Prevention of postischaemic lipid peroxidation and liver cell injury by iron chelation

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SUMMARY Liver ischaemia was induced by cross clamping the hilar pedicle for 30 minutes in groups of rats with or without treatment with the iron chelating agent desferrioxamine (deferrioxamine, DFR). The groups included eight animals each and were divided into the following treatment categories: control; ischaemia alone; ischaemia with subsequent reperfusion; ischaemia preceded by DFR, 60 mg/kg body weight; and reperfusion preceded by 20, 40, or 60 mg/kg DFR. The drug was given intravenously five minutes before either ischaemia or reperfusion. Malondialdehyde (MDA), a product of lipid peroxidation, and histopathological changes of liver tissue samples were used as indicators of hepatocellular injury. Lipid peroxidation (MDA concentration in $\mu\text{mol/kg}$ liver tissue) was highest (4.76 (1.19)) after ischaemia without reperfusion and less pronounced (2.87 (0.34)) after reperfusion. Both concentrations, however, were significantly ($p < 0.05$) higher than basal (control) values (1.78 (0.27)). At 60 mg/kg body weight, DFR treatment reduced MDA to basal or even lower concentrations in both situations (1.98 (0.08) and 1.26 (0.06), respectively) with a corresponding improvement in liver histopathology. Lower DFR doses were less protective. The data suggest that liver ischaemia is associated with free radical initiated, and apparently iron catalysed lipid peroxidation, which can be significantly decreased by iron chelation.

The harmful effects of complete interruption of blood flow to an organ has long been a topic of intense interest. Complete interruption of blood flow to the liver is often necessary during surgical intervention for trauma or when extensive resection of a tumour is performed.¹ With the recent development of liver transplantation, the effects of temporary ischaemia on restoration of liver function are of even greater importance. Under these conditions, the harmful consequences of liver ischaemia and subsequent reflow on liver function are often related to injury at the cellular and subcellular level (membrane integrity, mitochondrial function, protein synthesis, DNA, etc).²⁻⁶

Damage to cell and organelle membranes is probably the critical lesion that precedes irreversible cell injury.³ This has been largely attributed to peroxida-

tion of⁷ membrane lipids which can be initiated by oxygen free radical species such as the superoxide anion (O_2^-) and some of its active derivatives.⁸⁻¹⁰ Superoxide is generated during normal respiratory metabolism but is rapidly metabolised by the enzyme superoxide dismutase, which converts it to hydrogen peroxide (H_2O_2) that is in turn metabolised to water by catalase and glutathione peroxidase.¹¹ Some low molecular weight substances, such as vitamin E and ascorbic acid, can also serve as free radical scavengers. Free radicals beyond the capacity of cellular scavenging mechanisms can interact with many subcellular structures, such as membrane lipids, to form lipid peroxides and thus disrupt the integrity of the affected membranes.¹²

Lipid peroxidation takes place in a chain reaction through which a polyunsaturated fatty acid (PUFA) is oxidised to produce several intermediate lipid-based free radicals that in turn react with other PUFAs, thus propagating the process and possibly extending the damage to sites and dimensions beyond the initial insult.^{12,13} Initiation and propaga-

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tion of lipid peroxidation takes place more favourably in the presence of a transitional metal catalyst such as iron, which is quite ubiquitous in mammalian tissues.¹⁴ In addition, O_2^- is capable of releasing iron directly from ferritin (by reduction of ferric to ferrous form).¹⁵ Ferrous iron (Fe^{++}) can donate one electron to intermediate byproducts of lipid peroxidation, thus creating additional free radicals.¹² Similarly, low molecular weight chelates of iron, such as $ADP-Fe^{++}$, can undergo reactions with oxygen to generate active oxidation species that directly initiate lipid peroxidation in PUFAs.¹³

Available evidence indicates that free radical mediated lipid peroxidation takes place not only during reperfusion, when the sudden oxygen flux produces significant amounts of free radicals, but also during hypoxia itself despite the low oxygen tension.¹⁶ During hypoxia, cellular processes take place in a 'reducing' environment with anaerobic metabolism and low adenosine triphosphate (ATP) production.⁶ Under these conditions, there is an accumulation of hypoxanthine and increase in intracellular calcium with associated conversion of xanthine dehydrogenase to xanthine oxidase. The latter acts on xanthine and hypoxanthine, even under minimal oxygen tension, to produce superoxide radicals.¹⁶ Under hypoxic conditions, other substances like coenzyme Q, and even ascorbic acid, undergo autooxidation from their reduced forms to produce radical species capable of initiating lipid peroxidation.¹⁷ Hypoxia is also associated with a rapid release of iron from ferritin,¹⁸ which assists in propagating the process of lipid peroxidation even further.

Most recent studies that have been concerned with the role of lipid peroxidation in postischaemic injury have focused mainly on the role of reperfusion in its pathogenesis.¹⁴⁻¹⁶ The purpose of this study was to examine the extent of such injury in rat liver tissue after ischaemia alone or with subsequent reperfusion, and to explore the effect of iron chelation on its prevention or amelioration.

Methods

EXPERIMENTAL ANIMALS

Young male, Sprague-Dawley, rats each weighing approximately 250 g, were used after about one week of acclimatisation, during which they were provided with food and water *ad libitum*. Animals were divided into five experimental groups: group I. Animals killed after minimal or no stress. No surgery was performed on these animals before death. Data obtained from this group served as a base line; group II. Animals which were subjected to anaesthesia, laparotomy and 30 minutes of liver ischaemia after

which they were immediately killed (without reperfusion). They were intravenously injected with 0.5 ml normal saline five minutes before the induction of ischaemia; group III. Reperfusion was allowed in these animals after 30 minutes of liver ischaemia. They were intravenously injected with 0.5 ml normal saline, five minutes before reperfusion; group IV. Same as in group II but instead of saline, animals were injected with 0.5 ml solution containing DFR (Ciba-Geigy, Summit, NJ, USA), 60 mg/kg body weight; group V. Same as in III but instead of saline, animals were injected with DFR, 20 mg/kg (subgroup Va), 40 mg/kg (subgroup Vb), or 60 mg/kg (subgroup Vc).

Eight animals were used in each of groups I, II, and IV. In these groups all animals were killed by decapitation at the end of the procedure as outlined above. Their livers were immediately removed, placed on ice, dissected and representative samples used for assessing tissue injury by means of measuring lipid peroxidation and histopathological examination. For groups III and V, 16 animals were used in each group or subgroup: eight were killed two hours after the procedure and their liver tissue used for determining lipid peroxidation while the remaining eight were allowed to recover from surgery. These were killed 24 hours later and their livers used for histopathological examination. This delay was necessary since it usually takes more than eight to 12 hours before irreversible cell injury (necrosis) becomes detectable by light microscopy. Obviously, such a waiting period is incompatible with the experimental design of groups I, II, and IV where only ischaemia (without reperfusion) was intended. Hence, the same animal in each of these groups was used for both, the histological and the biochemical studies immediately after the ischaemic period while duplicate animals were used in the reperfused groups.

LIVER ISCHAEMIA

Rats were anaesthetised with an intraperitoneal injection of pentobarbital sodium, 40 mg/kg body weight. This was followed by 80 units of heparin intravenously. Liver ischaemia was induced by cross clamping of the entire hilar pedicle (hepatic artery, portal vein and common bile duct). In animals where reperfusion was intended, the clamp was released at the end of the ischaemic period. All intravenous injections were done via the right femoral vein.

LIGHT MICROSCOPY

Slices of liver tissue were fixed in 10% buffered formalin, routinely embedded in paraffin and 5 μ sections stained with haematoxylin and eosin (HE) and Periodic Acid-Schiff (PAS) methods. Slides were examined by light microscopy and the extent of

hepatocellular pathology evaluated. This was done in a double blind fashion using coded slides.

LIPID PEROXIDATION

Malondialdehyde (MDA) is the major product of peroxidation of polyunsaturated fatty acids and hence, it has been widely used as an index of free radical mediated lipid peroxidation.¹³ We measured MDA concentrations in liver tissue homogenates using the method described by Slater and Sawyer.¹⁹ Two grams of liver tissue were cut into small fragments, washed with normal saline and homogenised in 1 ml distilled water while on ice. After homogenisation, 4 ml 5% trichloroacetic acid were added and the homogenate thoroughly mixed. This was followed by centrifugation at 2000 g for 15 minutes 1.5 ml of the supernatant was mixed with 1.5 ml of 0.67% thiobarbituric acid and the mixture heated at 100°C for 30 minutes. The solutions were then cooled on ice for five minutes and the absorbance of the individual samples determined at 532 nm wavelength. The concentration of MDA (in $\mu\text{mol/kg}$ liver tissue) was calculated using an extinction coefficient of 153 000/ Mol/cm.

STATISTICAL ANALYSIS

The Student's *t* test and one-way analysis of variance were used to determine the level of significance for observed differences among the various groups.

Results

The results of MDA determinations are presented in Table 1. Liver ischaemia (group II) was associated

with a significant increase in MDA concentration over baseline (group I). Ischaemia followed by reperfusion (group III) was also associated with an MDA level that is still significantly higher than in group I but much lower than in group II. Treatment with DFR (60 mg/kg), five minutes before the induction of ischaemia resulted in a significant reduction of MDA level (group IV). When ischaemia was followed by DFR treatment and reperfusion (group V), MDA concentrations returned to near basal values (subgroups Va and Vb) or even lower (subgroup Vc).

The histopathological changes observed in representative sections from liver of animals killed 24 hours after ischaemia were assigned histological scores (HS) based on the extent of hepatocellular injury as follow: HS 0–1: No changes or reactive changes only (swelling, congestion, single cell drop-out and glycogen depletion). HS 2: Mild injury; one or more minute foci of necrosis, the largest involving approximately less than 1% of examined sectional area of the lobule. HS 3: Moderate; as in 2 but the necrotic foci are approximately 1–5% of the lobule. HS 4: Severe; as above but more than 5%.

Histological scores for the eight animals from which histological sections were studied in each group or subgroup are presented in Table 2.

Discussion

Our data show that liver ischaemia was associated with an increase in the concentration of MDA, a byproduct of lipid peroxidation. This indicates that free radical mediated peroxidation of membrane

Table 1 Malondialdehyde concentrations ($\mu\text{mol/kg}$ liver tissue) of individual animals from the various experimental groups. Significant difference ($p < 0.05$) is indicated by: *, from I; †, from II; ‡, from III

Animals	Experimental groups						
	Without DFR				With DFR		
	I Control	II Ischaemia	III Ischaemia + reperf.	IV 60 mg/kg + ischaemia	V Ischaemia + DFR + reperfusion		
					Va 20 mg/kg	Vb 40 mg/kg	Vc 60 mg/kg
1	2.76	8.62	3.51	2.07	2.02	1.72	1.11
2	1.02	1.01	2.00	2.20	2.23	3.13	1.15
3	1.29	5.27	2.52	1.72	1.36	1.58	1.44
4	3.07	8.18	2.54	2.01	2.15	1.55	1.03
5	1.47	4.20	3.42	1.76	1.51	2.42	1.35
6	2.08	1.11	4.04	2.31	2.21	2.96	1.41
7	1.20	8.39	3.73	1.65	2.10	1.17	1.50
8	1.35	1.30	1.20	2.12	1.30	2.11	1.09
Mean	1.78	4.76*	2.87*	1.98†‡	1.86†‡	2.08†	1.26†‡
SE	0.27	1.19	0.34	0.08	0.14	0.25	0.06

Table 2 Number of animals in each group assigned a particular score of liver pathology: 0–1, none or reactive changes; 2, mild; 3, moderate; 4, severe injury. Experimental setting for each group is as in table 1.

Group	Histological score			
	0–1	2	3	4
I	8*			
II	8*			
III		1	2	5
IV	8*			
Va	3	3	1	1
Vb	4	2	1	1
Vc	4	2	2	

*No changes detectable by light microscopy were expected as these animals were killed immediately after the procedure.

lipids might have been a key pathogenetic mechanism in the chain of events that eventually lead to hepatocellular necrosis which we observed histologically in surviving animals. Liver reperfusion was expected to promote an even higher degree of lipid peroxidation as a result of the sudden increase in oxygen uptake and free radical generation by liver cells. While this might, indeed, have transiently been the case, our data show that the 'net' lipid peroxidation product (MDA) after reperfusion, although high, was actually lower than that seen after ischaemia alone. The explanation for this seemingly unexpected result probably lies in two phenomena that might have been previously overlooked. First, the absence of blood flow during ischaemia leaves many of the freshly generated free radical species in place, thus allowing them to react with other molecules and enter in free radical producing chain reactions. Second, by halting the blood flow, liver tissue is deprived from numerous antioxidants or free radical scavengers that are ordinarily brought in by the circulating blood. These two phenomena are reversed upon reperfusion with a net result, apparently, favouring a modest reduction in the cumulative amount of local free radicals.

In both situations (ischaemia alone and ischaemia with subsequent reperfusion), however, it appears that iron is a key catalyst so that its neutralisation was associated with a significant reduction in lipid peroxidation. This was accomplished by using the iron chelating agent deferoxamine. A single dose of 60 mg/kg body weight, given either before the induction of ischaemia or before reperfusion, was sufficient to prevent most, if not all of the harmful lipid peroxidation. This was evident not only by comparing the concentrations of MDA in liver homogenates, but also by histopathological examination of liver tissue from surviving animals. In about 80% of these

animals, evidence of only minimal to moderate hepatocellular injury was found while about 60% of the nontreated animals showed evidence of severe injury. At this dose (60 mg/kg), DFR was relatively protective even against a more extended ischaemia. In an unpublished study, only three of 21 animals (14.3%) which we subjected to 45 minutes of liver ischaemia survived. Pretreatment of a similar group of animals with DFR resulted in eight (38.8%) survivals. Deferoxamine in doses higher than 60 mg/kg did not provide a significant additional protection. Lower doses (40 and 20 mg/kg), however, while relatively beneficial were less protective.

Measuring MDA concentrations in a variety of tissues has been widely used to evaluate the extent of lipid peroxidation and hence, free radical injury. Evidence of the latter in rat liver after surgically induced ischaemia was documented by Marubayashi *et al.*,^{7,20} who found a decrease in α -tocopherol and glutathione (intracellular free radical scavengers) and a slight increase in MDA concentrations. All of these changes, however, became more significant after restoration of blood flow. The discrepancy between their findings and ours may be because of the differences in experimental protocol as they added a 'portofemoral shunt' to the vessel clamping procedure. These authors and others¹ used low molecular weight free radical scavengers to ameliorate postischaemic liver injury with variable success. In other experiments, high concentrations of MDA were found in myocardial tissue which had been reperfused after prolonged regional ischaemia.²¹ Reduction in MDA production was achieved by administering SOD during reperfusion. Increased MDA in ischaemic myocardium was associated with an increase in low molecular weight chelate forms of iron²² which supports the proposed role of iron in catalysing lipid peroxidation during ischaemia/reperfusion. Similar results were observed in experiments with brain ischaemia and reperfusion in animals.²³ An increase in MDA concentrations in these experiments was prevented, like in our studies, by treatment with DFR.

Among several known metal chelators, DFR has proved most effective in inhibiting iron mediated free radical production. It is a specific chelator of Fe^{+++} and can be injected into humans and experimental animals where it easily penetrates the blood brain and cell membrane barriers.^{23–25} Iron bound in the DFR complex is chemically inert and iron dependent lipid peroxidation cannot occur in the presence of stoichiometrically adequate amounts of DFR.²⁴

Deferoxamine and other drugs, such as the xanthine oxidase inhibitor allopurinol,²⁶ which can prevent the formation of free radical species or their propagation, in the first place, may prove far more

beneficial than free radical scavengers which act upon already formed free radicals.

With the continuing necessity of intraoperative liver ischaemia for various surgical manipulations, experimental models similar to the one herein described have obvious practical implications, although more in depth studies are still needed.

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