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Studies on the biological effects of ozone: 10. Release of factors from ozonated human platelets

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In a previous work we have shown that heparin, in the presence of ozone (O_3), promotes a dose-dependent platelet aggregation, while after Ca^{2+} chelation with citrate, platelet aggregation is almost negligible. These results led us to think that aggregation may enhance the release of platelet components. We have here shown that indeed significantly higher amount of platelet-derived growth factor (PDGF), transforming growth factor β1 (TGF-β1) and interleukin-8(IL-8) are released in a dose-dependent manner after ozonation of heparinised platelet-rich plasma samples. These findings may explain the enhanced healing of torpid ulcers in patients with chronic limb ischemia treated with O_3 autohaemoteraphy (O_3-AHT).

Key words: ozone, platelets, aggregation, growth factors, inteleukin-8

Introduction

Ozone (O_3) can promote platelet aggregation particularly when heparin is used as an anticoagulant[1]: this observation is not surprising in the light of previous results[2] showing the role of reactive oxygen species (ROS) in platelet activation. In contrast, Ca^{2+} chelation with citrate markedly inhibits aggregation.[1] Thus, the selection of the most appropriate anticoagulant becomes crucial when blood is intended to be used for autotransfusion (O_3-AHT) after being briefly exposed to a gas mixture composed of about 97% oxygen and 3% ozone.[3] It is known that platelets are a rich source of several growth factors such as platelet-derived growth factor (PDGF),[4] transforming growth factor β1 (TGF-β1),[5] eicosanoids and interleukins (IL).[6]

PDGF and TGFβ1 promote wound healing and if, in the course of O_3 AHT, the reinfused platelets increase their release, it can be envisaged how this complementary therapy, besides improving oxygenation of hypoxic tissues, enhances healing of torpid ulcers in chronic limb ischemia. For this reason we have now investigated whether the use of heparin or citrate added to blood, before ozonation, affects the release of platelet factors differently.

Materials and methods

Ozone generation and measurement

O_3 was generated from medical grade O_2 using electrical corona arc discharge in the last generation O_3 generator (Model Ozonosan PM100K, Hansler GmbH, Iffezheim, Germany) which allows the gas flow rate and O_3 concentration to be controlled in real time by photometric determination at 253.7 nm as recommended by the Standardisation Committee of the International O_3 Association.

Reagents

Anticoagulants were either heparin (calcium salt, 30 IU/ml blood) normally used for therapeutic purposes (Calciparina, Italfarmaco) or ACD (Citric acid, Na citrate, Glucose) (Haemonetics, Braintree, USA). Adenosine diphosphate (ADP) was a product from Sigma Chemical Co. (St. Louis, Mo) and for the studying aggregation a 0.5 mM solution was freshly prepared.

Preparation of platelet rich plasma (PRP) samples

Both ACD and heparinised platelet rich plasma (PRP) were prepared from the same blood samples (60 ml) drawn, after informed consent, from five fasting (12 hours) non-smokers volunteers between the ages of 23 and 27 years, who were considered to be healthy and had not ingested platelet-active medication for at least two weeks.

Nine parts blood were anticoagulated with either one part ACD or with one part of saline containing heparin so that its final concentration was 30 IU/ml. Blood was centrifuged at 200 $\times g$ for 20 min and platelets were measured with a Coulter counter. An

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average platelet count of 3 × 10^8/ml plasma was used. A further centrifugation of PRP at 6000 × g for 15 sec gave a platelet-containing pellet and a supernatant platelet-free plasma used for biochemical determinations.

O_2 and O_3 delivery to biological samples

A predetermined volume of the O_2/O_3 gas mixture at three O_3 concentrations (20, 40 and 80 μg/ml per ml of PRP) was collected with a silicone coated disposable syringe and immediately introduced into a second syringe containing an equivalent volume of PRP via a 'y' connector. Final gas pressure remained at normal atmospheric pressure. In order to obtain reproducible results, it needs to be emphasised that O_3 is a very reactive gas so that extremely rapid and precise handling is required. The PRP samples were gently but continuously mixed with the gas for up to 30 sec and afterwards they were dispensed into test tubes for various analysis. Control samples were either untreated or mixed with an equal volume of O_2. After incubation each sample was immediately centrifuged at 10,000 × g for 20 min at 2°C and the supernatant platelet-free plasma was used for determining variations of thiobarbituric acid reactive substances (TBARS),[7] the total antioxidant status (TAS)[8] and of protein thiol groups (PTG) according to Hu.[9] An aliquot of the plasma samples was frozen at −70°C until determinations of several factors specified below were carried out.

Biochemical determinations

1. Thiobarbituric acid reactive substances (TBARS) determination: in order to evaluate the relevance of lipid peroxidation, TBARS were assessed according to Pompella *et al.*[7]
2. Total antioxidant status (TAS) in plasma samples was assessed according to Rice-Evans and Miller.[8]
3. Protein thiol groups (PTG) were measured in plasma according to Hu[9] using procedure 1 with 5,5′-Dithio-bis(2-Nitrobenzoic acid) DTNB dissolved in absolute methanol.

Immunoassay

Immunoassays of either human PDGF-AB or TGFβ1 (after activation of the latent TGFβ1 to the immunoreactive form) were carried out using Quantikine immunoassay kits produced by R&D System (Minneapolis, USA). On the basis of preliminary tests heparinised PRPs were diluted 1:20 while citrate PRPs were diluted 1:1 only. Possible release of thromboxane A2, a vasoconstrictor and aggregation-enhancer factor, was monitored by measuring the stable compound thromboxane B2 (TXB2) by using an immunoassay kit produced by R&D Systems. For this assay,

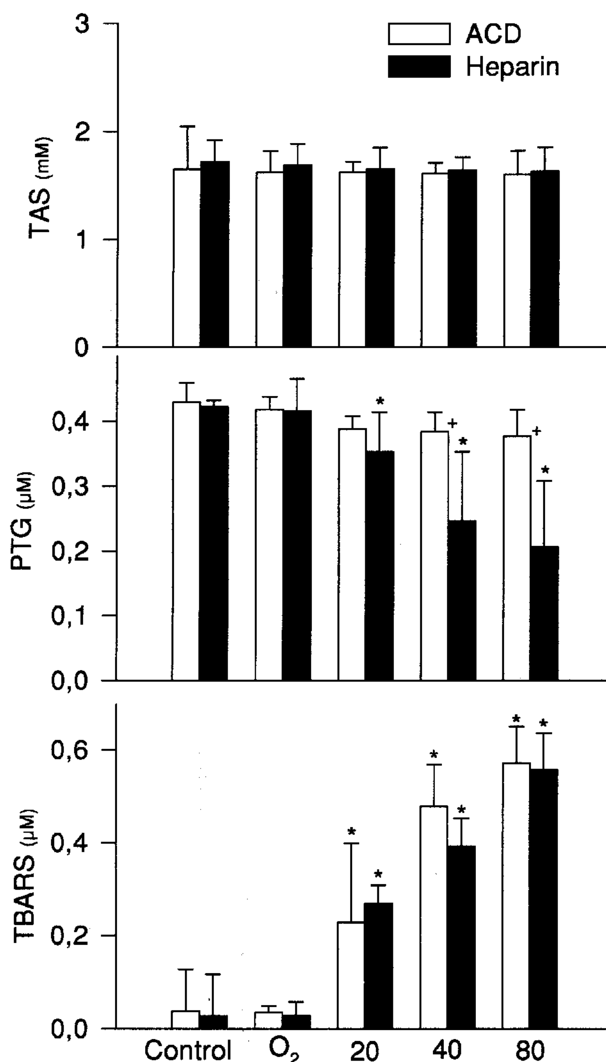

FIG. 1. Effect of 30 sec exposure of either O_2 or O_2–O_3 (20, 40 and 80 μg/ml per ml of plasma) on total antioxidant status (TAS), protein thiol group (PTG) and thiobarbituric acid reactive substances (TBARS) of the same human platelet rich plasma samples collected either in heparin or in ACD. The statistical significance has been indicated with a (*) for intergroup and with a (+) for intragroup analysis.

plasma samples were diluted 1:5. Immunoassay of IL-8 was carried out using Cytoscreen kit produced by Biosource International (Camarillo, CA, USA). Plasma samples were diluted 1:1 with the appropriate diluent. A 3-cycle automatic washing was routinely performed. Samples have been tested at least in duplicate against the appropriate standards.

Statistical analysis

Results obtained from five donors have been expressed as the mean ± the standard deviation of the mean (SD). A software package was used for data collection and statistical analysis (Statview SE, Abacus Concepts Inc., Berkeley, California). The significance of the differences between the means at different

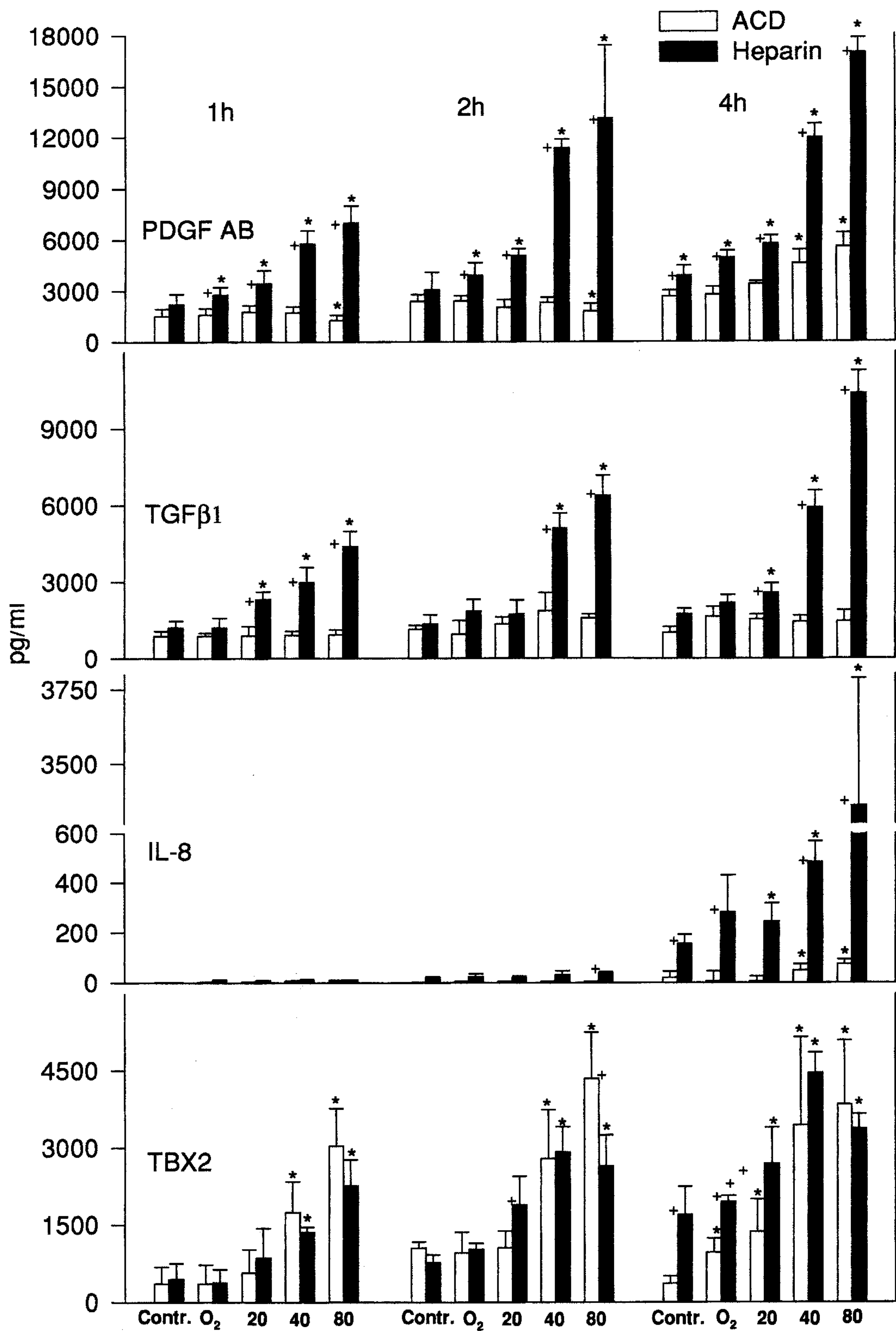

FIG. 2. Release of factors from human platelets during 1, 2 and 4 h incubation. The same PRP samples collected either in heparin or ACD were either not exposed (control), or exposed to O_2 alone, or O_2–O_3 at 20, 40 and 80 μg/ml concentration for 30 sec before incubation. The statistical significance has been indicated with a (*) for intergroup and with a (+) for intragroup analysis.

times in each group was analysed by one-way analysis of variance (ANOVA). The significance of the differences between means for the two groups at different times was analysed by Student's *t*-test. The level of statistical significance was set at $p < 0.05$ for both inter and intragroups analysis.

Results

Particularly PTG values decreased in approximate relation with the O_3 concentration while TBARS values increased several folds (Fig.1). Moreover we have observed that oxidation of PTG is far higher in heparinised that Ca^{2+} chelated samples suggesting that physiological Ca^{2+} levels favour ROS activity. Indeed intragroup analysis showed a significant difference at a medium (40 µg/ml) and at a high (80 µg/ml) O_3 concentration.

Fig.2 shows the strikingly and significant different release of PDGF AB, TGFβ1 and IL-8 from heparinised in comparison to Ca^{2+} chelated PRP samples. For the first two cytokines the difference is clear at all times, while for IL-8 it becomes evident only after 4 hours of incubation. As far as the release of TXB2 is concerned, heparin does not appear to have a determinant role and both anticoagulants have yielded significant differences after ozonation of PRPs.

Discussion

A modified form of AHT, by irradiating blood with ultraviolet light, was firstly proposed by Wehrli and Steinbart[10] but AHT became popular after Wolff[11] had shown that direct exposure of blood to a known dose of O_2–O_3 was very simple, practical and free of risk of contamination. Since that time, countless O_3-AHT sessions have been performed in Europe and in spite of a lack of double-blind, randomised studies, it seems that this approach can be useful in vascular diseases, particularly in chronic limb ischemia. Rokitansky *et al*[12] and Werkmeister[13] had shown that even at late stages (III and IV grade) of the disease, O_3-AHT, combined to topic application of ozone, can spare amputation and favour healing of torpid ulcers and necrotic areas. It is unfortunate that the results of these studies have been reported in a rather anecdotal form, so that during a revision [14–15] of this field, it was pointed out that not only it is urgent to perform controlled studies but to clearly understand mechanisms of action and explain why O_3-AHT enhances healing of ulcers.

A first important point that has never been clarified was which type of anti-coagulant: heparin or the usual sodium citrate would be more suitable. Indeed in a previous work[1], we have shown that heparin, in the presence of O_3, can promote platelet aggregation while, in contrast, Ca^{2+} chelation is practically ineffective. We then went to suspect that promotion of aggregation would favour the release of an array of intracellular components from platelets and we thought worth while to carry out a preliminary investigation.

We have now shown that two important healing factors, namely PDGF and TGFβ1, increase markedly during incubation particularly in heparinised PRP samples exposed to 40 and 80 µg/ml of O_3. If this happens in vivo, after reinfusion of ozonated blood in patients with chronic limb ischemia, it may indeed favour healing of necrotic ulcers. However, this assumption must be tempered by the previous finding[1] that platelet aggregation corresponds to either 20±6% or as much as 68±14% for O_3 concentrations of either 40 or 80 µg/ml, respectively. The former O_3 concentration still does allow an important release of growth factors with no risk of blood coagulation and therefore may represent the optimal O_3 concentration.

The fairly late release of IL-8 has been interpreted as due to the time lag necessary for the synthesis. It is known that induction of IL-8 by O_3, while is promoted by a temporary rise of H_2O_2[14–16] in cytoplasmic water via the activation of nuclear factor (NF)-κB, is inhibited by ROS scavengers.[17] As this chemokine is capable of initiating the chemotactic gradient that draws leukocytes from circulation into tissues, it may exert the additional role of favouring phagocytosis of bacteria and necrotic tissue present in torpid ulcers.

Release of TXB2, as the stable compound derived from thromboxane A2, appears as a drawback but we cannot draw a conclusion unless we carry out determination of other eicosanoids such as prostaglandin E2 and prostacyclin that induce vasodilation and inhibit aggregation. By using endothelial cells, work now in progress aims to clarify the role of O_3 activation of cyclooxygenase and nitric oxide syntethase.

On the basis of these results, we would like to evaluate comparatively the effectiveness of AHT in patients with chronic limb ischemia treated with either citrated or heparinised blood exposed to the mild O_3-AHT concentration of 40 µg/ml.

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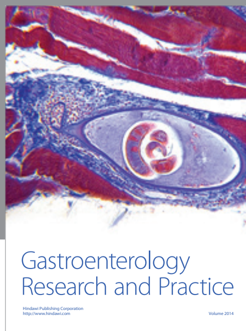

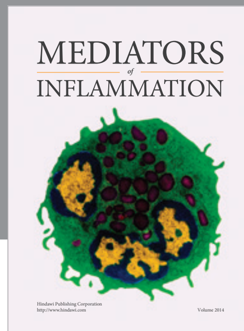

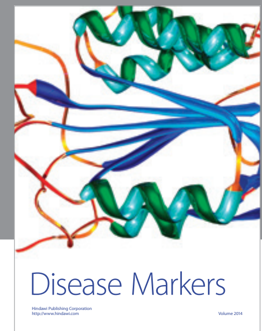

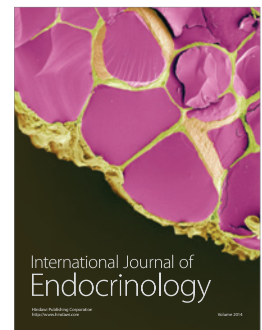

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