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EVIDENCE FOR THE INVOLVEMENT OF OXYGEN-DERIVED FREE RADICALS IN ISCHAEMIA-REPERFUSION INJURY

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Six patients undergoing vascular reconstructive surgery were examined for evidence of oxygen-derived free radical (OFR) damage to the protein, immunoglobulin G (IgG). OFR damage was determined as an increase in the fluorescence (ex 360 nm em 454 nm) to ultraviolet absorption (280 nm) ratio of IgG, representing N-Formyl kynurenine and other as yet unidentified fluorophores. The IgG ratio was found to increase slightly during ischaemia and to undergo marked elevation upon reperfusion (275 ± 405% baseline value at 40 min post-clamp; mean ± sd). A high ratio was maintained post-reperfusion, even after 60 min reperfusion. Determination of thromboxane B2, (TXB2), leukotriene B4, (LTB4) and 6-keto prostaglandin F1alpha, (PGF1a), revealed a decrease in their concentrations during ischaemia and a transient, marked increase on reperfusion. Only TXB2 concentrations were found to correlate with the IgG ratio (negative correlation, $p < 0.05$). No correlation was observed between von Willebrand antigen factor, a marker of endothelial cell damage and fluorescent IgG ratio. However, levels of the factor increased slightly during ischaemia and more sharply upon reperfusion. These preliminary results therefore suggest that a more likely source of the OFRs responsible for IgG damage is endothelial cell xanthine oxidase, rather than cyclo-oxygenase or lipoxygenase.

KEY WORDS: Ischaemia, reperfusion, fluorescence, IgG, oxygen radical

INTRODUCTION

Evidence for the involvement of OFR's in ischaemia-reperfusion injury was first presented by Granger *et al.* in 1981 who studied the phenomenon in a cat intestine model.[1] These workers demonstrated that administration of superoxide dismutase (SOD) just before reperfusion prevented injury to capillaries in the cat small intestine following 1 h of partial ischaemia. Many other organs have since been studied as models of ischaemia-reperfusion injury, including brain, kidneys, lung, intestinal mucosa, heart and liver[2-5] and there is a great deal of evidence implicating OFR's in tissue injury, particularly following the reperfusion process. Most of this evidence, however, is indirect and is based primarily on reduction of injury following the administration of known free-radical scavengers such as superoxide dismutase, catalase, glutathione and the iron chelators, desferrioxamine and transferrin.[6-9] Direct evidence for the involvement of OFR's has been obtained in a limited number of studies using electron spin resonance (ESR) to detect free

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TABLE I
Ischaemia-reperfusion study patient details

Subject	Age at operation yrs	Surgical procedure	Clamp time mins	Sampling site
1	63	Tube craft for AAA	79	inferior vena cava
2	73	Tube graft for AAA	102	inferior vena cava
3	48	A.F. bypass	R. Leg. 112 L. Leg. 146	common femoral vein
4	50	A.F. bypass	R. Leg. 94 L. Leg. 125	common femoral vein
5	61	A.F. & Left F. popliteal bypass	R. Leg. 79 L. Leg. 171	common femoral vein
6	66	AF-Iliac bypass	129	inf. v. cava pulmonary artery radial artery

AAA = Abdominal Aortic Aneurysmectomy
AF = Aorto-Femoral
F = Femoral
R = Right
L = Left

radicals and their adducts, both in animal models of ischaemia-reperfusion injury and human studies.[10-13]

We have decided to investigate the involvement of OFR's in a human model of ischaemia-reperfusion injury which arises following vascular surgery. During surgery for thoracoabdominal aneurysm repair, a significant number of patients develop severe coagulation disturbances shortly after declamping. Uncontrollable haemorrhage accounts for up to 26% of intraoperative and early post-operative deaths.[14] The precise nature of the coagulation disturbances has not been well documented, but it is thought that disseminated intravascular coagulation and secondary fibrinolysis may be primarily involved.[15] The severity of the coagulopathy is related to the duration of clamping and begins following clamp removal,[15] therefore it is possible that the coagulation disturbances may be mediated by OFR's. In support of this, disseminated intravascular coagulation has been closely linked with OFR generation in the endotoxic rat.[16] We have therefore investigated patients undergoing abdominal aortic aneurysmectomy and aorto-femoral bypass for evidence of OFR production during ischaemia-reperfusion and correlated these findings with platelet related factors TXB2, LTB4 and PGF1alpha.

METHODS

A total of six patients undergoing vascular surgery was studied. Patient details are recorded in Table 1. Blood samples were collected in both heparinised tubes and heparinised tubes containing salicylic acid at the following intervals; early and late pre-clamp, shortly after clamping; 10 min before clamp removal; immediately following declamp and at 10 min intervals up to 60 min declamp. In one individual, further samples were taken from the pulmonary and radial arteries at mid-clamp and for up to 6 h following declamp. Plasma was separated by centrifugation at 3000 rev/min for 5 min and stored at −70°C prior to analysis.

Determination of OFR production: generation of OFR's was measured indirectly by determination of IgG oxidation in plasma using the method of Lunec *et al.*, 1985,[17] a method previously used to determine IgG oxidation in rheumatoid sera and synovial fluid. Briefly, the method was as follows; plasma was diluted with phosphate buffered saline (1:20 dilution, v/v) and 50 μl aliquots injected onto a TSK-GEL G3000 SW gel permeation column, 7.5 × 300 mm. Elution of proteins was performed with a mobile phase of 0.067 M Phosphate buffer, pH 7 at a flow rate of 1 ml/min. Eluate was monitored by means of fluorometric and UV detectors in series. The fluorescence wavelengths were; excitation 360 nm, emission 454 nm, (specific for kynurenine and its metabolites) whilst the UV detection was at 280 nm (optimum wavelength for tryptophan detection).

Determination of TXB2, LTB4 and PGF1a; was by means of ELISA methodology using commercial kits supplied by Amersham International plc.

Determination of von Willebrand antigen factor; was performed using ELISA methodology.[18]

Statistical analysis utilised one way ANOVA, the Kruskal-Wallis test and two-tailed t-test.

RESULTS

The variation in the IgG ratio during ischaemia-reperfusion is shown for all subjects in Figure 1. Of the nine profiles analysed, seven showed an increase in the IgG ratio during the ischaemic period and eight revealed a sharp increase in the ratio immediately following declamp and the onset of reperfusion. In only three profiles had the ratio started to fall back towards baseline values by the end of sampling, i.e. about 60 min reperfusion.

Statistical analysis of the data from subjects 1–5, revealed no significant differences between the time points but a series of paired t-tests comparing base-line values (pre-clamp) with individual time points showed significantly higher ratios at all time points except the early clamp ($p < 0.01$ for immediate declamp/reperfusion sample; $p < 0.05$ for all other time points). Hence the most marked increase in the IgG ratio occurred immediately following the onset of reperfusion. Data from a single subject (subject 6), studied for up to 6 h following clamp removal, with sampling from the inferior vena cava, pulmonary and radial arteries, showed markedly similar profiles, with a consistent decrease in the IgG ratio during the ischaemic period followed by an abrupt increase on reperfusion. Changes in the IgG ratio plateaued at 90 min reperfusion (see Figure 1).

Linear correlation analysis of the relationship between duration of clamp time and maximum change in the IgG ratio indicated that there was no significant relationship between the two parameters.

In order to assess the possible role of arachidonic acid metabolism as a source of the OFR's generated during ischaemia-reperfusion, the metabolites, TXB2, LTB4 and PGF1alpha, were measured. Results of these determinations indicated that the concentrations fell during ischaemia but rose sharply and briefly following restoration of blood flow. Data from a representative subject, subject 3, is shown in Figure 2. However, the only significant correlation between the IgG ratio and arachidonic acid metabolites was found with TXB2 (negative correlation, $p < 0.05$, $r = .4216$).

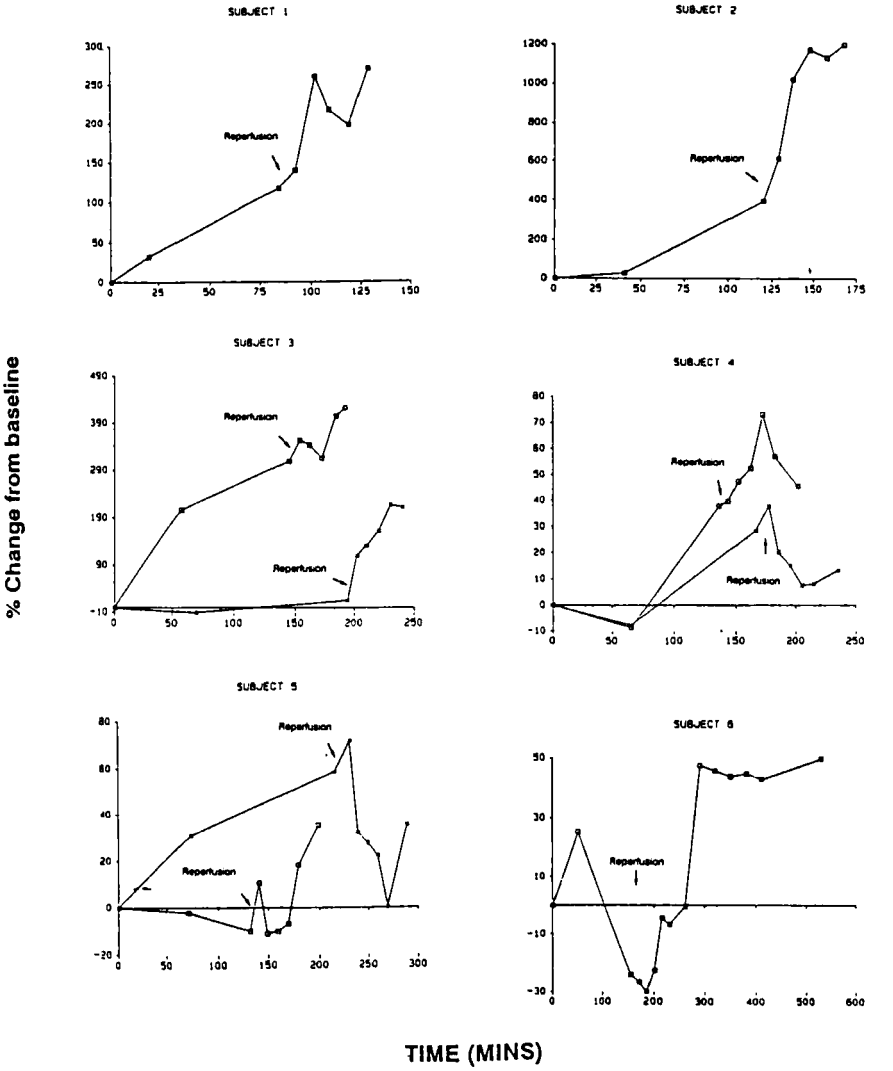

FIGURE 1 Changes in the IgG Fluorescence/UV ratio with time in six subjects undergoing ischaemia-reperfusion during vascular surgery. Plotted values represent the mean of duplicate estimations. All values are expressed as the percentage increase over the pre-clamp ratio.

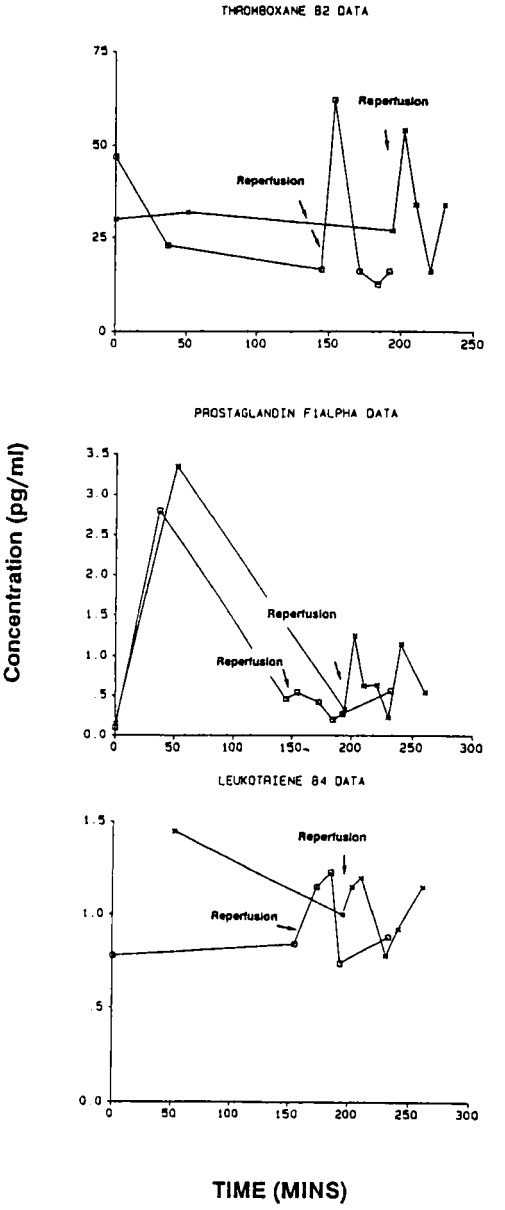

FIGURE 2 Changes in eicosanoids with time in a subject (subject 3) undergoing vascular surgery (aorto-femoral bypass). Plotted values are the means of duplicate estimations.

Similarly, there was no correlation between von Willebrand antigen factor and the IgG ratio.

DISCUSSION

We have demonstrated that, in this particular model of ischaemia-reperfusion injury, there is evidence for the generation of oxygen-derived free radicals (OFR's) during both the ischaemic and reperfusion periods. It is likely that the OFR's involved are hydroxyl and peroxy radicals as it has been reported that these radicals are linearly associated with the observed changes in the UV and fluorescence characteristics of IgG which occur during oxidation of the molecule. The superoxide anion does not generate autofluorescence in proteins.[19] The lack of significant differences between the sample time points can probably be explained by the large variation between subjects. This variation may be a reflection of the different surgical procedures undertaken in addition to expected inter-subject variation. The slight rise in the IgG ratio observed during ischaemia probably arises from collateral blood flow and may indicate that aortic clamping alone cannot produce total ischaemia in the lower limbs. It has been documented that ischaemic damage is directly related to the duration of ischaemia and inversely related to the chances of recovery following reperfusion.[20] However, the work reported here did not exhibit a correlation between the duration of ischaemia and the maximum change in the IgG ratio. In one subject who underwent ischaemic periods to both lower limbs, the longer ischaemic time was associated with higher IgG ratios but in two other such subjects, the reverse effect was seen.

The present study has demonstrated that OFR's are generated in our re-vascularisation model of ischaemia-reperfusion injury, with the largest effect seen immediately following reperfusion. This agrees with previous work with a human revascularisation model following similar surgical procedures, where plasma lipid peroxide levels showed a strong transient increase at one hour reperfusion.[21] It is possible that the increase in lipid peroxide levels occurred earlier then one hour post reperfusion, but this was the earliest time point used in the above study. Similarly, early, transient increases in myeloperoxidase activity and reduction in vitamin E concentration have been reported during human kidney transplantation although again, limited samples were taken post reperfusion.[22]

Regarding the effect of ischaemia-reperfusion on the metabolites of arachidonic acid, previous work has reported increased release of leukotrienes during ischaemia[23,24] and increased concentrations of PGF1a in ischaemia-reperfusion injury in rats.[25] Studies in humans have also reported increased levels of TXA2 in patients with myocardial infarction and elevated TXB2 levels in patients with unstable angina.[26,27] The results from the present study suggest transient increases in these three metabolic products following reperfusion, but the extent to which they were sustained could not be defined beyond 60 mins post declamp. All data obtained so far, however, indicate negative relationships between arachidonic acid metabolites and the IgG ratio, suggesting that these pathways are not the source of the OFR's which cause IgG oxidation.

Although no correlation could be demonstrated between von Willebrand antigen factor, an indicator of endothelial cell damage, and the IgG ratio, the general trend of results for this factor was a decrease in factor concentration on initiation of the clamp, a slight increase during ischaemia and a sharp increase on reperfusion. As

the concentration of von Willebrand antigen factor increased during the ischaemic period, as did the IgG ratio, it is tempting to suggest that the endothelial cells may have been the source of the OFR's generated during ischaemia. This would be consistent with the current hypothesis regarding the location of the xanthine oxidase enzyme, which is believed to be an ecto enzyme present on the outer surface of endothelial cells. However, further data must be accumulated before firm conclusions can be drawn in this regard.

The source of the free radicals which arise during both ischaemia and reperfusion is not yet clear. Although the evidence to date suggests that they do not arise from the metabolism of arachidonic acid by either the cyclo-oxygenase or lipoxygenase pathways, there is some indication that they may arise from endothelial cells, presumably via xanthine oxidase conversion of hypoxanthine to xanthine.

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