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THE EFFECTS OF IRON-MEDIATED OXIDATIVE STRESS IN ISOLATED RENAL CORTICAL BRUSH BORDER MEMBRANE VESICLES AT NORMOTHERMIC AND HYPOTHERMIC TEMPERATURES

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Experiments on renal cortical brush border membrane vesicles have been undertaken in order to assess the involvement of iron in oxidative stress at physiological temperatures and under conditions of hypothermia. A decrease in temperature stimulated iron-induced lipid peroxidation. The results are discussed in relation to the role of the oxidation state of the iron and iron(II)/iron(III) ratios in the initiation of peroxidative events.

KEY WORDS: Iron, lipid peroxidation, renal BBMV, hypothermia, iron II/iron III ratios.

INTRODUCTION

Clinical renal transplantation is a successful procedure for the treatment of end-stage kidney disease. However, several problems remain to be solved; one of these is the prevention of damage to the donated kidney in the period between harvesting and re-implantation in the chosen recipient patient (a time interval which may comprise many hours). There is increasing evidence that organs subjected to such periods of cold ischaemia (i.e. isolated from a blood supply and packed in ice) are susceptible to damage from a form of oxidative stress, both during the cold storage period and additionally during the time of resumption of blood supply.

Evidence of increased susceptibility to lipid peroxidation was demonstrated in homogenates of tissue from rabbit kidneys which had previously been subjected to periods of ischaemia both at 37°C and at 2°C.[1,2] These changes correlated with a reduction in antioxidant status of the tissues as displayed by the ratios of reduced to oxidised glutathione. A period of blood reperfusion after ischaemia further increased the production of fluorescent chromolipids (via Schiff's base formation) from peroxidation of homogenates.[3,4] Other workers reported improved recovery of renal function after ischaemia when anti-radical agents such as superoxide dismutase or allopurinol had been administered to animals during kidney ischaemia and/or at the time of blood reperfusion.[5,6] The consensus of many such studies is to invoke oxygen-derived free radical reactions in damage resulting from renal ischaemia and reperfusion. In experiments to investigate the pathogenesis of these interactions, we noted

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that this damage could be modified by the presence of desferroxamine, an iron-chelator, although complete protection was not achieved.[7] The role of iron in ischaemia/reperfusion damage during the cold storage period was a matter for question, since our early studies were performed on whole tissue homogenates incubated at 37°C after the cold period.[2]

The present studies were undertaken to assess the mode of action of exogenous iron in oxidative stress of a defined membrane fraction, namely, brush border membrane vesicles[8,9] (prepared from porcine kidneys) at cold temperatures (2°C) and at 37°C.

METHODS

Due to the complexity of mammalian kidneys and the variety of cell types present, a defined *in vitro* system of cortical brush border membrane vesicles was used, which enable experiments to be reproduced in terms of membrane concentration and membrane purity. Kidneys from anaesthetized adult male pigs were removed and flushed with a cold salt solution as in previous studies.[2,4] The cortices were removed by dissection and homogenised with 10 mM Hepes/50 mM solution of mannitol (1 g tissue/30 ml buffer). All solutions were maintained at 2°C throughout the preparation to minimise membrane damage. A differential centrifugation method[8] was then used to obtain brush border membrane vesicles (BBMV). The concentration of the vesicles was determined by the protein assay of Lowry *et al.*[10] and the purity of the vesicles was estimated by measuring the content of trehalase (a constituent enzyme of BBMV[8]) in the membrane fractions. Enrichments of approximately six-fold were obtained in the trehalase content at the BBMV fractions, when compared with the enzyme content of the original crude homogenate, which is comparable to other studies[9] The vesicles were then stored in liquid nitrogen.

BBMV were diluted 1:10 with phosphate-buffered saline to give a final protein concentration range of 0.6–1.6 mg/ml. 10 ml of this dilute mixture were used for each iron stress experiment. 100 μl of stock $FeSO_4$. $7H_2O$ was added to give a final concentration of 0.1 mM. This stock solution was made up in double-glass-distilled water immediately before use to minimise spontaneous oxidation to iron (III). Samples were incubated at either 2°C or 37°C for 90 minutes, aliquots were taken at appropriate time intervals and immediately assayed. Samples of brush border membrane vesicles (2 ml) were taken and the levels of thiobarbituric acid- reactive (TBAR) compounds were measured by the method of Yagi.[11] For this assay, reaction tubes were set up containing 7% (w/v) sodium lauryl sulphate (0.2 ml), 0.1 MHCl (2.0 ml), 10% (w/v) phosphotungstic acid (0.3 ml) and 0.67% (w/v) thiobarbituric acid (1.0 ml). To these were added 2 ml of the BBMV samples, and they were heated at 95°C for 60 minutes. The tubes were then cooled and 5 mls of butan-1-ol (gold label grade) were added to each, followed by mixing and centrifugation. The chromophore in the solvent phase was measured by spectrofluorimetry (λ ex 515 nm, λ em 540–560 nm). Lipids were extracted from the vesicles using chloroform/methanol.[12] Fluorescent chromolipids[13] were detected by measuring the relative fluorescence intensity with a Perkin-Elmer MPF 44B spectrofluorimeter (λ ex = 350 nm, λ em = 390–500 nm) standardised with quinine sulphate (10 ug/ml 0.1 M H_2SO_4). The concentrations of phospholipid extracted from the vesicles were measured using a phosphorus assay.[14]

Total iron levels in BBMV preparations were measured by atomic absorption spectroscopy at 248.3 nm using a Perkin-Elmer 3030 atomic absorption spectrometer.

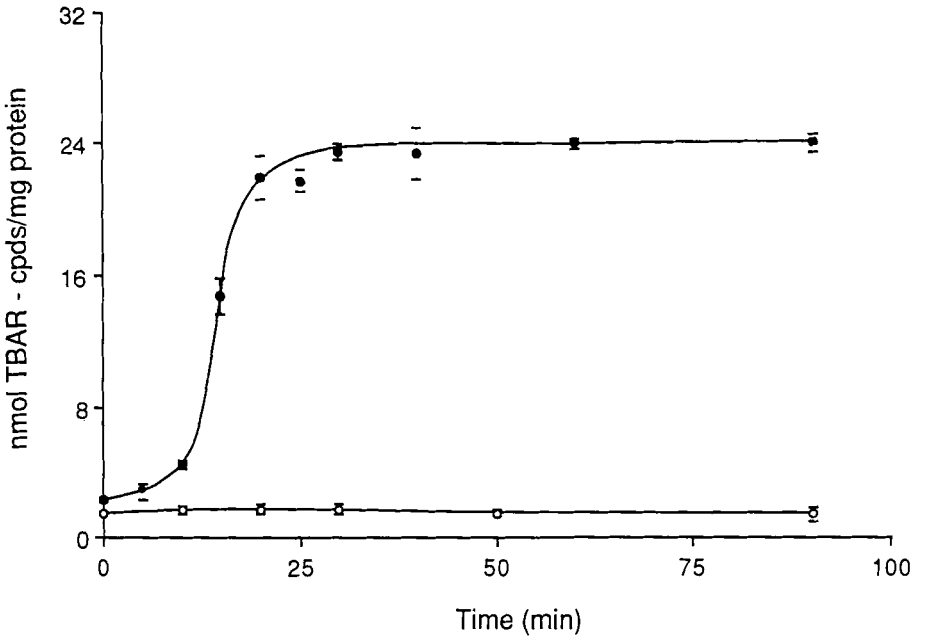

FIGURE 1 Time course of lipid peroxidation during a 90 minute period of BBMV (1.0 mg protein/ml) incubated at 2°C in the presence of either iron (II) (0.1 mM) –●–● or iron III (0.1 mM) –○–○–. Points are average values (n = 2) corrected for blank controls; variation shown by bars.

Samples of known protein concentration, were evaporated, resuspended in 2 ml of concentrated nitric acid and digested at 140°C for 20 min. Standards and blanks were incorporated.

RESULTS

Brush border membrane vesicles were incubated with iron (II) (0.1 mM) at 2°C for 90 min in order to establish the time course for production of TBAR-compounds in this non-reducing system.

After an initial lag period of about 10 minutes (Figure 1), the level of TBAR-compounds increased and was maximal after 40 minutes. Similar experiments were performed with iron (III) (0.1 mM) which did not show an increase in levels of TBAR-compounds. After 90 minutes the levels in the iron (III) incubation were the same as control membranes incubated in the absence of iron. Dose-response curves of a fixed concentration of BBMV (0.9 mg/ml) incubated with a range of initial concentrations of iron (II), from 0–0.1 mM, for 90 min, were investigated to determine an effective concentration with respect to lipid peroxidation at 2°C (Figure 2). An initial concentration of 100 μM iron (II) was found to give the highest levels of TBAR-compounds under these conditions. The effect of incubating a range of BBMV concentrations with initial concentrations of 0.1 mM iron (II) at 2°C was investigated to determine optimum vesicle concentrations (in effect, the optimum iron:lipid ratio) with respect to both TBAR-compounds and chromolipid production after 90 min.

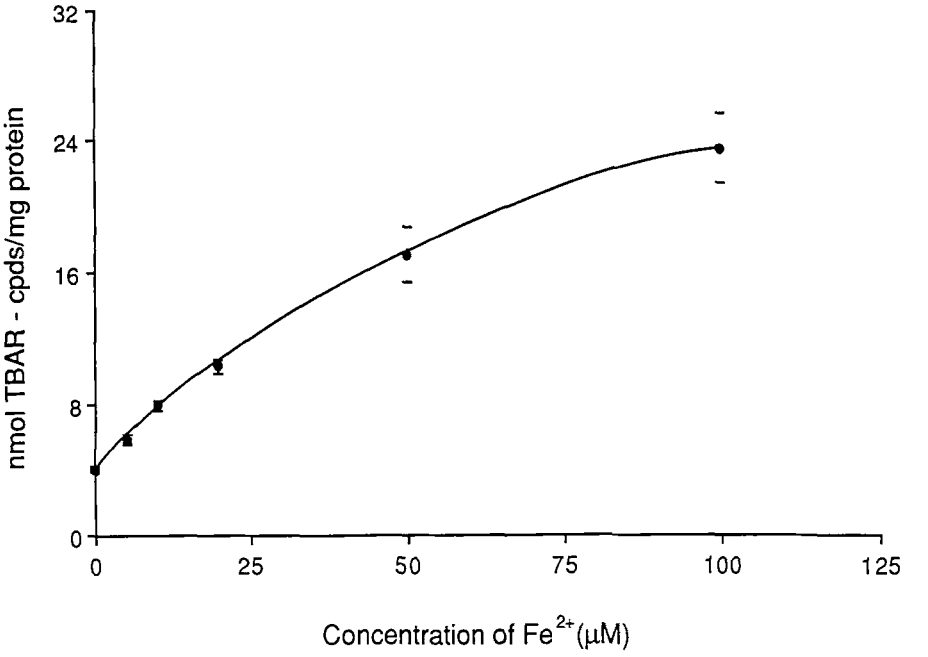

FIGURE 2 The variation in levels of TBAR-compounds with increasing initial concentrations of iron (II). In all cases BBMV (0.9 mg/ml) were incubated at 2°C for 90 minutes with the various initial concentrations of iron (II). Points are means (n = 2); variation shown by bars.

(Figures 3a and 3b). In each case higher levels were obtained from more dilute systems. (A range of 0.6–1.6 mg protein/ml was found to be most appropriate).

The effect of temperature on the extent of lipid peroxidation (Figure 4a and 4b) was investigated by incubating dilute BBMV with iron (II), initial concentration 0.1 mM, at 2°C and at 37°C. Control samples were incubated at the same time under similar conditions but in the absence of iron. Experiments were also performed in which desferrioxamine was added to the vesicles before the iron (II). Incubation of BBMV under iron stress at 37°C induced less lipid peroxidation in terms of TBAR-products (Figure 4a) than was observed at 2°C. Desferrioxamine suppressed lipid peroxidation as expected. The effect of temperature on iron-mediated lipid peroxidation in terms of the formation of chromolipid complexes was also investigated (Figure 4b). The levels of fluorescent chromolipids were greater in vesicles that had been incubated at 2°C than at 37°C, indicating again that lipid peroxidation had occurred to a greater extent at the lower temperature.

Endogenous iron levels in the BBMV preparations were assessed by atomic absorption spectroscopy and shown to be 3 nmol/mg protein. This low iron level did not contribute towards the oxidative stress as shown by the lack of response of incubated control membranes.

DISCUSSION

The results in the present study indicate that iron in the absence of reducing agents

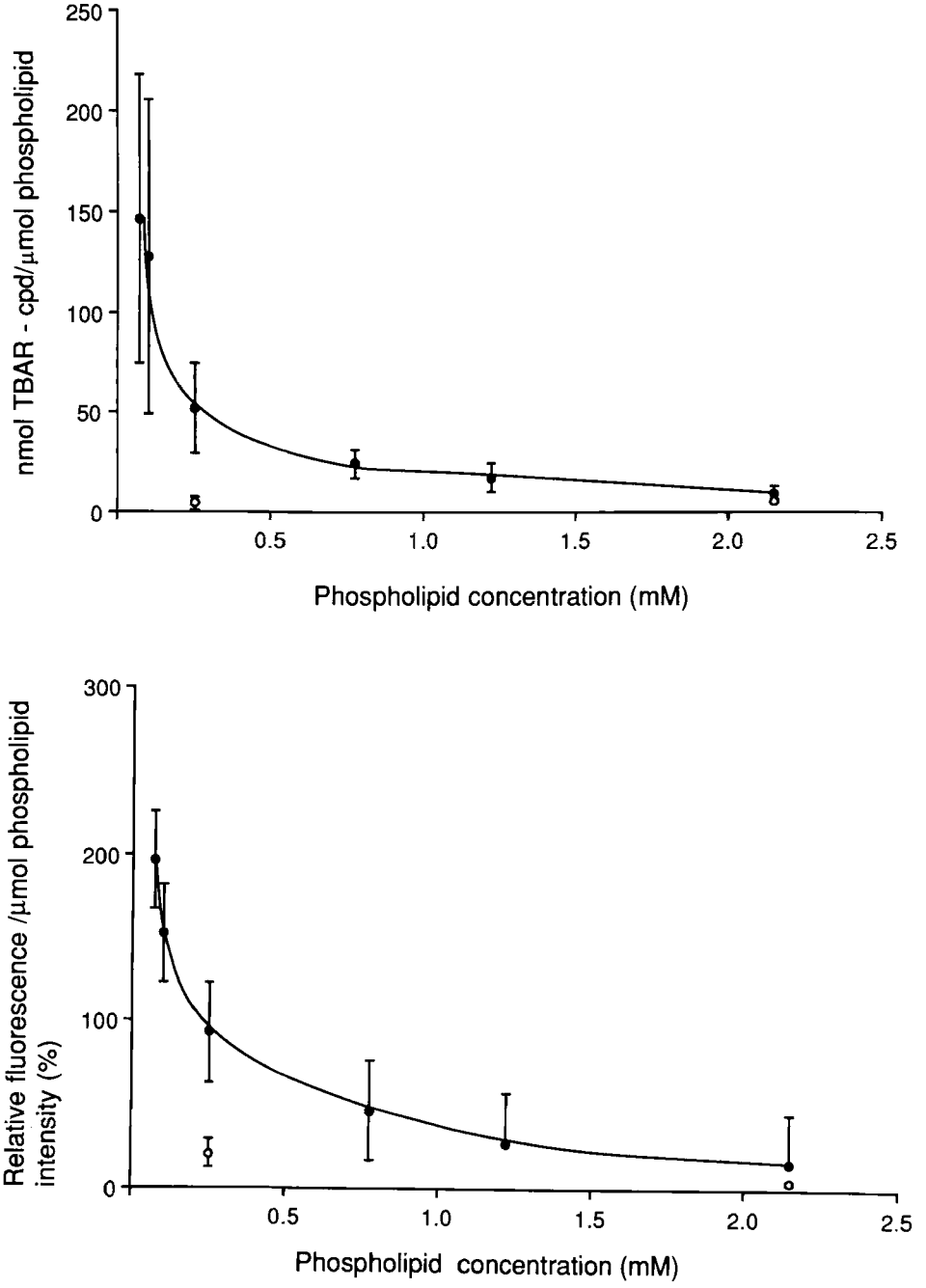

FIGURE 3 The effect of BBMV concentration on lipid peroxidation induced by iron (initial ferrous concentration 0.1 mM) after incubation at 2°C for 90 mins in terms of: (a) TBAR-products and (b) fluorescent chromolipids. —●—● iron-stressed, —○—○— controls Points represent means ± standard deviation (n = 3).

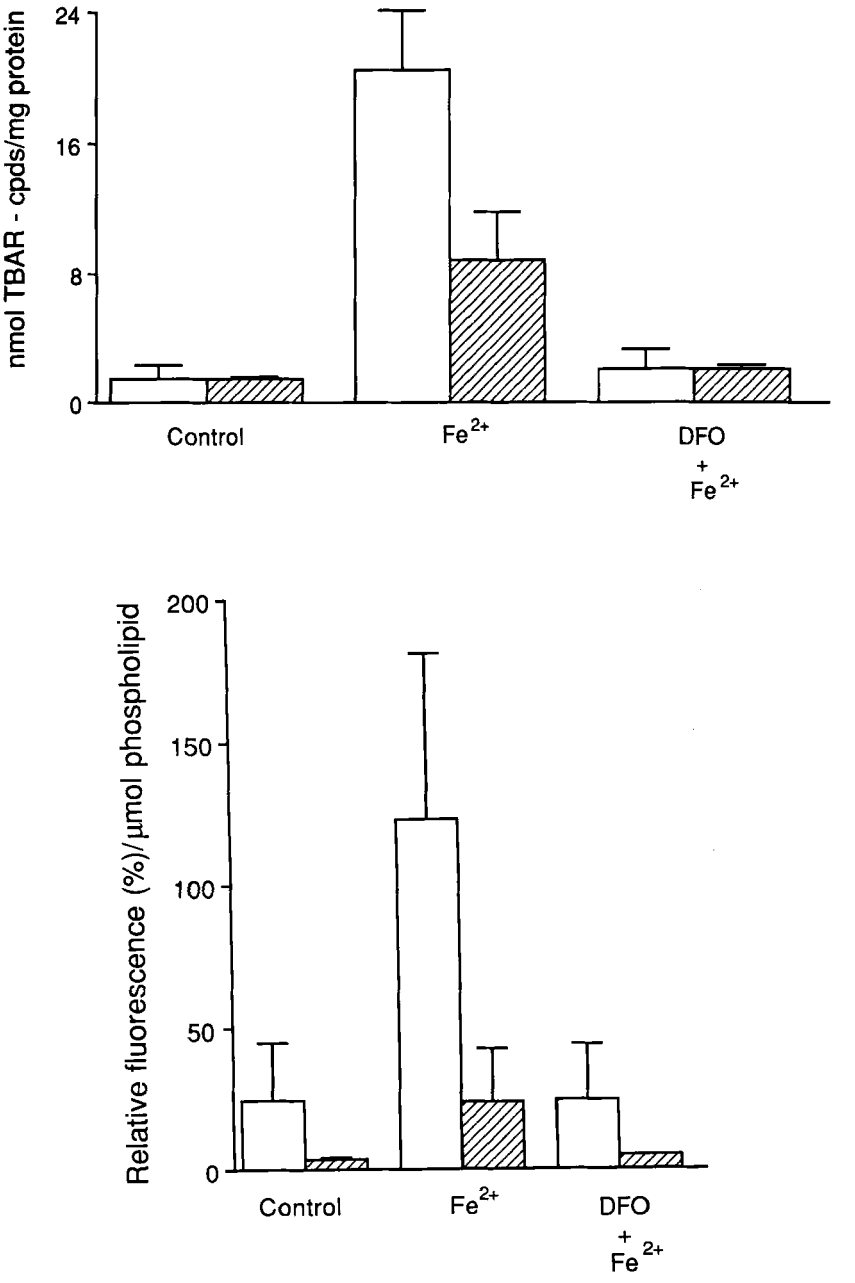

FIGURE 4 The effect of temperature on lipid peroxidation in (a) dilute BBMV (0.6–1.6 mg protein/ml) incubated with initial iron (II) (0.1 mM) ± desferrioxamine (1 mM) for 90 min at 2°C (open columns) and 37°C (hatched columns): (a) TBAR-products (b) fluorescent chromolipids. Mean values are shown ± standard deviation ($n \geqslant 5$). (Significance values from controls: (a) 2° $p = 0.001$, 37°C $p = 0.001$ (b) 2°C $p = 0.02$, 37°C $p = 0.15$) Significance values of iron-stress at 2°C from 37°C: (a) $p = 0.001$, (b) p 0.038.

can stimulate lipid peroxidation in BBMV, both at normal body temperatures and at 2°C.

The time course of TBAR-production when ferrous ions were added to membranes in the absence of reducing agents at 2°C indicate that there was a lag period of some 10 minutes, before levels of TBAR-product increased in an exponential fashion, to reach a plateau afer 40 minutes. This type of response is consistent with previous data on lipid peroxidation in microsomal preparations. The lag period in those studies[15] was attributed to the ability of inherent antioxidant defence mechanisms in the membrane to 'break' the chain of lipid peroxidation; once these inhibitors had been consumed, peroxidation was able to proceed. Some workers have implicated the importance of ferrous/ferric ratios in the time-dependency of the onset of lipid peroxidation,[16,17] attributing the lag period to the time required to achieve a catalytic ratio of the reduced and oxidised species. To investigate this our preliminary investigations have applied equimolar ratios of ferrous: ferric ions (50 μM) in BBMV stressing systems and the consequent elimination of the lag phase is in agreement with this interpretation. The total inhibition of lipid peroxidation in the presence of desferrioxamine (a ferric ion chelator) reinforces the importance of ferrous/ferric ratios in the peroxidation process.

The limitation of the maximal formation of peroxidation products was not consuption of available fatty acid substrates: when an additional bolus of ferrous ions or ascorbate was added to the BBMV preparation at the end of the 90 minute period there was an additional burst of lipid peroxidation, demonstrating that substrate availability was not the limiting factor.

The mechanism of initiation of iron-mediated lipid peroxidation in BBMV remains a matter of discussion. It has been proposed that ferrous ions may interact with molecular oxygen in solution to liberate O_2^-.[18] Slow, non-enzymatic dismutation of O_2^- to H_2O_2 in aqueous media can occur, providing substrate for the formation of $OH^\cdot$ by interaction with further ferrous ions. However, high concentrations of mannitol (up to 50 mM), had very little effect on lipid peroxidation in the renal BBMV (data not shown); this would argue that the classical role of $OH^\cdot$ cannot be assumed, unless the $OH^\cdot$ was being produced at a site not accessible to mannitol. Another proposition may be that the ferrous ions were causing breakdown of preformed lipid hydroperoxides; it has been suggested that tissues contain low levels of such hydroperoxides as a consequence of normal metabolism. However, ferric ions which can also catalyse breakdown of lipid hydroperoxides,[19] had little effect on renal BBMV, so that this does not apply here. A third proposition may be the direct interaction of ferrous ions with oxygen to generate an iron-oxy radical capable of initiation. Such activation of ground state oxygen by iron has been proposed previously[20] and the studies on iron-stress of hepatic lysomsomes[21] would tend to support this. In those systems, as in our present studies, addition of ferrous ions resulted in lipid peroxidation which could not be inhibited by mannitol or superoxide dismutase + catalase.

The study of the concentration-dependency of lipid peroxidation shows that at higher membrane concentrations or at lower iron concentrations for a fixed membrane concentration less peroxidation was evident. A similar relationship has been observed by others in microsomal membranes.[22] This implies that fatty acid substrate:iron ratios are important in the peroxidation process.

Perhaps the most interesting finding in our studies was the temperature-dependency of lipid peroxidation.[23] The difference observed between membranes of fixed concentrations, with standard ferrous ion stress at 2°C as opposed to 37°C suggests that

in cells and tissues held at low temperatures, the sensitivity to iron-catalysed oxidative stress is greater. This may have implications for many procedures where low temperatures are employed to store living cells, such as transfusion or transplantation. One possible explanation for this observation may be the effect on the solubility of oxygen as the temperature is reduced. Another possibility may be that the biophysical nature of the bilayer of the BBMV changed with reduced temperature in such a way as to enhance susceptiblity to oxygen and lipid peroxidation. For example, reduced temperature affects the fluidity of biomembranes, and the distribution of various molecular species within the plane of the membrane;[24] whether this has an enhancing effect on lipid peroxidation remains to be determined.

The present studies highlight several important technical points for the study of iron-linked oxidative stress in membranes *in vitro*. Careful consideration must be given to choice of iron and membrane concentrations, otherwise widely differing results may be achieved. The use of reduced temperatures to store membrane samples between different treatments must also be viewed with caution, since enhanced response may result.

In summary, renal BBMV underwent lipid peroxidation in the presence of iron and oxygen, and this was enhanced at low temperatures. The classical $OH^{\cdot}$ scavenger (mannitol) was ineffective in presenting the damage. The iron concentration (100 μM) added was high when compared to values expected for low molecular-weight iron or decompartmentalised iron in cells, but the system we have investigated was a non-regenerating system, so that lower initital ferrous ion concentrations, continually regenerated may be equally damaging. The studies suggest that further investigations into the damaging role of catalytically available iron complexes during organ storage for transplantation are warranted.

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