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

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Nonstandard abbreviations: DCFDA, 2',7'-Dichlorodihydrofluorescein diacetate;

DPPH, 1,1 diphenyl-2-picrylhydrazyl; $TRAP_{1-6}$, trombin receptor activating

peptide; Ferrozine, (3-(2-Pyridyl)-5,6-Bis(4-Phenylsulfonic Acid)-1, 2,4 triazine;

sCD40L, soluble CD40 ligand.

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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 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) oxidation. Incubation of endothelial cells with dipyridamole also attenuated t-butylhydroperoxide induced oxidative stress. Using a redox sensitive fluorescent dye, dipyridamole improved cellular activity after treatment with t-butylhydroperoxide. Incubation with dipyridamole did not alter platelet release of nitric oxide or hydrogen peroxide but significantly attenuated superoxide release. Using flow cytometry and confocal microscopy, dipyridamole decreased platelet ROS generation. Dipyridamole also suppressed platelet soluble CD40 ligand (sCD40L) release. In summary, at therapeutically relevant concentrations, dipyridamole suppresses 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.

INTRODUCTION

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 likely 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, clinical observations such as this 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 peroxyl 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 dipyridamole's antioxidant properties directly alter endogenous release of reactive oxygen and nitrogen species in the vasculature. As vessel patency, tone, and thrombosis may all be affected by endogenous formation of vascular ROS, the redox-dependent properties of dipyridamole were determined in both platelets and endothelial cells.

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MATERIALS AND METHODS

2',7'-Dichlorodihydrofluorescein diacetate (DCFDA), 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate, acetyl ester (CMDCFDA), sodium pyruvate, and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma Chemical Company (St. Louis, MO). Dipyridamole (2,6-bis(diethanolamino)-4,8-dipiperidino-pyrimido(5,4-d) pyrimidine) was obtained from Boehringer Ingelheim (Ridgefield, CT). Alamar Blue dye was purchased and is a proprietary product from Biosource International (US patent #5,501,959) (Camarillo, CA). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and glutamine were obtained from GIBCO-BRL (Gaithersburg, MD). Ferrozine (3-(2-Pyridyl)-5,6-Bis(4-Phenylsulfonic Acid)-1, 2,4 triazine, monosodium salt) was obtained from Bio Vectra (Oxford, CT). 1, 1 Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Wako Chemicals (Richmond, VA). Amplex Red assay kit for hydrogen peroxide measurements was obtained from Molecular Probes (Eugene, OR). Fibrinogen and human thrombin were purchased from Enzyme Research Laboratories (South Bend, IN). Thrombin receptor activation peptide (TRAP$_{1-6}$) was procured from Peninsula Laboratories (San Carlos, CA). T-butylhydroperoxide was obtained from Aldrich Chemical Company (Milwaukee, WI). Dihydrorhodamine (2-(3, 6 diamino-9H-xanthen-9-yl)-benzoic acid, methyl ester) and Spermine nonoate (1, 3-propanediamine, N-[4-[1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino] butyl]) were procured from Cayman Chemicals (Ann Arbor, MI).

Direct Antioxidant Properties of Dipyridamole

The antioxidant activity of dipyridamole was determined by two methods: by measuring its effect on the scavenging capacity of free radical DPPH and 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). Diphenyl-2-picryl-hydrazyl (DPPH) is a stable free radical and DPPH 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 previously described(Ligeret et al., 2004) by adding DPPH to increasing concentrations of dipyridamole and measuring the decrease in absorption at 515 nm over time.

The measurement of Fe^{3+} 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 following conversion of Fe^{3+} to Fe^{2+} as previously described(Ligeret et al., 2004). Briefly, 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 Fe^{2+}-ferrozine complex formation monitored spectrophotometrically at 560 nm as a function of time. Control samples were run in parallel with DMSO and/or dipyridamole without $FeCl_3$ or ferrozine.

Platelet Isolation and Endothelial Cell Culture

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Blood from healthy human volunteers on no medications or vitamin supplements was drawn in a syringe containing 10% sodium citrate. The Boston Medical Center Ethics Committee on Human Research approved the study protocol and all patients gave their written, informed consent to participate.

Platelet rich plasma was prepared following centrifugation of blood (150xg). Washed platelets were prepared as previously described(Freedman et al., 1996a; Freedman et al., 1996b). Platelet pellets were resuspended in HEPES buffer (pH 7.4) for subsequent analysis. Platelet counts were determined in a Coulter Counter (model ZM, Coulter Electronics, Miami, Fl).

Bovine aortic endothelial cells (BAEC) were cultured in DMEM containing 10% FBS, 2.8 mM L-glutamine, 100 u/ml penicillin, and 100 µg/ml Streptomycin as previously described(Freedman et al., 1995).

Measurement of Reactive Oxygen Species

Confluent endothelial cells washed with fresh DMEM were incubated with DMEM pre-mixed with DCFDA (10 μM) with and without dipyridamole and incubated for 2 hours and fluorescence readings were taken immediately after adding fresh DMEM. Fluorescence was recorded in a microplate reader (Molecular Devices) over time following excitation at 485 nm and emission at 535 nm. To normalize fluorescence, cellular proteins were measured using the Biorad D_c protein assay kit (Biorad, 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 minutes at 37°C in Krebs buffer in

presence of 10µM CMDCFHDA (carboxymethyl derivative of DCFDA) along with DMSO (vehicle control) or 10µM dipyridamole. Cells were subsequently washed and then treated with 1u/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 DMEM and incubated with and without dipyridamole. Increasing concentrations of t-butylhydroperoxide were added and incubated for 2 hours 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). Following 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 (550nm excitation/570nm emission) in a fluorescent microplate reader. Cells were normalized by protein content as described above using the Biorad protein assay kit. Potential endothelial cell toxicity due to t-butylhydroperoxide was assessed using a lactate dehydrogenase (LDH) assay(Lautraite et al., 2003).

Measurement of Platelet Nitric Oxide Production and Aggregation

We adapted a NO-selective micro-electrode (Inter Medical Co., Ltd., Nagoya, Japan) for use in a standard platelet aggregometer (Payton Associates, Buffalo, N.Y.) in order to monitor platelet NO production and aggregation simultaneously, as previously described(Freedman et al., 1996a; Freedman et al., 1996b). Platelet NO production was rigorously quantified as the integrated area under the curve of the signal detected by the micro-electrode following platelet activation with 20 μM TRAP. As compared to 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, Ann Arbor, Michigan).

Measurement of Platelet Superoxide Production

Lucigenin-derived chemiluminescence was used to estimate aggregation-dependent superoxide production from stimulated platelets using a lumiaggregometer (whole blood lumiaggregometer, model 500-CA, Chronolog Corp., Haveltown, PA) as previously detailed(Freedman and Keaney, 1999). Washed platelets (4 x 10^5 platelets/μL), preincubated with lucigenin (bis-N-Methylacridinium Nitrate) (250 μM) in Hepes buffer for 3 minutes, were placed into the lumiaggregometer (aggregometer with luminescence detection). After stabilization of the signal, PMA (0.1 μM)-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).

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Platelet Generation of Hydrogen Peroxide

In situ generation of stimulation induced platelet hydrogen peroxide was carried out in presence of dipyridamole using a sensitive hydrogen peroxide detection kit (Amplex Red, Molecular Probes, Eugene,OR). Reactions were carried out in HEPES buffer with 20 μM dipyridamole. Amplex red reagent was added, readings were taken at 560nm excitation/590nm emission, and fluorescence was monitored as a function of time following addition of 0.1μM PMA. In separate experiments, catalase was utilized 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 minutes in presence of vehicle control or varying concentrations of dipyridamole. After TRAP stimulation for 3 minutes, the samples were immediately analyzed in a Fluorescence Activated Cell Sorter (Dako Cytomation). 10–50,000 platelet specific events 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. Briefly, freshly isolated platelets in HEPES buffer were incubated in presence of redox sensitive probe dihydrorhodamine (DHR, 10μM) for 10 minutes in presence and absence of 20μM dipyridamole. Confocal images were captured at different time points before and after TRAP (20μM) stimulation. Images were processed following laser background subtraction using NIH image software.

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Platelet Soluble CD40 Ligand Release

Washed human platelets ($2x10^8$/ml) were stimulated with thrombin (0.2 u/ml) for 2 hrs in the presence of varying concentration of dipyridamole at room temperature. Platelet supernatant was collected by centrifugation. The released sCD40L was measured by ELISA (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 paired *t* test. A statistically significant difference was assumed with a value of *P*<0.05. All data are expressed as the mean ± SEM.

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; the effect on the scavenging capacity of free radical DPPH and 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 (Figure 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 (Figure 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 if 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 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 Figure 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 increased at 1, 2, and 3 hours after washing (data not shown). Representative confocal images are shown in Figure 2b.

t-Butylhydroperoxide is used to generate oxidative stress in various biological systems(Lautraite et al., 2003). As shown in Figure 3a, the addition of t-butylhydroperoxide leads to enhanced oxidative stress and this effect is attenuated following 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 (Figure 3b). Alamar Blue is a redox sensitive dye, 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 incubated with t-butylhydroperoxide were tested for release of LDH. No significant toxicity was observed at all 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 if these effects are specific for the endothelial cells or also relevant in platelets, 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 as 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 (Figure 4a). This effect was noted over a range of dipyridamole concentrations (2-100 µM; data not shown).

To determine if dipyridamole alters 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 (Figure 4b) or hydrogen peroxide (data not shown) as measured by the Amplex Red Assay over a range of concentrations. Platelet

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generation of ROS following 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 (Figure 5a). Washed platelets were also incubated in presence of redox sensitive probe dihydrorhodamine (DHR, 10µM) for 10 minutes in presence and absence of 20µM dipyridamole. Confocal images (Figure 5b) were captured at different time points before and after TRAP (20µM) stimulation (displayed images were taken 10 minute after TRAP stimulation). Presence of dipyridamole attenuated TRAP activation induced platelet fluorescence induction (Figure 5b).

To determine if 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 hrs in presence of increasing concentrations of dipyridamole. Platelet supernatant was collected and the released sCD40L were measured by ELISA. As seen in Figure 6, incubation with dipyridamole leads to a modest but significant suppression of sCD40L release from activated platelets.

DISCUSSION

Platelet and vascular stimulation leads to release of ROS that are known to induce vasoconstriction, 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. Additionally, at therapeutically relevant concentration (3.5μM) (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 (≥20μM, 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 0.5-20μM dipyridamole, displayed a wide range of antioxidant/anti-inflammatory properties both in platelets and endothelial cells.

Data suggests 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 (Figure 4) would support the previous suggestions as well as our own findings (Figures 1a and 1b) that dipyridamole has a direct antioxidant effect.

Oxidant stress can lead to endothelial dysfunction and this, in turn, 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). While dipyridamole did not alter endogenous release of platelet derived NO(Figure 4b), the findings of our study are consistent with these previous observations(Aktas et al., 2003) as the dipyridamole-dependent enhancement of exogenous NO could be mediated through the suppression of platelet superoxide release (Figure 4a) and 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/inflammatory interactions that are known to be important in acute and chronic cardiovascular disease. Flow cytometry data generated in the AGATE 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 ($\geq 1\mu M$) 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 eNOS uncoupling contributing to the endothelial balance between NO and superoxide(Kalinowski and Malinski, 2004). Release of several ROS, including superoxide, hydroxyl radical, and H_2O_2 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 isoforms 1 and 2, cytochrome P450 epoxygenase isoform 2C9 (CYP2C9), xanthine oxidase (XO), uncoupled endothelial NO synthase (eNOS), and mitochondrial respiration contribute to the production of superoxide, H_2O_2 and hydroxyl radicals. Like endothelium-derived ROS, platelet-derived ROS potentially stem from enzymatic sources, including

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cyclooxygenase-1, xanthine oxidase, mitochondrial respiration, or uncoupled eNOS. The platelet isoform of NAD(P)H-oxidase has gained the most attention because it can be activated by 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 as dipyridamole induced inhibition of NADPH oxidase activity has been demonstrated in a diabetic animal model (Onozato et al., 2003).

A recent study demonstated that platelet NADPH oxidase subunit, gp91phox, regulates the expression of CD40L(Pignatelli et al., 2004). 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.

ACKNOWLEDGEMENTS

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Footnotes

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FIGURE LEGENDS

Figure 1a. The antioxidant property of dipyridamole effectively reduces the stable free radical DPPH. The reduction of DPPH (100 μM) was monitored in presence of increasing concentrations of dipyridamole. The absorption change at 515 nm is plotted for the 60 minutes data point (n=5; *$P<0.05$).

Figure 1b. The effect of dipyridamole on Fe^{3+} reduction using the FRAP assay. Reduction of ferric (Fe^{3+}) ions was monitored by ferrous-ferrozine complex formation. Increasing concentrations of dipyridamole were added to a solution containing $FeCl_3$ and ferrozine. Absorption at 560 nm is plotted as a function of incubation time (n=4).

Figure 2a. Dipyridamole decreases basal ROS generation in endothelial cells. Confluent BAECs were treated with increasing concentrations of dipyridamole and DCFDA for two hours and DCFDA fluorescence was measured immediately after washing (n=3; *$P<0.001$ vs. control) and two hours after washing.

Figure 2b. Presence of dipyridamole attenuates stimulation dependent endothelial reactive oxygen species generation. Representative confocal images of endothelial cells in presence of 10μM CMDCFDA after stimulation (1u/ml thrombin, 10min) in presence of either vehicle control or 10μM dipyridamole.

Images were captured in two photon confocal microscopy and processed using NIH image software and displayed with pseudo color assignment.

Figure 3a. Dipyridamole attenuates t-butylhydroperoxide-induced oxidative stress in endothelial cells. BAECs were incubated for 2 hours with t-butylhydroperoxide and DCFDA (10µM) in presence and absence of 5 µM dipyridamole.   DCFDA fluorescence was measured over time (0-2 hrs. with the T=0 time period shown; n=5; *P=0.007, ** P=0.001). Similar results were observed after 1 hr and 2 hr incubation (data not shown).

Figure 3b. Dipyridamole improves endothelial activity after exposing to t-butylhydroperoxide-induced oxidative stress. t-Butylhydroperoxide treated endothelial cells were evaluated for metabolic activity using the redox sensitive dye Alamar blue (10%). Dipyridamole attenuates the t-butylhydroperoxide-induced oxidative stress (550nm/570nm; n=5; *P<0.001 vs. control).

Figure 4a. The effect of dipyridamole on stimulation-dependent platelet superoxide release. Washed human platelets ($4x10^8$/ml) were incubated with either vehicle control (DMSO) or 20 µM dipyridamole for 10 minutes. Platelet aggregation induced superoxide release was measured in a lumiaggregometer where 0.1 µM PMA was used as agonist (n=5; *P<0.001).

<u>Figure 4b.</u> The effect of dipyridamole on platelet aggregation induced nitric oxide (NO) release. Washed human platelets were incubated with different concentrations of dipyridamole (DPD) or vehicle control for 5 minutes followed by measurement of TRAP (Thrombin Receptor Activation Peptide) induced NO release using a NO selective microelectrode (n=5; P=ns).

<u>Figure 5a</u>. Dipyridamole decreases stimulation induced platelet reactive oxygen species generation in a concentration dependent manner. By flow cytometry, there is an increased median platelet fluorescence following TRAP stimulation (20 µM). Presence of dipyridamole from 20 µM to 100 µM significantly attenuates this ROS generation (* $P<0.05$, n=3).

<u>Figure 5b</u>. Confocal images of platelets using redox sensitive probe dihydrorhodamine (DHR) demonstrate attenuation of reactive oxygen species generation by dipyridamole. Confocal images were captured after TRAP (20µM, 10min) stimulation in the presence or absence of dipyridamole (DPD, 20µM).

<u>Figure 6</u>. Dipyridamole attenuates sCD40L release from thrombin activated platelets. Washed human platelets (2×10^8/ml) in HEPES buffer were incubated for 2 hours at room temperature with 0.2 u/ml thrombin in presence of varying concentration of dipyridamole. Platelet supernatants were measured for sCD40L release (n=6, ^+P=0.007 vs. thrombin, * $P<0.001$ vs. thrombin).
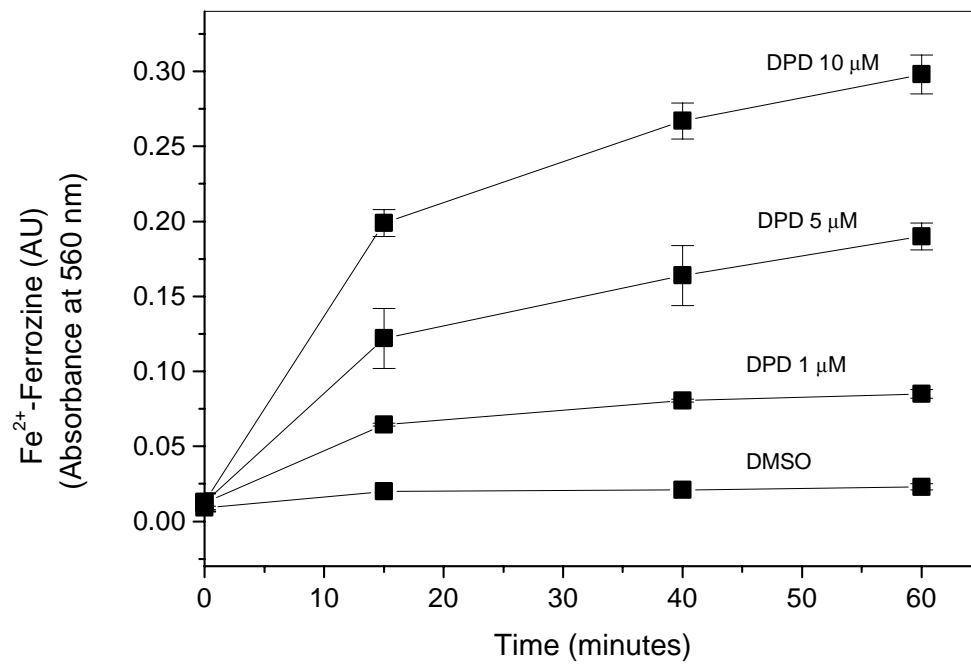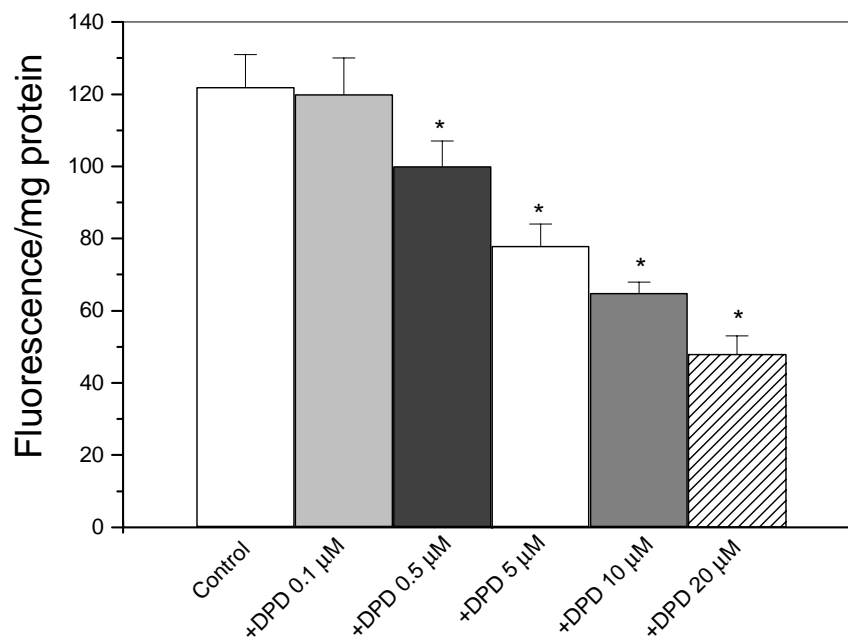
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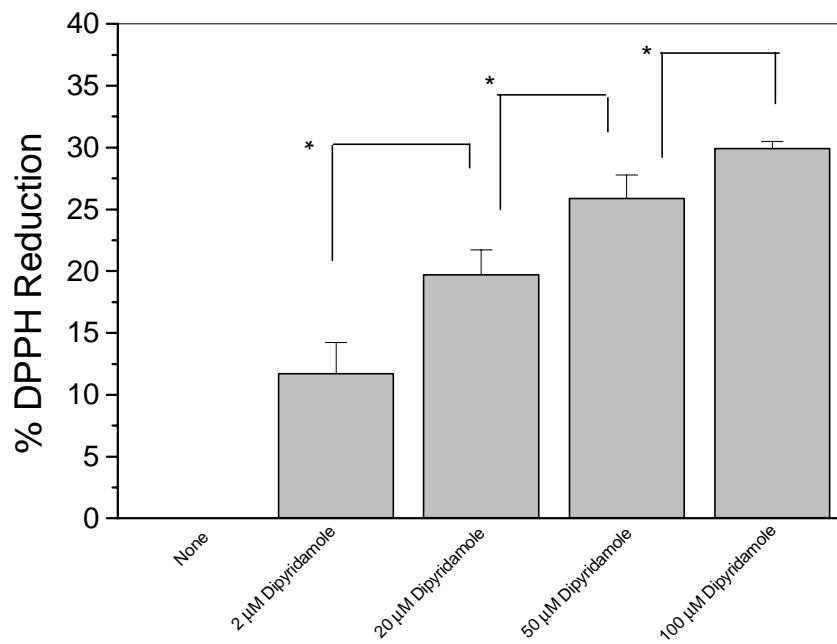
Figure 1a

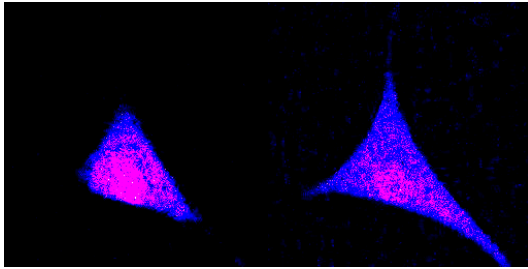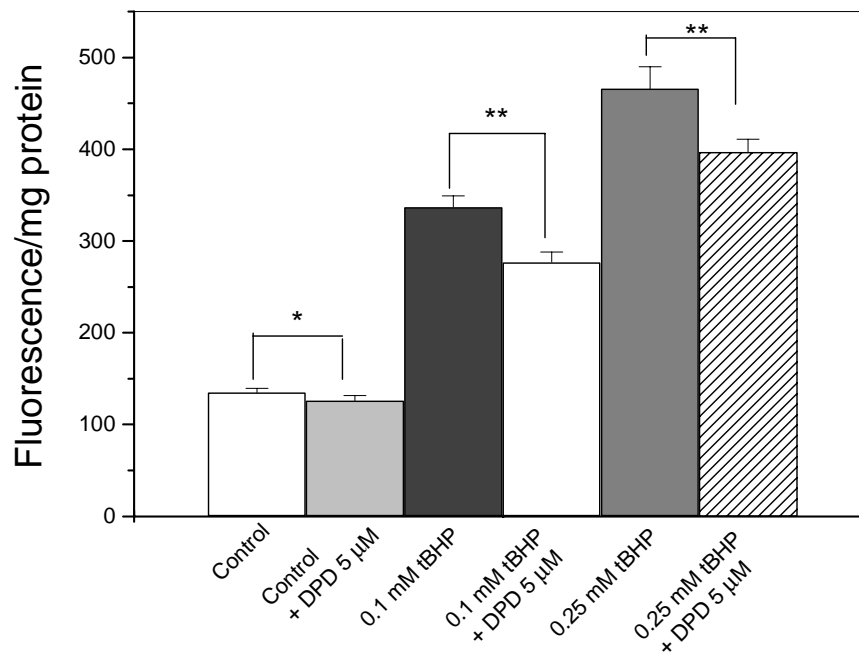Figure 1b

Figure 2a

Figure 2b
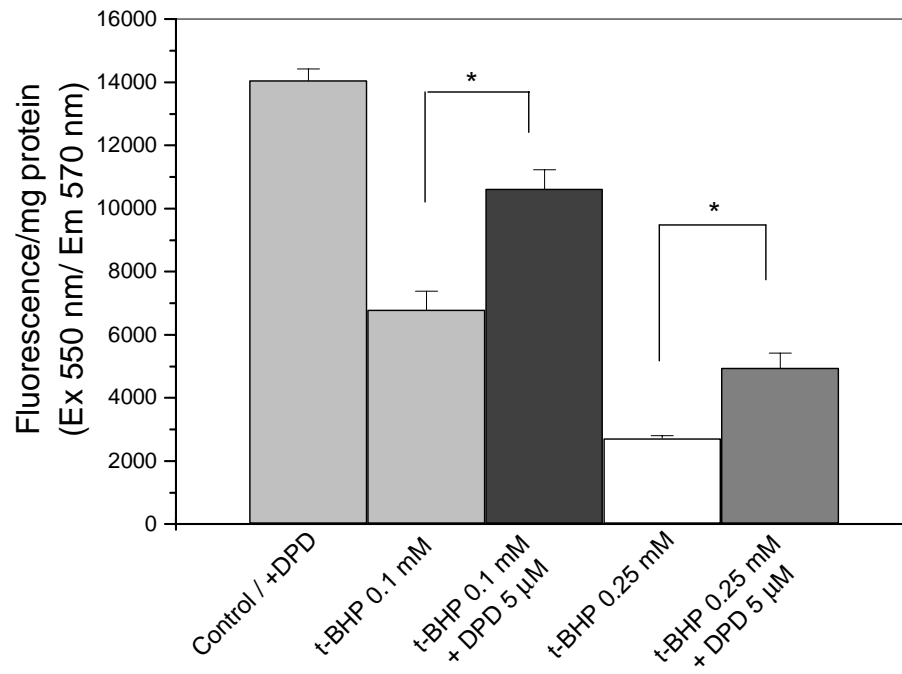
ECs+Thrombin   Thrombin+10µM DPD
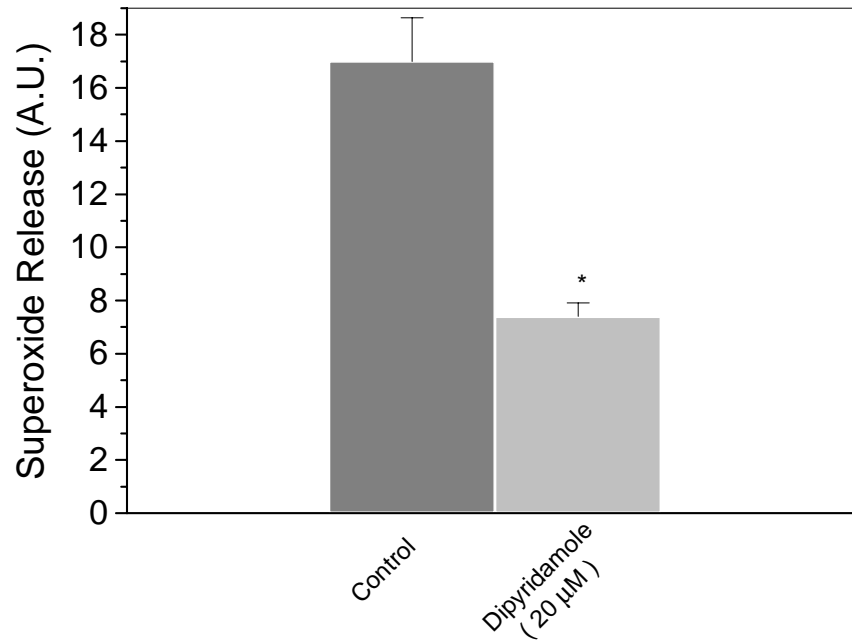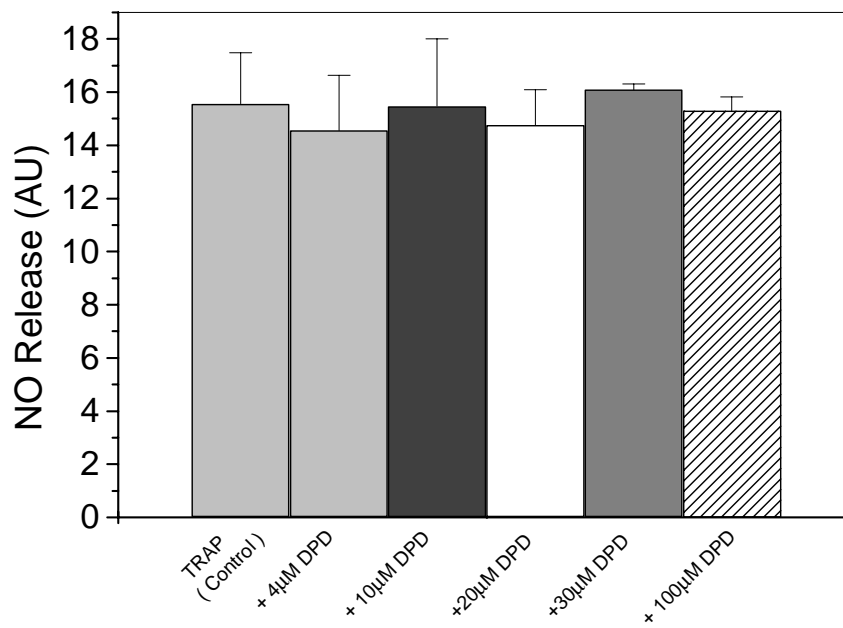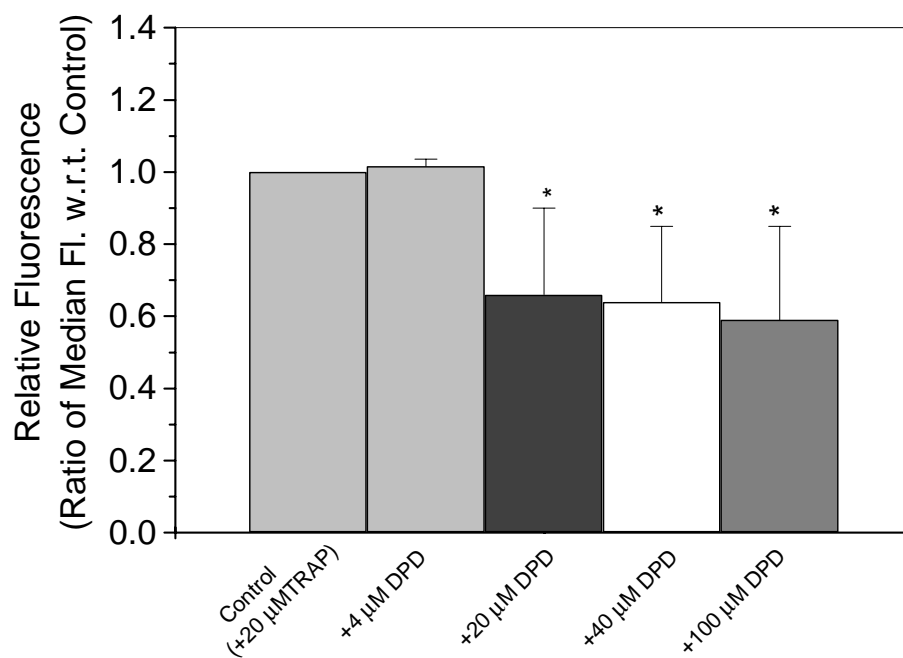
Figure 3a

Figure 3b

Figure 4a

Figure 4b

Figure 5a.

Figure 5b

Platelets+TRAP   TRAP + DPD
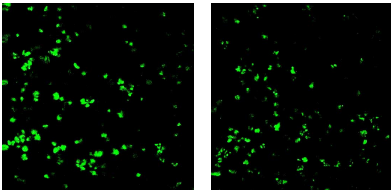
Figure 6