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IBUPROFEN PROTECTS RAT LIVERS FROM OXYGEN-DERIVED FREE RADICAL-MEDIATED INJURY AFTER TOURNIQUET SHOCK

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Rats subjected to tourniquet shock suffer a severe form of circulatory shock, tissue and organ oxidative stress, and final multiple system organ failure (MSOF) and death of the animals within 24 h of tourniquet release. The oxidative damage observed in hind-limb muscle tissue after reperfusion does not by itself account for the final systemic and lethal MSOF. We have postulated that organ failure has its genesis in a primary perfusion abnormality, e.g. the hind limbs, which is followed by secondary hypoperfusion of other organs, such as the liver, as has been shown to be the case in several septic shock models. It has also been shown that injured or necrotic tissue can activate neutrophils, Küpffer cells, platelets, and both the complement and coagulation cascades. In turn, complement activation also leads to neutrophil and Küpffer cell activation as assessed by their capacity to generate oxyradicals. Herein we have evaluated the potential protective effect of ibuprofen on hepatic oxygen-derived free radical production, as well as its effects on both polymorphonuclear leucocyte (PMN) activation and liver infiltration. The protective effect of ibuprofen on hepatic oxidative injury was assessed by determining total thiol groups (SH), thiobarbituric acid-reactive substances (TBARS), and by the release of aspartic acid (AsT) and alanine (AlT) aminotransferases in control animals, in animals subjected to 5 h of tourniquets, and in animals after 2 h of hind-limb reperfusion. Liver infiltration by PMNs was determined by histology after staining with eosin-hematoxylin, and PMN activation by their capacity to reduce nitro blue tetrazolium (NBT). Our results show that total hepatic thiol content decreased significantly, over and above the the normal circadian decrease in liver glutathione, after the 5 h tourniquet period (from 6.16 ± 0.97 to 4.07 ± 0.21 μmoles/g w.w). The decrease in liver thiols in animals pretreated with ibuprofen was not significantly different from that in control animals (from 5.76 ± 0.21 to 4.69 ± 0.19 μmoles/g w.w), and could be accounted for by the circadian effect. A further significant decrease was observed in the control (3.01 ± 0.12 μmoles/g w.w), but not in the ibuprofen pretreated rats (4.65 ± 0.16 μmoles/g w.w), after the 2 h reperfusion period. TBARS production remained essentially unchanged during the tourniquet period in both the control and ibuprofen pretreated animals (average 260 nmoles/g w.w), but increased significantly after hind-limb reperfusion in the control animals (386.9 ± 18.5 nmoles/g w.w), but not in the ibuprofen treated rats (267.2 ± 7.4 nmoles/g we.w). The protective effect of ibuprofen was also evident in plasma aminotransferase levels (AsT and AlT) which increased 14 and 6-fold, respectively, during the experimental period in the untreated rats, and only 6 and 3-fold in the animals pre-treated with the drug. No significant differences were observed in PMN liver infiltration in any of the animals, nor at any of the different time periods under study. Nevertheless, our results indicate that there is a 3-fold increase (over control values) in the number of circulating activated PMNs after hind-limb reperfusion in the non-protected control rats, and only a 2-fold increase in those protected by ibuprofen. It is concluded that ibuprofen: a) protects rat livers from the oxidative stress which results after 2 h of reperfusion of rat hind limbs subjected to 5 h of ischemia by means of tourniquets, and b) significantly decreases the number of NBT-positive PMNs in the systemic circulation after hind-limb reperfusion.

KEY WORDS: Oxidative stress; ibuprofen; oxyradicals; liver; tourniquet shock; ischemia; reperfusion; free radicals.

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INTRODUCTION

We have previously shown that two hours of reperfusion of rat hind limbs which had been subjected to 5 hours of bilateral tourniquets, results in severe oxygen stress which leads to serious injury of the gastrocnemius muscles as assessed by a decreased oxygen consumption, loss of total thiol groups, edema, the presence of TBARS in the femoral vein,[1] and by the release of lactic dehydrogenase isoenzymes,[2] all of which lead to the death of the animals within 24 hours of hind-limb reperfusion. We also demonstrated that significant protection was afforded by the pretreatment of the animals with allopurinol.[3]

In an attempt to understand the pathogenesis of the shock syndrome, in both experimental animals and in the clinical setting, we postulated a model that suggested that local episodes of ischemia/reperfusion, such as in the hind limbs during the application of tourniquets, could result in the eventual hypoperfusion of other organs, and that these would eventually be subjected to tissue injury through the generation of oxygen-derived free radicals.[4] In agreement with this hypothesis we have shown that oxygen-derived free radicals mediate a severe form of liver injury, as assessed through the determination of total thiol groups (SH), thiobarbituric acid reactive substances (TBARS), and by the release of aminotransferases (AsT and AlT), 2 hours after the release of the bilateral hind-limb tourniquets.[5] These results were in agreement with those of Lefer and Ma[6] who showed that splanchnic artery occlusion followed by reperfusion resulted in, among other pathophysiological events, liver injury and that the extent of the injury could be ameliorated by the prior administration of SOD. Similar results were obtained by Schirmer *et al.*[7] who showed that femur fracture, associated with soft-tissue trauma, as opposed to that without soft-tissue injury, resulted in liver hypoperfusion.

Tissue ischemia is a major factor in the pathogenesis of several life-threatening disease (e.g., myocardial infarction, shock, cardiac arrest, stroke), and substantial evidence suggests an important role for reactive oxygen species in the extensive tissue damage that occurs during organ reperfusion. Although the source(s) generating these oxygen-derived free radicals are still the subject of debate, growing evidence suggests that polymorphonuclear inflammatory cells (PMNs) play a key role in the pathogenesis of these diseases in the heart,[8] intestine,[9] and liver.[10,11,12]

Ibuprofen, a nonsteroidal anti-inflammatory agent, is known to inhibit lipid peroxidation, cyclooxygenase metabolism, and to be a free radical scavenger.[11,13] It improves hemodynamic functions, prevents acidosis, and increases survival[14,15,16] in haemorrhagic and endotoxic shock models. Nevertheless, it can also be deleterious in that it stimulates the permanent adherence of leukocytes to liver endothelial cells.[17] Ibuprofen has also been shown to inhibit oxygen-derived free radical production by peritoneal macrophages, Küpper cells, monocytes, and neutrophils in response to a variety of stimuli.[13,18] Consequently, the precise biochemical mechanisms involved in these protective effects of ibuprofen are probably multifactorial, and involve the inhibition of arachidonate metabolism and oxidant injury. This study was designed to evaluate the potential protective effect of ibuprofen on liver oxidant injury observed after the reperfusion of rat hind legs previously subjected to 5 hours of ischemia through the application of bilateral tourniquets.

MATERIALS AND METHODS

Reagents

Ibuprofen, NBT, 5,5′-dithiobis(2-nitrobenzoic) acid, and thiobarbituric acid were obtained from Sigma Chemical Co (St. Louis). Plasma aminotransferase levels (AsT and AlT) were assayed with commercial kits (Boehringer, Mannheim GmbH).

Experimental Protocols

As previously reported,[1,2,5] bilateral rubber band tourniquets (20 × 5 cm) were placed for 5 hours, under ketamine (10 mg/Kg body wt) and xylazine (2.5 mg/Kg body wt) anaesthesia, beneath the skin of male Sprague-Dawley (250–300 g) rats who had had free access to food and water. Rats were separated into two groups (I & II). Group II animals were pretreated with ibuprofen (12.5 mg/Kg body wt) administered intraperitoneally 24 and 2 hours prior to the application of the tourniquets, and again at 2 and 4 h into the tourniquet period. Group I animals underwent the same protocol but were injected with solvent (ethanol-water 3:1). A third of the animals were sacrificed before the placement of tourniquets (controls), a second group immediately after the release of the rubber bands (tourniquet group), and the remaining animals 2 h after hind-limb reperfusion (reperfusion group). In order to discard a possible effect of the solvent (ethanol-saline) in which ibuprofen was dissolved, a completely new group of animals were given two 12,5 mg/Kg body weight doses of ibuprofen (12 hours apart) intragastrically on the day prior to the experiment, and every 4 h, starting 2 h before tourniquet application, on the day of the experiment.

Methods

Liver thiol levels, represented by reduced glutathione, free cysteine and protein cysteines, and TBARS levels were determined as previously indicated[5] in the right lateral lobes of livers excised, under ether anaesthesia, at the different time periods indicated above; the former by the method of Ellman[20] with 5,5′-dithiobis (2-nitrobenzoic acid), and the latter with thiobarbituric acid as described by Okhawa *et al.*[21] The results of these determination are expressed as "per wet weight" of liver tissue and not, as is probably more correct, as "per mg of protein or DNA", because we have shown (unpublished observations) that liver tissue protein content does not differ significantly between samples obtained from sham operated control animals (10.3 ± 0.5 mg of protein/g wet weight) and from the group of animals subjected to five hours of tourniquet application followed by two hours of hind limb reperfusion (10.8 ± 0.6 mg of protein/g wet weight). Alanine (AlT) and aspartic acid (AsT) aminotransferases were assayed in blood samples obtained from the abdominal aorta with commercial kits. See reagents. Neutrophil infiltration of hepatic tissue was determined in tissue samples obtained from control, tourniquet and reperfusion group animals. These were fixed in formaldehyde, included in paraffin, and finally cut and stained with eosin-hematoxylin. Superoxide anion production by activated PMNs was assessed by the reduction of tetrazolium blue (NBT) in freshly obtained heparinized venous blood samples as described by Barroso-Aranda and Schmid-Schönbein.[22] Specifically, 0.1 ml of fresh heparinized blood (10 U/ml) was transferred into a siliconized concave microslide and mixed with an equal volume of a 1:1 solution of 0.2% NBT in saline adjusted to pH 7.2 with phosphate buffer. The microslides were placed in a Petri dish containing wet

cotton wool to maintain humidity and incubated at 37°C for 30 min. After 15 min at room temperature, the samples were spread with cover-slips and stained with Wright's stain. One hundred neutrophils were counted at ×100 oil-objective magnification.

Statistics

The results are presented as the mean ± SE. Students t-test p values of less than 0.05 were considered significant.

RESULTS

Protective Effects of Ibuprofen on Hepatic Total Thiol Levels

As shown in Figure 1, there were no significant differences in total thiol levels of both solvent (6.16 ± 0.97 μmoles/g wet tissue, bar I) and ibuprofen pretreated animals (5.76 ± 0.25 μmoles/g wet tissue, bar II) before the ischemic period. These decreased significantly (4.07 ± 0.21 μmoles/g wet tissue) in the untreated, but not in the ibuprofen treated rats (4.69 ± 0.19 μmoles/g wet tissue), after the five hour tourniquet period. There was a further significant decrease in these levels after the 2 h reperfusion period in the untreated rats (3.01 ± 0.12 μmoles/g wet tissue), as opposed to the ibuprofen treated animals (4.65 ± 0.16 μmoles/g wet tissue).

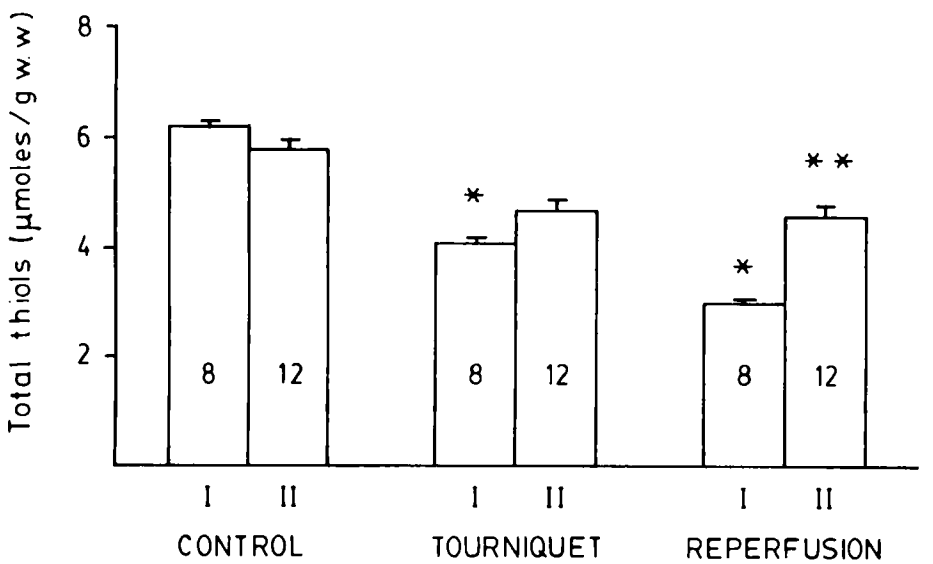

FIGURE 1 Effect of ibuprofen on rat liver total thiol content during 5 h of bilateral hind-limb tourniquets (tourniquet group) and after 2 h of hind-limb reperfusion following tourniquet release (reperfusion group) compared to sham-operated control animals. The bars labelled I and II represent animals pre-treated with saline and ibuprofen, respectively. The significant decreases in liver SH contents in the tourniquet and reperfusion group of animals can be accounted for by the normal circadian decrease in GSH levels over this 5 h period (10 AM–3 PM). The number of animals in each group is represented by numbers placed in the different columns. *$p < 0.001$ with respect to sham operated animals (control rats). **$p < 0.005$ when compared to bar I of the reperfusion group of rats.

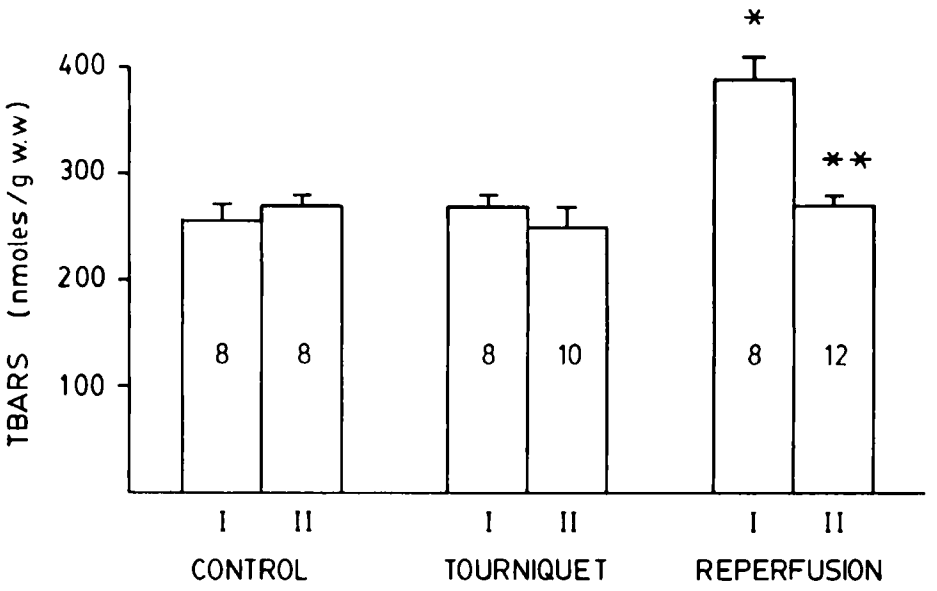

FIGURE 2 Protective effect of ibuprofen on rat liver TBARS production after 2 h of hind-limb reperfusion following the 5 h tourniquet period. The number of animals in each group is indicated by numbers placed in the different columns. *$p < 0.001$ when compared to sham operated animals (control rats). **$p < 0.005$ when compared to bar I of the reperfusion group of rats.

Protective of Ibuprofen on Hepatic TBARS Levels during the Tourniquet and after the Reperfusion Period

As shown in Figure 2, TBARS levels in the control and tourniquet groups of rats were essentially the same (average 260.3 ± 15.7 nmoles/g w.w) in both the solvent and ibuprofen pretreated rats. These levels increased significantly in the untreated animals (to 386.9 ± 18.5 nmoles/g w.w), as compared to the ibuprofen-treated rats (267.2 ± 7.4 nmoles/g w.w), after the two hour reperfusion period.

Control experiments, in which ibuprofen was administered intragastrically through a cannula, showed that there were no significant differences between the TBARS levels determined in the control, tourniquet, or reperfusion groups animals and the results shown in Figure 2, i.e., with the rats that received the intraperitoneal solution of the drug dissolved in the 3 to 1 ethanol-saline mixture (results not shown).

Protective Effect of Ibuprofen on Aminotransferase Serum Levels

Figure 3 shows that plasma AsT levels increased (14-fold) from 60.8 ± 9.3 U/L in the control animals to 827.5 ± 9.8 U/L in the unprotected rats, and only 6-fold (from 87.1 ± 6.2 to 534.2 ± 37.9 U/L) in the ibuprofen pre-treated rats, after the 2 h reperfusion period. A similar protective effect of the drug was seen when plasma AlT levels were determined. These were not significantly different in the normal and drug treated rats before tourniquet application (17.3 ± 1.8 and 21.0 ± 1.6 U/L, respectively), and increased (6 and 3-fold) after the 2 h reperfusion period to 96.8 ± 2.9 U/L in the untreated rats and to only 64.0 ± 5.1 U/L in the ibuprofen treated animals.

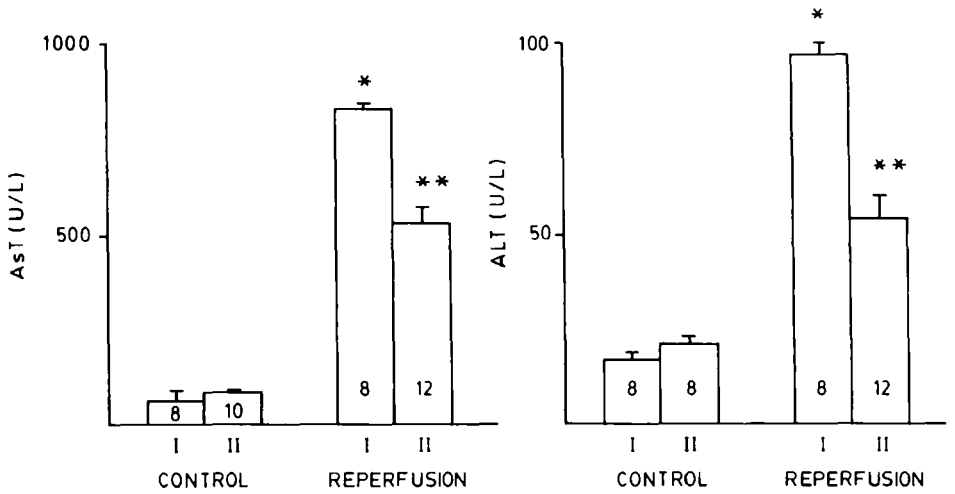

FIGURE 3 Serum levels of aspartic acid (AsT) and alanine (AIT) aminotransferases in control and reperfusion group animals in the presence and absence of ibuprofen. The number of animals in each group is represented in the different columns. *$p < 0.005$ when compared to saline and ibuprofen pre-treated sham operated control animals (control). **$p < 0.005$ in AsT levels, and 0.005 in AIT plasma levels, when compared to saline pre-treated rats after two hours of hind-limb reperfusion (bar I of the reperfusion group).

Protective Effect of Ibuprofen on Neutrophil Activation

The Table shows that the number of activated PMN's increased 3-fold (from a value of 27 ± 1 cells per 100 cells in the optical field to 78 ± 2 activated cells) after 5 h of tourniquet followed by the two hour reperfusion period in the saline pre-treated animals, and that ibuprofen pre-treatment significantly decreased the number of activated cells (55 ± 2%) after the same time period.

DISCUSSION

The objective of the present study was to further our previous work where we demonstrated that the initial reperfusion period of rat hind legs, subjected to 5 h of bilateral tourniquets, was characterized by an enhanced loss of liver thiols, and subsequent liver damage as evidenced by the release of aminotransferases,[5] presumably through a Küpffer cell-induced oxidant stress, as has been shown to be the case in rat livers

TABLE

Number of activated polymorphonuclear leukocytes per 100 cells in blood samples taken from control and 2 h reperfusion group animals pre-treated with saline or ibuprofen. *$p < 0.0005$ when saline pre-treated reperfusion group rats are compared to those of normal control rats, and **$p < 0.005$ when the comparison is made with the saline pre-treated reperfusion group.

GROUP	n	ACTIVATED CELLS (%)
CONTROL	10	27 ± 1
SALINE	10	78 ± 2*
IBUPROFEN	10	55 ± 2**

subjected to ischemia/reperfusion.[12] In this study we investigated the potential protective effect of ibuprofen, a nonsteroidal anti-inflammatory agent, on liver injury caused by oxygen-derived free radicals. More and more, macrophages and neutrophils are being implicated as mediators of tissue injury in inflammatory diseases ranging from reperfusion injury, ulcerative colitis, skin disorders, to the adult respiratory distress syndrome.[23,24] In these, as in other inflammatory disorders, important components of the pathological processes are being attributed to the neutrophils ability, first to adhere to endothelial cells, and then to release reactive oxidizing chemicals through the activation of their NADPH oxidases, or through the release of microbicidal peptides, proteins, and enzymes from their intracellular granules, all of which contribute to the destruction of normal cells and connective tissues. We have recently shown that oxygen-derived free radicals, as assessed by the prior administration of allopurinol or of an antioxidant mixture composed of superoxide dismutase, catalase, and dimethylsulphoxide, are involved in the pathogenesis of liver injury of rats subjected to ischemia-reperfusion of their hind limbs by means of tourniquets.[5] These latter results agree with those of Jaeschke's[10,11,12,25,26] and others,[27,28,29] who have consistently shown that Küpffer cells and PMNs are responsible for the oxidative stress that leads to liver injury in rats subjected to different protocols of ischemia-reperfusion.

Ibuprofen has been shown to have beneficial effects by eliminating the increase in plasma levels of arachidonic acid derivatives seen in several shock states,[17,29,30] and by decreasing serum fluorescent products of lipid peroxidation.[31] Ibuprofen has also been demonstrated to inhibit neutrophil chemotaxis,[32] to inhibit their aggregation and degranulation,[33] as well as their adherence to nylon-wool columns in vitro,[34] which is analogous to cell adherence to vascular endothelium *in vivo*. Our present histological results (not shown) showed no differences in the number of PMNs present in liver tissue samples from the control, tourniquet, or reperfusion group of rats, either in the presence or absence of ibuprofen, suggesting that in this model, neutrophil infiltration of the liver, if it occurs, is a much later event. These observations agree with the results of Jaeschke *et al.*,[11,12] who showed that two phases of liver injury could be identified. In the first, and although PMNs start to accumulate, they showed that the resident macrophages (Küpffer cells) were responsible for oxygen free radical production, and that it was the activity of these cells that was responsible for the reperfusion injury to the liver.[12] The initial phase of injury is followed by a later progression phase in which an 80% increase in neutrophils could be observed.[11] On the other hand, our results clearly show that the percentage of activated circulating PMN's increases significantly after 2 h of hind-limb reperfusion, and that ibuprofen was again protective. In an haemorrhagic shock model, it was shown that the number of NBT-positive PMNs correlated in a singular manner with irreversibility of the outcome.[22] Our present results show that about a quarter of the circulating PMNs, in both the ibuprofen-pretreated and non-treated rats, were activated in the control animals before tourniquet application, and that this number increased dramatically (to around 78%) after the two hour reperfusion period in the non-treated animals, and that there was a significant decrease in the number of activated neutrophils in the presence of the drug. Though the factors that determine the basal physiological level of leukocyte activation are still uncertain, these cells have been shown to be activated by direct receptor action of activators such as complement, endotoxin, N-formylated peptides, and others,[35] by depletion of deactivators such as adenosine;[36] by contact activation with endothelium; and by mechanical shear. Any of these factors may be operative in our shock model, but we feel that one good candidate for the activation of these leukocytes, and of resident Küpffer cells, could be due to complement activation by cellular proteins[24] released

from the postischemic limbs, as has been shown after coronary artery occlusion[37] and in post-ischemic cardiac lymph.[38] Cellular debris reaching the liver after hind-limb reperfusion constitutes a second candidate for Küpffer cell activation and subsequent superoxide anion generation. This statement is supported by the work of Bautista *et al.*[39] who clearly showed that liver macrophages were activated after the intravenous administration of latex particles, and that ibuprofen inhibited the production of superoxide anions by the resident Küpffer cells. Both of these alternatives require further study.

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