Free Rad. Res., Vol 34, pp. 461-466
Reprints available directly from the publisher
Photocopying permitted by license only

The Inhibition of Oxygen Radical Release from Human Neutrophils by Resting Platelets is Reversed by Administration of Acetylsalicylic Acid or Clopidogrel

CHRISTINA M. REINISCH[a], STEFAN DUNZENDORFER[a], CHRISTOPH PECHLANER[a], GIOVANNI RICEVUTI[b] and CHRISTIAN J. WIEDERMANN[a,*]

[a]*Medical Intensive Care Unit, Division of General Internal Medicine, Department of Medicine, University of Innsbruck, Anichstrasse 35, 6020 Innsbruck, Austria, and and* [b]*Division of Internal Medicine and Nephrology, Department of Internal Medicine and Therapeutics, University of Pavia, Viale Liberta 17, I-27100 Pavia, Italy*

Accepted for publication by Prof. A. Bast

(Received 4 February 2000; In revised form 21 July 2000)

Resting platelets inhibit oxygen radical release from neutrophils. Antiplatelet therapy may support this function by preventing platelet activation. Whether antiplatelet agents affect the antioxidative action of resting platelets in the absence of platelet activation is unknown. The effect of acetylsalicylic acid or clopidogrel administration on the antioxidative action of resting platelets was therefore studied in ten healthy volunteers. Preparations of resting platelets were obtained from 5 subjects each – before, during and after an eight-day course of daily treatment with 100 mg of acetylsalicylic acid or 75 mg of the thienopyridine clopidogrel. Human peripheral blood neutrophils were pretreated with the platelets at a ratio of 1/50 for 45 min; then formyl-Met-Leu-Phe-triggered oxygen radical release was measured fluorometrically. The inhibitory effect of platelets on oxygen radical release from neutrophils which was seen before treatment was abolished by antiplatelet therapy with either of the drugs, and inhibition was restored gradually after discontinuing acetlsalicylic acid / clopidogrel intake. Results suggest that the protective role of resting platelets in controlling oxygen radical release from neutrophils in the absence of platelet activation may be impaired by antiplatelet therapy.

Keywords: Oxygen radicals, Neutrophils, Platelets, Interaction, Aspirin, Clopidogrel, Primary prevention

INTRODUCTION

Resting platelets limit the release of oxygen radicals from chemoattractant-stimulated neutrophils[1]. This mechanism may contribute to the prevention of excessive damage to host tissues in the vasculature. On the other hand, upon activation, platelets stimulate neutrophils and monocytes to release an increased amount of superoxide anion[2]. As normally circulating platelets are resting and can be activated by haemostatic as well as proinflammatory stimuli like

* Correspondence: Professor Christian J. Wiedermann, MD, Department of Internal Medicine, University of Innsbruck, Anichstrasse 35, 6020 Innsbruck, Austria. Phone: +43/512/504–4180, Fax: +43/512/504–674180, E-Mail: Christian.Wiedermann@uibk.ac.at

ADP or thrombin and interleukin-1 or interferon-γ, respectivly, they may participate in the regulation of oxygen radical release from neutrophils in contrary ways depending on the state of platelet activation. Antiplatelet drugs act in an antithrombotic and anti-inflammatory fashion by preventing or diminishing platelet activation [3]. Whether antiplatelet therapy affects functions of resting platelets is currently unknown.

Because of the increasing use of antiplatelet drugs in primary prevention of cardiovascular disorders and certain types of cancers[4], we wondered if antiplatelet therapy influences the capacity of platelets to inhibit oxygen radical release from neutrophils. In the present in vivo study, the effect of acetylsalicylic acid (ASA) or clopidogrel on the inhibitory action of resting platelets on neutrophils was therefore tested.

MATERIALS AND METHODS

Study protocol

Study subjects were ten healthy volunteer physicians: 7 men, age 33 years (25–45) [mean (range)]; 3 women, age 27 years (24–32). Five of them received 100 mg of ASA and the other five 75 mg of clopidogrel daily, for a total of 8 days. After an overnight fast, blood was collected for isolation of platelets from an antecubital vein immediately before start of treatment, on day 7 of treatment, and on 4 occasions thereafter (day 9, 12, 14 and 16) using a 10% citrate solution as anticoagulant.

Platelet preparation

As blood sampling demands the insertion of a needle into the blood vessel thereby causing a destruction of the endothelial wall of the blood vessel, the sampling procedure may activate platelets. Therefore, a standardized blood sampling procedure known to minimize platelet activation was used[5]. In addition, vascular endothelial growth factor (VEGF) levels in plasma that are low but significantly rise upon platelet activation[6] were measured in platelet-free plasma throughout the study and no changes were found (data not shown). This observation indicates that platelets that were finally tested for interaction with neutrophils were not activated by the sampling procedure. Preparation of platelets was performed as described[1]. Briefly, by slow centrifugation (200 × g, 10 min) erythrocytes and leucocytes were removed and the platelet rich-plasma was collected. The platelets were then pelleted by centrifugation of platelet-rich plasma. Before testing for interaction with human neutrophils, platelet preparations of each of the two treatment groups were pooled.

Neutrophil isolation

Neutrophils were obtained from the peripheral blood of healthy volunteers (anticoagulated with 1.6 mg EDTA/ml blood) after discontinuous density gradient centrifugation on Percoll by dextran sedimentation and centrifugation through a layer of Ficoll-Hypaque, followed by hypotonic lysis of contaminating erythrocytes using sodium chloride solutions as described [7].

Respiratory burst of neutrophils

Neutrophil respiratory burst activity was measured by an assay using 2′,7′-dichlorofluorescin diacetate (DCFH-DA; Molecular Probes, Eugene, OR, USA). This assay is based on the oxidation of non-fluorescent DCFH-DA to highly fluorescent 2′,7′-dichlorofluorescein (DCF) both intracellularly and extracellularly, and has been previously validated for the quantification of superoxide anion radicals[8,9]. Neutrophils ($8x10^6$ cells/ml) were incubated without or with platelets at a ratio 1/50 for 45 min in a humidified incubator (95% air – 5% CO_2, 37°C). After washing, 100µl/well (96-well plate; Falcon® 3072) of $2x10^5$ PMNL were immersed in a $1x10^5$mol/L solution of DCFH-DA in phenol-red-free HBSS without Ca^{2+} and Mg^{2+}

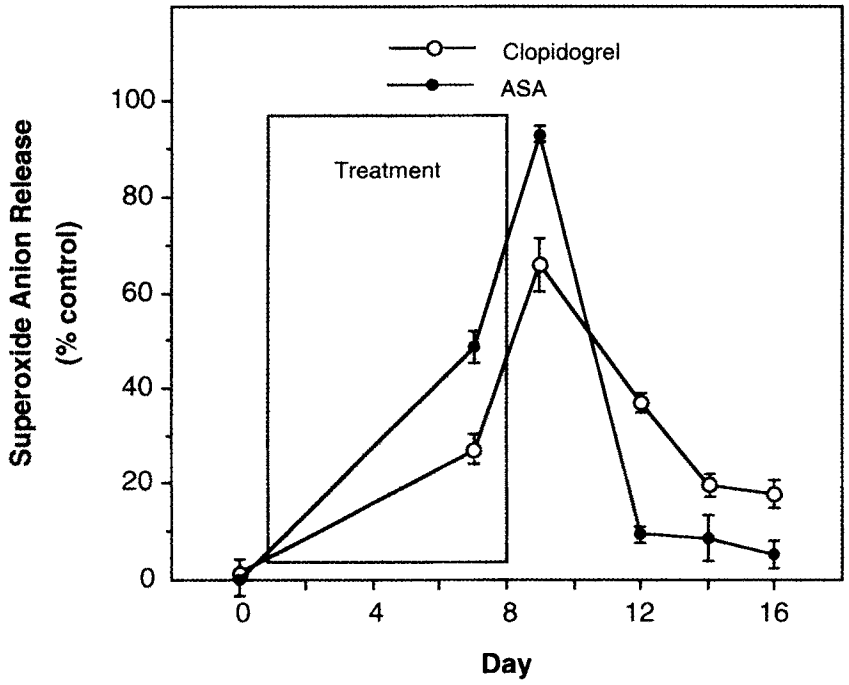

FIGURE 1 Inhibition of 1 μM fMet-Leu-Phe-triggered release of oxygen radicals from human neutrophils by resting platelets. Platelets were obtained from two treatment groups of 5 healthy subjects each before, during and after administration of ASA [100mg/d] or clopidogrel [75mg/d] and pooled for fluorimetric analysis of their effects on neutrophil respiratory burst activity. For 45 min at 37°C, neutrophils were pretreated with resting platelets from ASA- or clopidogrel-treated subjects at a ratio of 1/50, followed by washing. Then respiratory burst of neutrophils was triggered and oxygen free radical release measured after 10 min. Results are the mean ± SEM percentage levels of normalized fluorescence arbitrary units (FAU) obtained from experiments performed in replicates of eight. In the absence of resting platelets, fMet-Leu-Phe-triggered oxygen radical release (100 %) was 265 ± 50 FAU (mean ± SEM; n = 10). Shaded area represents the treatment period

(Gibco BRL Life Technologies, Vienna, Austria) containing 1 μmol/L of fMLP, as a triggering agent. The plates were covered with lids and placed in a humidified incubator at 37°C (5% CO_2) for 10 minutes as described [7]. Fluorescence activity was determined at 485/20 nm excitation and 530/25 nm emission wavelengths using the CytoFluor® Multi-Well Plate Reader Series 4000 (PerSeptive Biosystems, Inc., Framingham, MA, USA).

Statistics

The StatView software package (Abacus, Berkeley, CA) was used to calculate statistics including non-parametric multiple group comparison for independent variables (Kruskal Walis test).

RESULTS AND DISCUSSION

As shown in figure 1, pretreatment of neutrophils with resting platelets inhibited f-Met-Leu-Phe-triggered respiratory burst activity as compared to neutrophils which were preincubated with the medium only in the absence of platelets. Under treatment with antiplatelet agents, platelets lost their inhibitory action and oxygen free radical release from neutrophils could be observed again after treatment with

ASA or clopidogrel in 93% and 66% of controls, respectively. Eight days after the end of treatment, as newly formed platelets entered circulation and the drug-treated pool of platelets gradually disappeared, by interaction with platelets oxygen radical release from chemoattractant-stimulated neutrophils decreased again. Treatment with the antiplatelet drugs ASA or clopidogrel thus abrogates the inhibitory effect of platelets on the neutrophil oxygen radical release.

The effect of antiplatelet treatment may not only be due to alterations of platelet/neutrophil interactions but also be related to direct action of the antiplatelet drugs or their metabolites present in plasma on neutrophils. We have, therefore, tested the effects on formylpeptide-triggered respiratory burst of pretreatment of neutrophils with platelet-free plasma that was obtained before, during and after administration of ASA or clopidogrel. Pretreatment for 10 min of neutrophils with normal plasma, followed by washing, reduces respiratory burst activity in a concentration-dependent manner, with maximum inhibition reached at plasma concentrations of 10 percent or higher (Fig. 2). Oxygen free radical production of formylpeptide-triggered neutrophils after pretretment with 10% plasma from study subjects before treatment, however, was not significantly altered by treatment with ASA (n=5) or clopidogral (n=5) (Fig. 3). Data show that with this experimental design ASA and clopidogrel are not active on neutrophils as such. Also, possible disturbance by the antiplatelet drugs of the fluorimetric assay of oxygen free radical production appears unlikely.

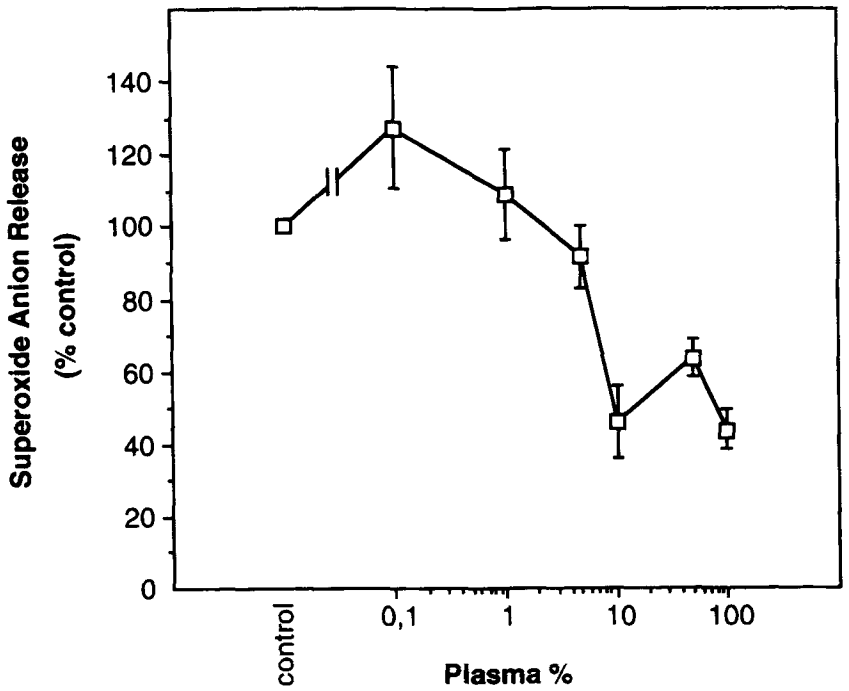

FIGURE 2 Inhibition of 1 μM fMet-Leu-Phe-triggered release of oxygen radicals from human neutrophils by normal human plasma. For 45 min at 37°C, neutrophils were pretreated with plasma, followed by washing. Then respiratory burst of neutrophils was triggered and oxygen free radical release measured after 10 min. Results are the mean ± SEM (n=3) percentage levels of normalized fluorescence arbitrary units (FAU). In the absence of plasma, mean fMet-Leu-Phe-triggered oxygen radical release (100 %) was 722 FAU (n = 3). P-trend, < 0.05

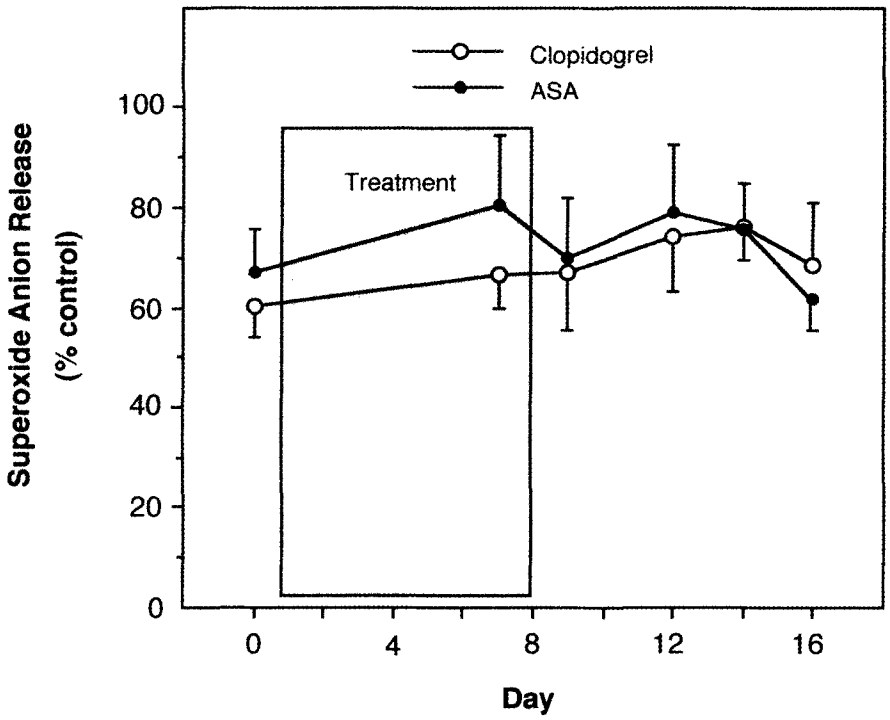

FIGURE 3 Inhibition of 1 μM fMet-Leu-Phe-triggered release of oxygen free radicals from neutrophils by plasma samples that were obtained from two treatment groups of 5 healthy subjects each, before, during and after administration of ASA [100mg/d] or clopidogrel [75mg/d] and tested for their effects on neutrophil respiratory burst activity. For 45 min at 37°C, neutrophils were pretreated with 10% plasma, followed by washing. Then respiratory burst of neutrophils was triggered and oxygen free radical release measured after 10 min. Results are the mean ± SEM percentage levels of normalized fluorescence arbitrary units (FAU) (n=5, each). In the absence of plasma, fMet-Leu-Phe-triggered oxygen radical release (100 %) was 323 ± 109 FAU and 226 ± 64 (mean ± SEM; n = 5) for clopidogral and ASA, respectively. Shaded area represents the treatment period. P-trend, >0.05, each

Mechanisms that may be involved in the observed effect of ASA- or clopidogrel-treated, resting platelets remain speculative. Previously, the inhibitory action of resting platelets on neutrophil respiratory burst was suggested to be associated with an increased generation of adenosine which activates an autoregulatory inhibitory pathway [1]. The recent observation that adenosine A receptor activation, through a mechanism involving a cyclic AMP-protein kinase A signalling pathway, inhibited the fMLP-triggered respiratory burst suggests that adenosine may act as an inflammatory agent modulating the respiratory burst during the different phases in neutrophil activation [10].

Adenosine is an endogenous nucleoside released from metabolically active cells and generated extracellularly via the degradation of released ATP and ADP [11]. Neutrophils degrade ATP and ADP released by platelets, in part by ADPase on the leukocyte membrane [12]. Thus, inhibition of spontaneous ATP and ADP release from platelets by antiplatelet agents [3] may have abrogated the inhibitory effect of resting platelets on neutrophil respiratory burst as is reported in the present study.

Our observations suggest that treatment with antiplatelet drugs may affect antioxidative mechanisms [3]. As the inhibition of premature oxygen radical release from neutrophils by resting platelets may be physiologically protective [1], its abrogation by ASA or clopidogrel may be disadvantageous. In particular, in primary prevention, with most of the circulating platelets presumably resting, antiplatelet medication may increase the oxygen radical burden to blood elements and vascular tissues.

In the light of the controversial data available on use of antiplatelet drugs in primary prevention[13], our observation uncovers a novel unwanted side effect of antiplatelet therapy. The use of antiplatelet drugs in primary prevention should therefore be based on the clinical picture until it is possible to clearly assess the balance of benefits and risks.

References

[1] T. Bengtsson, S. Zalavary, O. Stendahl and M. Grenegard (1996) Release of oxygen metabolites from chemoattractant-stimulated neutrophils is inhibited by resting platelets: Role of extracellular adenosine and actin polymerization. *Blood*, 87, 4411–4423.

[2] K. Nagata, T. Tsuji, N. Todoroki, Y. Katagiri, K. Tanoue, H. Yamazaki, N. Hanai and T. Irimura (1993) Activated platelets induce superoxide anion release by monocytes and neutrophils through p-selectin (CD62). *Journal of Immunology*, 151, 3267–3273.

[3] K. Schrör (1995) Antiplatelet drugs: a comparative review. *Drugs*, 50, 7–28.

[4] D.T. Lush (1999 Preventing heart disease and cancer. What randomized, primary-prevention studies show. *Postgraduate Medicine*, 106, 143–148.

[5] K.H. Grotemeyer (1991) The platelet-reactivity-test--a useful "by-product" of the blood-sampling procedure? *Thrombosis Research*, 61, 423–431.

[6] J.P. Maloney, C.C. Silliman, D.R. Ambruso, J. Wang, R.M. Tuder and N.F. Voelkel (1998) In vitro release of vascular endothelial growth factor during platelet aggregation. *American Journal of Physiology*, 275, H1054-H1061.

[7] S. Dunzendorfer, P. Schratzberger, N. Reinisch, C.M. Kähler and C.J. Wiedermann (1997) Effects of thalidomide on neutrophil respiratory burst, chemotaxis and transmigration of cytokine- and endotoxin-activated endothelium. *Naunyn Schmiedebergs Archives of Pharmacology*, 356, 529–535.

[8] A.R. Rosenkranz, S. Schmaldienst, K.M. Stuhlmeier, W. Chen, W. Knapp and G.J. Zlabinger (1992) A microplate assay for the detection of oxidative products using 2′,7′-dichlorofluorescin-diacetate. *Journal of Immunological Methods*, 156, 39–45.

[9] C.P. Wan, E. Myung and B.H. Lau (1992) An automated micro-fluorometric assay for monitoring oxidative burst activity of phagocytes. *Journal of Immunological Methods*, 159, 131–138.

[10] S. Zalavary and T. Bengtsson (1998) Modulation of the chemotactic peptide- and immunoglobulin G-triggered respiratory burst in human neutrophils by exogenous and endogenous adenosine. *European Journal of Pharmacology* 354, 215–225.

[11] B.B. Fredholm (1997) Purines and neutrophil leukocytes. *General Pharmacology*, 28, 345–350.

[12] Y. Zhang, J. Palmblad and B.B. Fredholm (1996) Biphasic effect of ATP on neutrophil functions mediated by P2U and adenosine A2A receptors. *Biochemical Pharmacology*, 51, 957–965.

[13] C.H. Hennekens (1999) Update on aspirin in the treatment and prevention of cardiovascular disease. *American Heart Journal*, 137, S9-S13.