentrations of dipyridamole (≥1 μM) induce suppression of the platelet inflammatory protein sCD40L, although we cannot conclusively say that this effect is mediated through the antioxidant properties of this drug. Thus, further study of adhesion molecules and inflammatory-mediated cellular cross-talk may be warranted.

Recent studies show that reduction of NO bioavailability is associated with an increase in endothelial production of superoxide (Kalinowski and Malinski, 2004). Superoxide in both the platelet and endothelial cell is generated by NAD[P]H oxidase and may trigger endothelial NO synthase (eNOS) uncoupling, contributing to the endothelial balance between NO and superoxide (Kalinowski and Malinski, 2004). Release of several ROS, including superoxide, hydroxyl radical, and H₂O₂, from platelets are reported, both from unstimulated and after stimulation with agonists, such as collagen or thrombin (Krotz et al., 2004). Several enzymatic systems contribute to the production of ROS, thereby influencing platelet activity. In the endothelium, NAD(P)H oxidase, cyclooxygenase isozymes 1 and 2, cytochrome P450 epoxygenase isozyme 2C9 (CYP2C9), xanthine oxidase, uncoupled eNOS, and mitochondrial respiration contribute to the production of superoxide, H₂O₂, and hydroxyl radicals. Similar to endothelium-derived ROS, platelet-derived ROS potentially stem from enzymatic sources, including cyclooxygenase-1, xanthine oxidase, mitochondrial respiration, or uncoupled eNOS. The platelet isofrom of NAD(P)H-oxidase has gained the most attention, because it can be activated by platelet activation. Platelets have been reported to possess NAD(P)H-oxidase activity (Seno et al., 2001; Krotz et al., 2002), and many of its subunits have been found at the protein level (Krotz et al., 2004). Dipyridamole may modulate NADPH oxidase activity, because dipyridamole-induced inhibition of NADPH oxidase activity has been demonstrated in a diabetic animal model (Onozato et al., 2003).

A recent study (Pignatelli et al., 2004) has demonstrated that platelet NADPH oxidase subunit, gp91phox, regulates the expression of CD40L. Thus, it is possible that dipyridamole, through its inhibitory property on NADPH oxidase, may alter platelet ROS generation as well as platelet release of sCD40L. In summary, at therapeutically relevant concentrations, dipyridamole suppresses stimulation-dependent platelet-superoxide generation, formation of ROS in platelets and endothelial cells, and improves cellular redox status. These data suggest that the antioxidant properties of dipyridamole have a direct effect on vascular cells and suppress the endogenous release of vascular reactive oxygen and inflammatory species shown to be relevant in the development of atherothrombotic diseases.

Acknowledgments

We thank Dr. Wolfgang G. Eisert for helpful discussions.

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