LIPOLYSIS GENERATES PLATELET DYSFUNCTION AFTER *IN VIVO* HEPARIN ADMINISTRATION

Short Title: Heparin, Lipolysis And Platelet Dysfunction

Elijah W. Muriithi, Philip R. Belcher, Stephen P. Day, Mubarak A. Chaudhry, *Muriel J. Caslake and David J. Wheatley.

*University of Glasgow, Departments of Cardiac Surgery and *Biochemistry, Royal Infirmary, Glasgow. G31 2ER. United Kingdom*

This Study Was Supported By The Royal College Of Surgeons Of England

KEYWORDS: Impedance Aggregometry, Lipase, Lipoprotein, Cardiac Surgery and Hirudin

Corresponding Author

Dr E.W. Muriithi

Current Address

Department Of Cardiothoracic Surgery

Royal Sussex County Hospital,

Eastern Road,

Brighton.

BN2 5BE

United Kingdom

Telephone +44 (0) 1273 696955 Bleep 8185

Facsimile +44 (0) 1273 664464

email: elijah.muriithi@brighton-healthcare.nhs.uk

LIPOLYSIS GENERATES PLATELET DYSFUNCTION AFTER *IN VIVO* HEPARIN ADMINISTRATION

ABSTRACT

Heparin, when administered to patients undergoing operations using cardiopulmonary bypass, induces plasma changes that gradually impair platelet macroaggregation, but *in vitro* heparinisation of whole blood does not. The plasma changes induced by *in vivo* heparin continue to progress in whole blood *ex vivo*. Heparin releases several endothelial proteins including lipoprotein lipase, hepatic lipase, platelet factor-4 and superoxide dismutase. These enzymes, which remain active in plasma *ex vivo*, may impair platelet macroaggregation after *in vivo* heparinisation and during cardiopulmonary bypass. In the current study proteins were added to hirudin-anticoagulated blood (200 U ml^{-1}) from healthy volunteers *in vitro* and whole blood impedance aggregometry assessed the platelet macroaggregatory responses to *ex vivo* stimulation with collagen (0.6 μg ml^{-1}). Over a 4 hour period human lipoprotein lipase and human hepatic lipase reduced the platelet macroaggregatory response from (mean ± 1SD) 17.0 ± 2.3 to 1.6 ± 1.3 and 1.2 ± 0.6 Ohms respectively (all $p<0.01$; n=6). Other lipoprotein lipases also impaired platelet macroaggregation but platelet factor-4 and superoxide dismutase did not. Platelet macroaggregation had an inverse linear correlation with plasma non-esterified fatty acid concentrations ($r^2=0.69$; two sided $p<0.0001$; n=8), suggesting that heparin-induced lipolysis inhibits platelet macroaggregation. Lipoprotein degradation products may cause this inhibition by interfering with eicosanoids and other lipid mediators of metabolism.

INTRODUCTION

Platelet dysfunction is a major contributor to the bleeding diathesis that increases transfusion requirements in up to 29% of patients undergoing operations using cardiopulmonary bypass [1]. The platelet function defect that develops during cardiopulmonary bypass is the inability to form large stable aggregates (macroaggregates) [2-4] while the formation of small aggregates (microaggregates) is not impaired [2-4].

While heparin has proaggregatory effects that augment platelet microaggregation [5,6], we and others have shown that heparin impairs platelet function before the start of extracorporeal circulation [4,7]. It has also been shown that while intravenous heparin markedly inhibits platelet macroaggregation, this inhibition is not reproduced by *in vitro* heparinisation of whole blood [4,8,9]. Platelet secretion of 5-hydroxytryptamine is similarly impaired by heparin *in vivo* but not *in vitro* [10]. These contrasting effects of heparin *in vivo* and *in vitro* suggest possible endothelial involvement in heparin-platelet interactions.

In earlier studies we showed that *in vivo* heparinisation gradually induced platelet dysfunction by causing plasma changes [9]; these plasma changes continued to develop *ex vivo*, after heparinisation *in vivo* [9]. We also observed that this action of heparin was not dose related when heparin doses of 30 u kg^{-1} or more were given[9]. When fully developed, the effect almost completely abolished platelet macroaggregation [9]. In contrast the plasma obtained from blood in which platelets were dysfunctional, after heparinisation

in vivo, inhibited platelet macroaggregation *in vitro*, without delay [9]. These observations also support the suggestion of previous workers that the platelet defect associated with cardiopulmonary bypass is extrinsic to the platelet [11] and suggest that intermediaries may act on plasma components to produce inhibitory substances, rather than inhibiting platelets directly. Micro- and macroaggregation in blood from unheparinised subjects was not affected by delay [12,13].

Heparin releases several endothelial proteins into the plasma [14], including lipoprotein lipase and hepatic lipase. These enzymes continue to hydrolyse plasma lipoproteins *ex vivo* after heparinisation *in vivo* [15,16]. Several products of lipoprotein hydrolysis inhibit platelet macroaggregation; these include free fatty acids (with unsaturated free fatty acids having a more pronounced effect) [17,18], Apolipoprotein E, [19], HDL [20] and lysophospholipids [21]. Other heparin-releasable endothelial proteins include platelet factor-4 and superoxide dismutase [14]. We hypothesised that heparin-induced lipolysis may cause platelet dysfunction and therefore investigated the effect of these proteins on platelet macroaggregation in whole blood.

METHODS

We carried out a series of four *in vitro* studies. In Study 1, the effects of human post-heparin lipases on platelet macroaggregation were studied in blood from 6 volunteers. In Study 2, the effects of lipoprotein lipase, extracted from bovine milk, on platelet macroaggregation was studied in blood from 12

volunteers. In Study 3 the effects of *Pseudomonas spp.* lipoprotein lipase on non-esterified fatty acid concentrations and platelet macroaggregation were studied in blood from 8 volunteers, while those of human post-heparin hepatic lipase were studied in in blood from 5 volunteers. In Study 4, the effects of human platelet factor-4 and of superoxide dismutase of bovine erythrocyte origin, on platelet macroaggregation, were studied in blood from 3 volunteers. All studies were carried out in accordance with the Declaration of Helsinki (1989) of the World Medical Association and with the approval of the Royal Infirmary of Glasgow Research Ethics Committee (Project No 97SC001 (896) on May 7th 1997). Informed consent was obtained from all participants.

Blood Sampling

Venous blood was taken, using a 19 G needle, from the antecubital fossa of healthy volunteers, who had not taken non-steroidal anti-inflammatory drugs or other antiplatelet medication for at least 7 days. Blood was anticoagulated with r-hirudin (200 U ml^{-1}) in siliconised glass containers, as previously described [13]. In earlier studies we demonstrated that both platelet aggregatory responses [12] and existent platelet microaggregates [13] are stable under these conditions, for up to 24 hours.

We elected to study platelet aggregation in hirudin-anticoagulated whole blood as hirudin maintains normocalcaemia and has no known direct action on platelets. Platelet aggregation has classically been studied in citrate-anticoagulated blood or platelet rich plasma. Chelating agents, such as EDTA and citrate, cause hypocalcaemia and therefore may not give a true picture of

platelet responses [22,23]. Hypocalcaemia favours thromboxane A_2 production and thromboxane A_2-dependent secretion during aggregation induced by weak agonists, such as ADP or adrenaline [24]. Hirudin specifically inhibits thrombin but has little or no effect on platelet activation induced by other agonists, such as ADP and collagen *in vitro* [25]; hirudin does not inhibit *in vivo* propagation of platelet rich thrombus [26]. For these reasons hirudin is suitable as an *in vitro* anticoagulant for studying platelet aggregation [27,28]. We have previously studied platelet dysfunction occurring during cardiopulmonary bypass using hirudin anticoagulation [2,4,9].

Protein Addition

Four heparin-releasable proteins were studied. Lipoprotein lipase and hepatic lipase (for reasons discussed in the introduction), superoxide dismutase because of its known enzymatic activity in blood, and platelet factor-4 because of its known association with the platelet. These proteins were added to achieve concentrations similar to those observed previously after *in vivo* heparinisation. The concentrations were: platelet factor-4 50 U ml^{-1} [29], superoxide dismutase 100 U ml^{-1} [30] and total lipase activity 100 U ml^{-1} [31]. The blood was then placed in a waterbath at 37^{o}C and aliquots were sampled at set time points for aggregometry and for non-esterified fatty acid assays.

Impedance aggregometry was performed on 0.5 ml aliquots of blood immediately after sampling. For assay of non-esterified fatty acids, in Study 3, 2.5 μg of paraoxon, a cholinesterase inhibitor that also has non-specific inhibitory effects on lipases [16], was added to 1 ml of the blood immediately

after sampling. These samples were then centrifuged at 3000 rpm and 4 ^{o}C for 15 minutes, and the supernatant plasma stored at -70 ^{o}C. The samples were analysed in bulk at the end of the studies.

Protein Sources

Human hepatic and lipoprotein lipases were extracted from post-heparin plasma, as described below. Superoxide dismutase and lipoprotein lipase enzymes, the former of bovine erythrocyte origin and the latter extracted from bovine milk or from *Pseudomonas spp.* cultures, were purchased from Sigma-Aldrich Company Ltd., Poole, UK. Human platelet factor 4 was kindly donated by the National Institute for Biological Standards and Control, London, UK. Our preliminary studies showed that the TRIS buffers, hypertonic saline and ammonium sulphate, that the bovine and human enzymes were preserved in, impaired platelet macroaggregation in some individuals. We therefore dialysed these enzymes against normal saline before adding them to blood.

Post-Heparin Lipase Extraction

Patients

Supplies of human post-heparin hepatic lipase and lipoprotein lipase for laboratory use were not commercially available, therefore 2 patients undergoing elective coronary artery bypass grafting using cardiopulmonary bypass were recruited for extraction of post-heparin lipases. Informed consent was obtained from these patients. Platelet function was not studied in these patients, their heparinised blood was solely used as a source of lipolytic enzymes. These patients were not on aspirin, other non-steroidal anti-

inflammatory drugs, platelet suppressants, steroids, warfarin, intravenous nitrates or heparin. Anaesthetic premedication was with temazepam, and induction by propofol. Anaesthesia was maintained with propofol infusion and opiates. Heparinisation was with porcine heparin (Leo Laboratories, Risborough, UK; 300 U kg^{-1}) given through a central venous cannula just before cannulation of the aorta. 50 ml of blood were sampled 10 minutes after heparinisation but before the start of extracorporeal circulation.

Lipase Extraction

Hepatic and lipoprotein lipases were extracted by a stepwise elution through a heparin sepharose column as previously described [32]. Briefly post-heparin blood was anticoagulated with r-hirudin 200 U ml^{-1} and placed on ice immediately after sampling. The separation process was carried out in refrigerated room at 4 ^{o}C. Half the heparin content, as estimated by a protamine titration, using the Hepcon® system (Medtronic Ltd, Watford, UK), was neutralised *ex vivo* with protamine. This neutralisation reduced the heparin concentration to levels closer to those in the original description of this extraction procedure [32], as the heparin in solution may compete with that in the column for binding of the lipases and reduce the efficiency of extraction. Plasma was separated after centrifugation at 3000 rpm for 15 minutes.

Heparin sepharose columns were equilibrated with 5 mM Sodium barbital, pH 7.4 containing 0.15 M of sodium chloride. Aliquots of 4 ml post-heparin plasma were diluted 1:1 with 5 mM sodium barbital buffer, pH 7.4, containing

0.45 M sodium chloride. The separation columns were loaded with 8 ml of diluted plasma. Elution through the columns was by washing with 8 ml fractions. The first fraction was eluted with 5 mM sodium barbital, pH 7.4 containing 0.3 M sodium chloride, the next two with 5 mM sodium barbital at pH7.4 with 0.72 M sodium chloride, and the final two with 5 mM sodium barbital at pH7.4 with 1.5 M sodium chloride.

The fractions were then dialysed against 3.8 M ammonium sulphate and then against 0.1 M phosphate buffer, pH 7.4. This method of extraction has a high yield of hepatic lipase in Fraction 3 and lipoprotein lipase in Fraction 5 [32].

Impedance Aggregometry in Whole Blood

An impedance aggregometer (Chronolog 500-VS, Chronolog Corporation, Havertown, PA, USA) measured electrical impedance changes in whole blood. Aliquots of 500 μl of whole blood were diluted with the same volume of saline (0.9%) in plastic cuvettes and equilibrated at 37 ^{o}C before measurement. The macroaggregatory response to 0.6 μg collagen (Hormon Chemie, Munich, Germany) was read as the scale deflection in centimetres at 5 minutes. The aggregometer was calibrated according to the manufacturer's instructions so that a 20 Ohm change in electrical impedance would give a deflection of 14 cm, giving a conversion factor of 1.43 Ohms per centimetre.

Collagen was chosen as the *in vitro* agonist, for aggregometry, because it is the principle agonist the platelet encounters in damaged vessel walls during primary hemostasis. Later, during secondary hemostasis, thrombin is also an

important platelet agonist. Furthermore platelet macroaggregation in response to low concentrations of collagen is largely the result of platelet secretion of thromboxane A2, and platelet release of ADP and serotonin. [33,34]; the involvement of these three other important endogenous platelet agonists makes the study of responses to low-dose collagen stimulation *in vitro* a useful global means of assessing platelet responses to physiologically relevant stimulation [2,4,9,12,34]. High concentrations of collagen can induce a full platelet aggregatory response independently of this autocrine positive feedback, however this may be the result of excessive and therefore probably non-physiological stimulation. To ensure that stimulation remained within a physiological range a collagen concentration of 0.6 μg ml^{-1} was used; we had previously determined that this was just below the minimum concentration that elicited a maximal macroaggregatory response [4].

Non-Esterified Fatty Acid Assays

Non-esterified fatty acid levels were serially determined as a measure of lipase enzyme activity. As most enzyme assays use the rate of release of breakdown products, under specific conditions, to generate an index of enzyme activity, the titres of these breakdown products may more accurately reflect enzyme activity. These assays were performed using NEFA C® test kits marketed by Wako Chemicals GMBH, Neuss, Germany. Briefly, this is a two reaction assay, performed on 96 well microtitre plates. The first reaction is the acylation of coenzyme A (CoA) by fatty acids in the presence of acyl-CoA synthetase. This was achieved by incubating 5 μl of plasma with 100 μl of Reagent A, which contains acyl-CoA synthetase, ascorbate oxidase, CoA,

ATP and 4-aminoantipyrine. In the second reaction acyl-CoA is oxidised by acyl-CoA oxidase to produce hydrogen peroxide, which in the presence of peroxidase causes oxidative condensation of 3-methyl-N-ethyl-(B-hydroxy-ethyl)-aniline with 4-aminoantipyrine to form a purple pigment. This was effected by, adding 200 μl of Colour Reagent B to the wells; this reagent contains acetyl-CoA oxidase and peroxidase. The optical density of the resultant solution was then read at 550 nm using a Dynatech MR 5000 platelet reader (Dynatech Corporation, Burlington, MA. USA). The non-esterified fatty acid concentrations were read directly from a calibration curve that was prepared using the standards that were supplied with the kit.

Materials

Heparin Sepharose CL-6B, purchased from Amersham Pharmacia Biotech AB, Uppsala, Sweden. Sodium barbital, Hydrochloric acid, and Ammonium sulphate, all purchased from BDH Laboratory Supplies, Poole, UK. Sodium di-hydrogen phosphate, Formachem (Research International) Ltd., Strathaven, UK. Di-sodium hydrogen phosphate, Fissons Scientific Equipment, Loughbrough, UK. Dialysis tubing, pore size 12 000-14 000 Daltons, Medicell International Ltd., London, UK. Paraoxon, Sigma-Aldrich Company Ltd., Poole, UK.

Statistical Analysis

Data are expressed as means ± 1 standard deviation. Comparisons were made using two level analyses of variance (anovar) with Bonferroni correction for multiple comparisons. Analyses were performed using Arcus Quickstat

Biomedical software (Addison Wesley Longman trading as Research Solutions, Cambridge, UK).

RESULTS

Study 1

Human Post-Heparin Hepatic Lipase

The human post-heparin hepatic lipase extract reduced the platelet macroaggregatory response to collagen 0.6 μg ml^{-1} from 17.0 ± 2.3 Ohms to 4.8 ± 3.7 Ohms after 2 hours and to 1.2 ± 0.6 Ohms after 4 hours ($p<0.0001$ and $p=0.01$ respectively; n=6). See Figure 1.

Human Post-Heparin Lipoprotein Lipase

Human post-heparin lipoprotein lipase extracts appeared to have a different timescale from hepatic lipase for reducing the platelet macroaggregatory response. The response fell from 17.0 ± 2.3 Ohms to 9.3 ± 5.2 Ohms after 2 hours and to 1.5 ± 1.3 Ohms after 4 hours (all $p<0.0001$; n=6). See Figure 1.

Study 2

Bovine Milk Lipoprotein Lipase

Lipoprotein lipase from bovine milk reduced the platelet macroaggregatory response less markedly than the human enzymes from 17.6 ± 3.1 Ohms to 12.8 ± 7.3 Ohms after 2 hours and to 5.8 ± 5.3 Ohms after 4 hours ($p=0.06$ and $p=0.02$ respectively; n=12). See Figure 2. The subjects studied appeared to comprise two distinct groups; a quick responding group and a slow responding group. See Figure 2. In the quick responders the platelet

macroaggregatory response fell from 16.4 ± 1.6 Ohms to 7.7 ± 6.6 Ohms over the first 2 hours and to 4.3 ± 3.4 Ohms over the next 2 hours (p=0.06 and p=0.02 respectively; n=6). However, in the slow responders the platelet macroaggregatory response of 18.8 ± 3.8 Ohms was essentially unchanged over the first 2 hours but then fell to 7.3 ± 6.7 Ohms over the next 2 hours (p=0.3 and p=0.05 respectively; n=6). See Figure 2.

Study 3

Pseudomonas Spp. Lipoprotein Lipase

Platelet Macroaggregation

This bacterial lipoprotein lipase inhibited platelet macroaggregation more rapidly than either the human or the bovine isoenzymes, reducing the response to 0.6 μg ml^{-1} collagen from 16.4 ± 3.3 Ohms to 1.3 ± 1.1 Ohms within an hour (p<0.0001; n=8). See Figure 3. The bacterial lipoprotein lipase which was supplied as a lyophilised powder and therefore did not require dialysis, appeared to be the most potent both in inducing lipolysis and inhibiting platelets. Dialysing out preservatives may have impaired the activity of the other enzymes studied.

Non-Esterified Fatty Acid Release

Non-esterified fatty acid generation was measured in the samples to which the Pseudomonas spp. lipoprotein lipase had been added. The release of non-esterified fatty acids was most marked in the first 15 minutes, increasing from 0.13 ± 0.33 mmol l^{-1} to 2.29 ± 0.86 mmol l^{-1} (p=0.004; n=8). The apparent increase over the last 15 minutes, from 3.26 ± 1.04 mmol l^{-1} to 3.59

± 1.04 mmol l^{-1}, failed to reach statistical significance (p=0.06; n=8) suggesting that an equilibrium level or maximal generation was approaching. See Figure 3. There was a significant inverse linear correlation between the non-esterified free fatty acid concentrations and the platelet macroaggregatory response (r^2=0.69; two sided p<0.0001; n=8). See Figure 3.

Human hepatic lipase

The effects of hepatic lipase on non-esterified fatty acid levels and platelet macroaggregation were also studied. In this additional group of volunteers human hepatic lipase reduced the platelet macroaggregatory response, less markedly than in the earlier subjects. The response fell from 13.7 ± 1.2 Ohms to 9.2 ± 1.5 Ohms after 2 hours and to 7.6 ± 2.1 Ohms after 4 hours (p=0.06 and p=0.2 respectively; n=5). See Figure 4. Over the same period the non-esterified fatty acid levels rose from 0.26 ± 0.08 mmol l^{-1} to 0.41 ± 0.01 mmol l^{-1} and then to 0.53 ± 0.08 mmol l^{-1} respectively (p=0.08 and p=0.2 respectively; n=5). See Figure 4. There was once again a significant inverse linear correlation between the non-esterified fatty acid levels and the platelet macroaggregatory response (r^2=0.81; two sided p=0.03; n=5). See Figure 4.

Study 4

Platelet Factor-4 and Superoxide Dismutase

Platelet factor-4 and superoxide dismutase did not affect platelet macroaggregation in whole blood from healthy volunteers *in vitro*, even after incubation at 37^{o}C for 3 hours. See Table 1. No synergistic actions of these

enzymes that inhibited platelet macroaggregation were detected, even in the presence of heparin. See Table 2.

DISCUSSION

Lipolytic enzymes are released from the endothelium into the plasma, by *in vivo* heparinization. These lipases and similar enzymes, derived from other sources, impaired platelet macroaggregation when added to whole blood *in vitro*. This inhibition was similar to that previously observed after *in vivo* heparinisation [9]. Platelet dysfunction correlated strongly with plasma non-esterified fatty acid levels suggesting that products of plasma lipoprotein hydrolysis impaired platelet macroaggregation; the correlation between plasma lipase activity and bleeding times previously observed in heparinised rabbits [35], supports this hypothesis. The other heparin-releasable proteins studied did not affect platelet macroaggregation.

Heparin And Lipid Homeostasis

Lipoprotein lipase is bound to the endothelial surfaces of arteries and capillaries by heparan sulphate. Hepatic lipase is similarly anchored on the luminal aspect of liver sinusoids. The location of these enzymes couples lipoprotein hydrolysis with the uptake of lipids and apolipoproteins. This helps keep plasma concentrations of these lipoprotein breakdown products low, favouring further lipoprotein degradation. Dislocation of these enzymes, by heparin, uncouples lipoprotein hydrolysis from product uptake, thus increasing plasma concentrations of lipoprotein breakdown products, like non-esterified fatty acids [31,36]. Accumulation of lipoprotein breakdown products inhibits

further lipid breakdown [37]; this inhibition may occur in both the extracellular and the intracellular compartments, as small non-polar molecules rapidly cross cell membrane lipid bilayers.

Mechanisms By Which Lipolysis May Affect Platelet Aggregation

Platelet agonists are classified as weak or strong, depending on their ability to stimulate platelet secretion and aggregation independently of autocrine positive feedback [38]. Thromboxane A2, an eicosanoid (lipid mediator derived from arachidonic acid) is a strong platelet agonist that is secreted during the autocrine positive feedback induced by weak agonists. The alterations in pasma lipids caused by lipolysis may interfere with the metabolism of thromboxane and its precursors in several ways:

Arachidonic acid is stored in cell membranes, it is the most abundant fatty acid in platelet membranes [38]. Integrin-controlled phospholipases [38] mobilise large quantities of arachidonic acid in response to stimulation; this occurs in two stages during platelet activation [39]. Arachidonic acid is obtained either from dietary sources or synthesised in the liver and transported to the tissues that utilise it, as a constituent of plasma lipoproteins, only trace quantities of it are found in the free form. Hepatic lipase and lipoprotein lipase release arachidonic acid from plasma lipoproteins [40]. Arachidonic acid released from plasma lipoproteins during intravascular lipolysis will cross cell membranes, thus bypassing the rate limiting (integrin-controlled) step in eicosanoid synthesis. Lipolysis also increases the concentrations of lysophospholipids and non-esterified fatty acids, which will

inhibit the hydrolysis of membrane phospholipids, by mechanisms previously described [37].

Lipolysis may therefore increase the basal or resting thromboxane production while retarding its generation in response to stimuli. Increased thromboxane production occurs after *in vivo* heparin administration [41,42]. Thromboxane concentrations correlated with free fatty acid concentrations in one of these studies [41]. The previously observed decreased secretion of thromboxane in shed blood after heparinisation [7] suggests reduced generation in response to stimulation.

Lipolysis may also interfere with platelet macroaggregation because other fatty acids normally present in lipoproteins, like eicosapentanoic acid, compete with arachidonic acid for enzyme binding sites [43,44]. Eicosanoid receptor responses may also be affected by metabolites of arachidonic acid [45] and other fatty acids [43,44].

Cardiopulmonary Bypass

During cardiopulmonary bypass plasma lipoprotein breakdown product concentrations rise markedly after heparinisation and remain elevated throughout the period of extracorporeal circulation [46,47]. Haemodilution, which occurs during cardiopulmonary bypass, may amplify the effects of lipolysis by increasing the amount of lipids that are aqueous (or free) as opposed to protein bound. Thus the metabolic effects of heparin-induced lipolysis may be more pronounced during cardiopulmonary bypass than they

are in other clinical situations.

Limitations Of Study

We did not specifically control for the effects of time on platelet macroaggregation, however in previous studies we noted that platelet macroaggregatory responses were preserved in hirudin-anticoagulated whole blood for up to 24 hours [12]. In hirudin-anticoagulated whole blood to which heparin was added *in vitro* these responses were stable for at least three hours [9]. The finding in the current study that platelet responses were stable over three hours after addition of heparin, platelet factor 4 and superoxide dismutase also suggests that deterioration in platelet responses was not caused by the delay or the enzyme vehicles.

We did not investigate specific lipoprotein breakdown products for inhibitory effects on platelet macroaggregation, however previous studies have already shown that several of these products inhibit platelets in a dose dependant manner [17,18,19,20]; it is therefore likely that multiple inhibition occurs simultaneously. The generation of other lipoprotein breakdown products is likely to correlate with that of non-esterified fatty acids and hence their levels would also correlate with platelet dysfunction.

We are uncertain as to why platelet macroaggregation deteriorated faster in blood from some individuals than others. This may be because they had fewer available lipid binding sites on their plasma proteins, or alternatively the faster response may be related to the constituents of their lipoproteins.

Conclusion

Heparin releases lipolytic enzymes from the endothelium, these enzymes hydrolyse plasma lipoproteins. Lipolysis in whole blood caused platelet dysfunction that correlated with the concentration of lipoprotein degradation products. This suggests that lipoprotein degradation products may cause the platelet dysfunction that occurs in heparinised subjects, including those undergoing operations using cardiopulmonary bypass.

ACKNOWLEDGEMENTS

Dr Karin Obermeier of Roussel Deutschland GmbH for kindly donating the hirudin used in these studies. Dr Elaine Gray and Dr Trevor Barrowcliffe of the National Institute for Biological Standards and Control, London, UK for their generous donation of Platelet Factor 4 concentrates. Prof C. J. Packard of the Department of Biochemistry, University of Glasgow, Royal Infirmary, Glasgow, for his advice and support. Dr Gillian M. Bernacca of the Department of Cardiac Surgery, University of Glasgow for assisting with the statistics.

REFERENCES

1. Belisle S. and Hardy J.F.. (1996) Hemorrhage and the use of blood products after adult cardiac operations: myths and realities. Ann. Thorac. Surg. **62**:1908-17.

2. Menys V.C., Belcher P.R., Noble M.I., *et al.*. (1994) Macroaggregation of platelets in plasma, as distinct from microaggregation in whole blood (and plasma), as determined using optical aggregometry and platelet counting respectively, is specifically impaired following cardiopulmonary bypass in man. Thromb. Haemost.**72**:511-8.

3. Kawahito K., Kobayashi E., Iwasa H., Misawa Y. and Fuse K.. (1999) Platelet aggregation during cardiopulmonary bypass evaluated by a laser light-scattering method. Ann. Thorac. Surg. **67**:79-84.

4. Belcher P.R., Muriithi E.W., Milne E.M., Wanikiat P., Wheatley D.J., and Armstrong R.A.. (2000) Heparin, Platelet Aggregation, Neutrophils And Cardiopulmonary Bypass. Thromb. Res. **98**:249-56.

5. Muriithi E.W., Belcher P.R., Rao J.N., Chaudhry M.A., Nicol D., and Wheatley D.J.. (2000) The effects of heparin and extracorporeal circulation on platelet counts and platelet microaggregation during cardiopulmonary bypass. J. Thorac. Cardiovasc. Surg.**120**:538-43.

6. Thomson C., Forbes C.D. and Prentice C.R.. (1973) The potentiation of platelet aggregation and adhesion by heparin in vitro and in vivo. Clin. Sci. Mol. Med. **45**:485-94.

7. Khuri S.F., Valeri R., Loscalzo J.*, et al.*. (1995) Heparin causes platelet dysfunction and induces fibrinolysis before cardiopulmonary bypass. Ann. Thorac. Surg. **60**:1008-14.

8. Besterman E.M. and Gillett M.P.. (1973) Heparin effects on plasma lysolecithin formation and platelet aggregation. Atherosclerosis **17**:503-13.

9. Muriithi E.W., Belcher P.R., Day S.P., Menys V.C., and Wheatley D.J.. (2000) Heparin induced platelet dysfunction and cardiopulmonary bypass. Ann. Thorac. Surg. **69**:1827-32.

10. Heiden D., Mielke C.H.J. and Rodvien R.. (1977) Impairment by heparin of primary haemostasis and platelet [14C]5- hydroxytryptamine release. Br. J. Haematol. **36**:427-36.

11. Kestin A.S., Valeri C.R., Khuri S.F., *et al.*. (1993) The platelet function defect of cardiopulmonary bypass. Blood **82**:107-17.

12. Belcher P.R., Muriithi E.W., Day S.P., and Wheatley D.J.. (2001) Platelet Aggregatory Responses To Low Dose Collagen Are Maintained In

Hirudin-Anticoagulated Whole Blood For 24 Hours When Stored At Room Temperature. Platelets **12**: 34-36

13. Muriithi E.W., Belcher P.R., Menys V.C., *et al.*. (2000) Quantitative detection of platelet aggregates in whole blood without fixation. Platelets **11**:33-7.

14. Novotny W.F., Maffi T., Mehta R.L. and Milner P.G.. (1993) Identification of novel heparin-releasable proteins, as well as the cytokines midkine and pleiotrophin, in human postheparin plasma. Arterioscler. Thromb. **13**:1798-805.

15. Pearce G.A. and Brown K.F.. (1983) Heat inhibition of in vitro lipolysis and 14C ibuprofen protein binding in plasma from heparinized uraemic subjects. Life. Sci.. **33**:1457-66.

16. Zambon A., Hashimoto S.I. and Brunzell J.D.. Analysis of techniques to obtain plasma for measurement of levels of free fatty acids. J. Lipid. Res. **34**:1021-8.

17. MacIntyre D.E., Hoover R.L., Smith M., *et al.*. (1984) Inhibition of platelet function by cis-unsaturated fatty acids. Blood **63**:848-57.

18. Siafaka-Kapadai A. and Hanahan D.. (1993) An endogenous inhibitor of PAF-induced platelet aggregation, isolated from rat liver, has been identified as free fatty acid. Biochim. Biophys. Acta. **1166**:217-21.

19. Riddell D.R., Graham A. and Owen J.S.. (1997) Apolipoprotein E inhibits platelet aggregation through the L- arginine:nitric oxide pathway. Implications for vascular disease. J. Biol. Chem. **272**:89-95.

20. Desai K., Bruckdorfer K.R., Hutton R.A. and Owen J.S.. (1989) Binding of apoE-rich high density lipoprotein particles by saturable sites on human blood platelets inhibits agonist-induced platelet aggregation. J. Lipid. Res.**30**:831-40.

21. Besterman E.M. and Gillett M.P.. (1971) Inhibition of platelet aggregation by lysolecithin. Atherosclerosis **14**:323-30.

22. Glusa E.. (1991) Platelet aggregation in recombinant-hirudin-anticoagulated blood. Haemostasis **21 Suppl 1**:116-20.

23. Lages. B. and Weiss H.J.. (1981) Dependence of human platelet functional responses on divalent cations: aggregation and secretion in heparin- and hirudin-anticoagulated platelet-rich plasma and the effects of chelating agents. Thromb. Haemost. **45**:173-9.

24. Packham M.A., Bryant N.L., Guccione M.A., Kinlough-Rathbone R.L. and Mustard J.F.. (1989) Effect of the concentration of Ca2+ in the suspending medium on the responses of human and rabbit platelets to aggregating agents. Thromb. Haemost. **62**:968-76.

25. Kirchmaier C.M., Bellinger O.K., Schulz B. and Breddin H.K.. (1991) Effect of recombinant hirudin on cytosolic Ca^{2+} concentrations using different platelet stimulators. Haemostasis **21 Suppl 1**:121-6.

26. Belcher P.R., Drake-Holland A.J., Hynd J.W. and Noble M.I. (1996) Failure of thrombin inhibition to prevent intracoronary thrombosis in the dog. Clin. Sci. **90**:363-8.

27. Wallen N.H., Ladjevardi M., Albert J. and Broijersen A.. (1997) Influence of different anticoagulants on platelet aggregation in whole blood; a comparison between citrate, low molecular mass heparin and hirudin. Thromb. Res. **87**:151-7.

28. Heptinstall S., White A., Edwards N., *et al.*. (1998) Differential effects of three radiographic contrast media on platelet aggregation and degranulation: implications for clinical practice? Br. J. Haematol.. **103**:1023-30.

29. Dawes J., Pumphrey C.W., McLaren K.M., Prowse C.V. and Pepper D.S.. (1982) The in vivo release of human platelet factor 4 by heparin. Thromb. Res. **27**:65-76.

30. Karlsson K. and Marklund S.L.. (1987) Heparin-induced release of extracellular superoxide dismutase to human blood plasma. Biochem. J. **242**:55-9.

31. Malmstrom R., Packard C.J., Caslake M., *et al.*. (1999) Effect of heparin-stimulated plasma lipolytic activity on VLDL APO B subclass metabolism in normal subjects. Atherosclerosis **146**:381-90.

32. Boberg J., Augustin J., Baginsky M.L., Tejada P. and Brown W.V.. (1977) Quantitative determination of hepatic and lipoprotein lipase activities from human postheparin plasma. J. Lipid. Res. **18**:544-7.

33. Kinlough-Rathbone R.L., Packham M.A., Reimers H.J., Cazenave J.P. and Mustard J.F.. (1977) Mechanisms of platelet shape change, aggregation, and release induced by collagen, thrombin, or A23,187. J. Lab. Clin. Med. **90**:707-19.

34. Menys V.C.. (1993) Collagen induced human platelet aggregation: serotonin receptor antagonism retards aggregate growth in vitro. Cardiovasc. Res. **27**:1916-9.

35. Barrowcliffe T.W., Merton R.E., Gray E. and Thomas D.P.. (1988) Heparin and bleeding: an association with lipase release. Thromb. Haemost. **60**:434-6.

36. Yang J.Y., Kim T.K., Koo B.S., Park B.H. and Park J.W.. (1999) Change of plasma lipoproteins by heparin-released lipoprotein lipase. Exp. Mol. Med. **31**:60-4.

37. Bengtsson G. and Olivecrona T.. (1980) Lipoprotein lipase. Mechanism of product inhibition. Eur. J. Biochem. **106**:557-62.

38. Brass L.F. (1995). 98 Molecular Basis of Platelet Activation. In Haematology: Basic Principles and Practice. (Hoffmann R., Benz Jr.E.J., Shattill S.J., Furie B., Cohen H.J. and Silberstein L.E. eds). , p. 1536-52 Churchill Livingstone. London.

39. Ozaki Y., Matsumoto Y., Yatomi Y., Higashihara M. and Kume S.. (1991) Two-step mobilization of arachidonic acid in platelet activation induced by low concentrations of TP 82, a monoclonal antibody against CD9 antigen. Eur. J. Biochem. **199**:347-54.

40. Melin T., Qi C., Bengtsson-Olivecrona G., Akesson B., Nilsson A.. (1991) Hydrolysis of chylomicron polyenoic fatty acid esters with lipoprotein lipase and hepatic lipase. Biochim. Biophys. Acta.**1075**:259-66

41. Lewy R.I.. (1980) Effect of elevated plasma-free fatty acids on thromboxane release in patients with coronary artery disease. Haemostasis **9**:134-40.

42. Landolfi R., De Candia E., Rocca B., *et al.*. (1994) Effects of unfractionated and low molecular weight heparins on platelet thromboxane biosynthesis "in vivo". Thromb. Haemost. **72**:942-6.

43. Dyerberg J., Bang H.O., Stoffersen E., Moncada S. and Vane J.R.. (1978) Eicosapentaenoic acid and prevention of thrombosis and atherosclerosis? Lancet **2**:117-9.

44. Fischer S. and Weber P.C.. (1983) Thromboxane A3 (TXA3) is formed in human platelets after dietary eicosapentaenoic acid (C20:5 omega 3). Biochem. Biophys. Res. Commun. **116**:1091-9.

45. Ruf A., Mundkowski R., Siegle I., Hofmann U., Patscheke H. and Meese C.O.. (1998) Characterization of the thromboxane synthase pathway product 12- oxoheptadeca-5(Z)-8(E)-10(E)-trienoic acid as a thromboxane A2 receptor antagonist with minimal intrinsic activity. Br. J. Haematol. **101**:59-65.

46. Nuutinen L.S., Mononen P., Kairaluoma M. and Tuononen S.. (1977) Effects of open-heart surgery on carbohydrate and lipid metabolism. J. Thorac. Cardiovasc. Surg. **73**:680-3.

47. Yokota H., Kawashima Y., Takao T., Hashimoto S. and Manabe H.. (1997) Carbohydrate and lipid metabolism in open-heart surgery. J. Thorac. Cardiovasc. Surg. **73**:543-9.

Figure 1

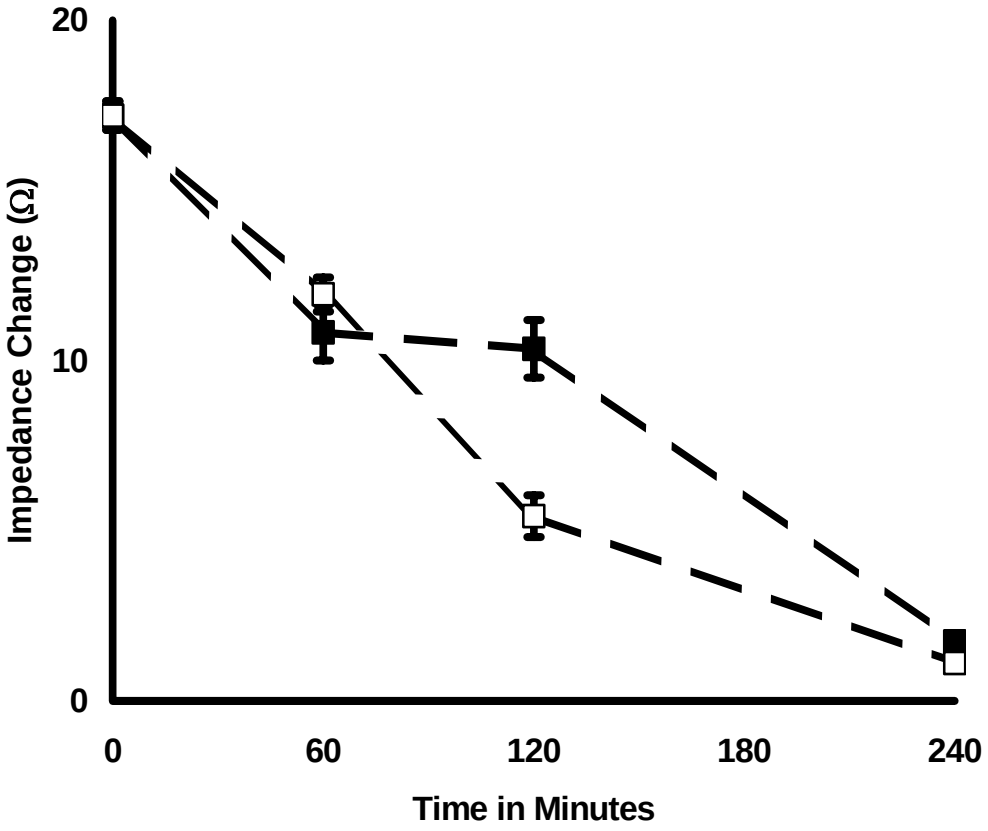


Figure 2

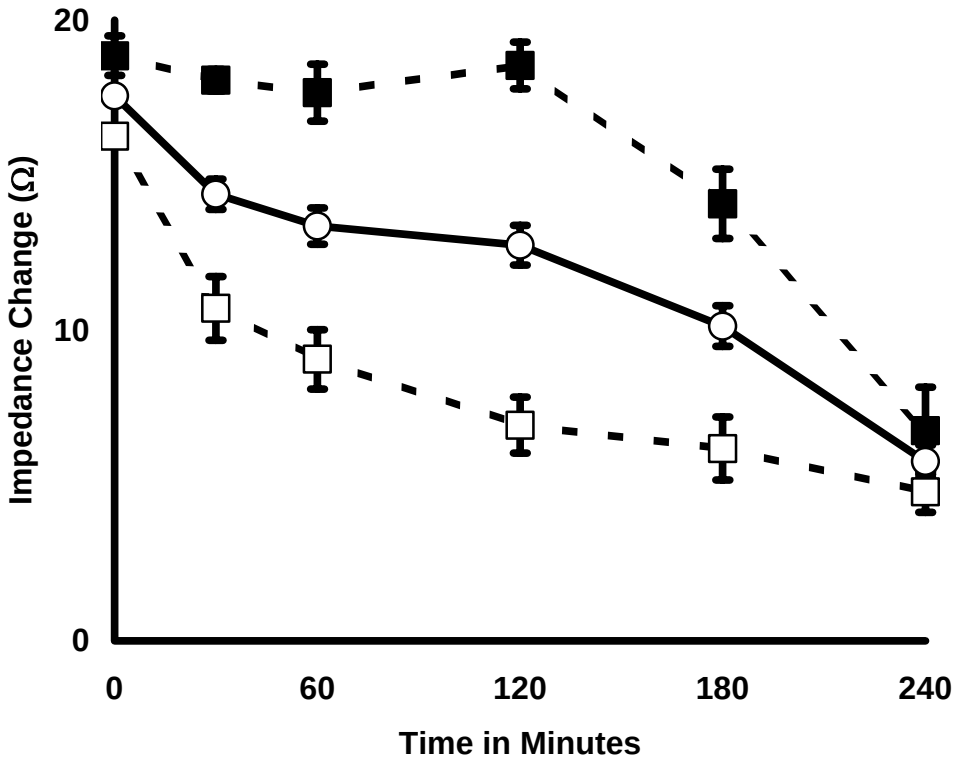


Figure 3

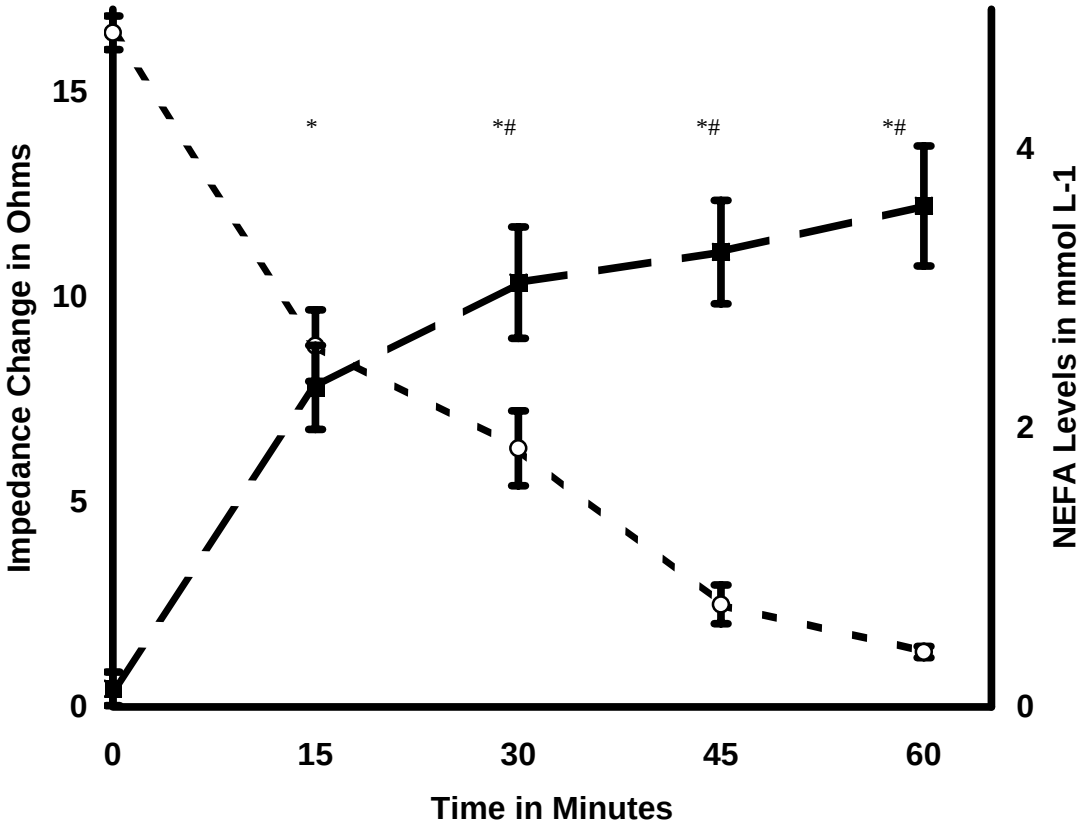
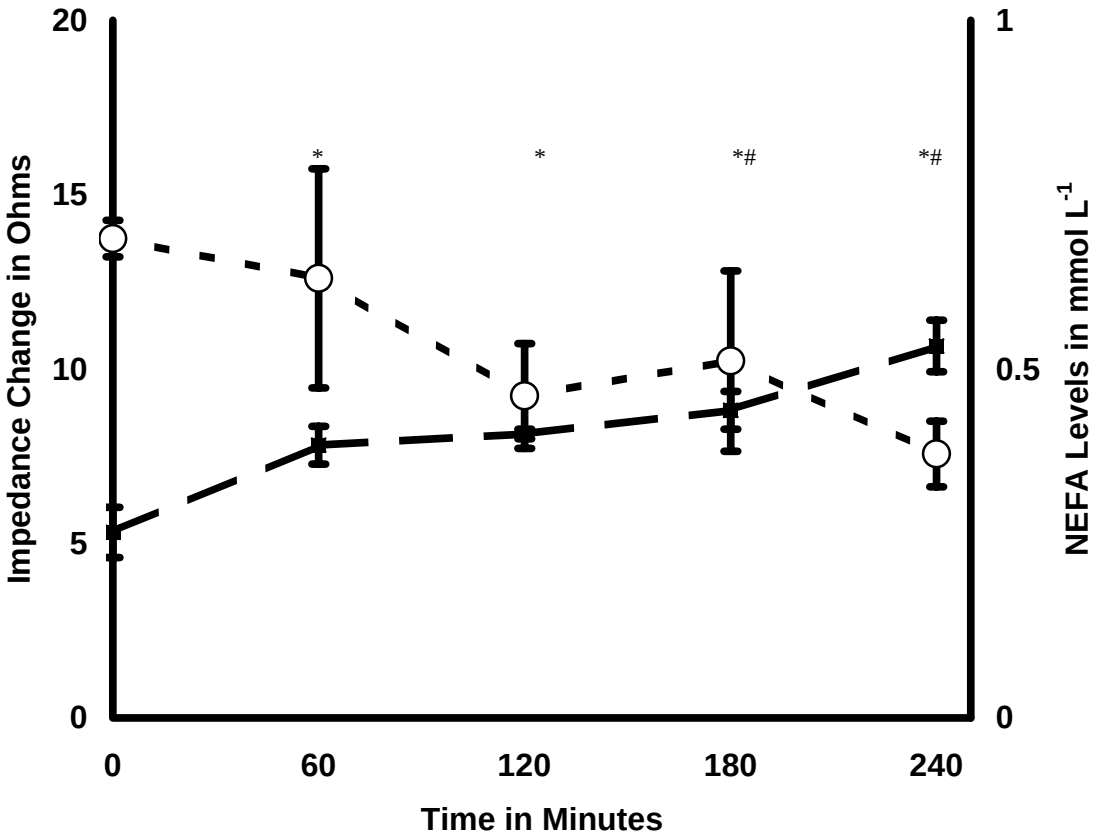

Impedance Change in Ohms
NEFA Levels in mmol L-1
Time in Minutes
0
5
10
15
0
2
4
0
15
30
45
60
*
*#
*#
*#

Figure 4


20
15
10
5
0
1
0.5
0
*
*
*#
*#
0
60
120
180
240
Time in Minutes
Impedance Change in Ohms
NEFA Levels in mmol L-1

LEGENDS

Figure 1

The Effect Of Human Post-Heparin Lipoprotein Lipase And Hepatic Lipase On Whole Blood Impedance Aggregometry In Response To *Ex Vivo* Stimulation With Collagen (0.6μg ml^{-1})

Mean change in impedance in Ohms; Error bars represent 1 standard error of the mean. Filled squares lipoprotein lipase (n = 6): Open squares hepatic lipase (n=6).

Figure 2

The Effect Of Bovine Milk Lipoprotein Lipase (100 u ml^{-1}) On Whole Blood Impedance Aggregometry In Response To *Ex Vivo* Stimulation With Collagen (0.6μg ml^{-1})

Mean impedance change in Ohms; Error bars represent 1 standard error of the mean. Open circles represent all subjects (n = 12); Filled squares represent the sub-group of slow responders (n = 6): Open squares represent the sub-group of fast responders (n=6).

Figure 3

The Effects Of Pseudomonas Spp. Lipoprotein Lipase (100 u ml^{-1}) On Non-Esterified Fatty Acid Levels And Impedance Aggregometry In Response To *Ex Vivo* Stimulation With Collagen 0.6 μg mL^{-1}, In Whole Blood

Data are mean with error bars representing 1 standard error of the mean (n=8). NEFA = non-esterified fatty acids. Filled squares represent NEFA levels, Open circles represent impedance changes. *p<0.05: NEFA *vs.* baseline: #p<0.05 Impedance change *vs.* baseline. For correlation see text.

Figure 4

The Effects Of Human Hepatic Lipase On Non-Esterified Fatty Acid Levels And Impedance Aggregometry In Response To *Ex Vivo* Stimulation With Collagen 0.6 μg mL^{-1}, In Whole Blood

Data are mean with error bars representing 1 standard error of the mean (n=5). NEFA = non-esterified fatty acids. Filled squares represent NEFA levels, Open circles represent impedance changes. *p<0.05: NEFA *vs.* baseline: #p<0.05 Impedance change *vs.* baseline. For correlation see text.

Table 1.

The Effects Of Heparin, Platelet Factor 4 And Superoxide Dismutase On Whole Blood Impedance Aggregometry.

Protein	Reading	A	B	C
Control	Immediate	13.1	25.0	20.0
Heparin 4 u ml^{-1}	Immediate	14.0	25.0	20.0
	3 hour	13.6	24.3	18.6
PF4 100 u ml^{-1}	Immediate	16.4	22.0	17.4
	3 hour	15.6	26.6	16.6
SOD 100 u ml^{-1}	Immediate	16.6	25.1	26.9
	3 hour	14.0	24.6	14.9

Macroaggregatory responses to collagen 0.6 μg in whole blood from 3 healthy volunteers (A, B and C). Impedance changes in Ohms. PF4 = Platelet Factor 4; SOD = Superoxide Dismutase.

Table 2.

The Effect Of Different Combinations Of Heparin, Platelet Factor 4 And Superoxide Dismutase On Whole Blood Impedance Aggregometry.

Combination	Reading	A	B	C
Control		13.1	25.0	20.0
H+P	Immediate	18.6	23.4	20.3
	3 hour	14.3	24.3	19.6
H+S	Immediate	17.9	26.6	27.3
	3 hour	14.3	24.3	19.6
P+S	Immediate	14.9	27.0	19.4
	3 hour	13.1	26.1	18.3
H+P+S	Immediate	17.1	26.0	24.6
	3 hour	12.9	25.4	21.4

Platelet macroaggregation in response to stimulation with *ex vivo* collagen 0.6 μg ml^{-1}, in whole blood from 3 healthy volunteers. Impedance changes in Ohms. H =Heparin 4 u ml^{-1}; PF4 = Platelet Factor-4 100 u ml^{-1}; SOD = Superoxide Dismutase 100 u ml^{-1}.