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# THE IMPORTANCE OF IRON, CALCIUM AND FREE RADICALS IN REPERFUSION INJURY: AN OVERVIEW OF STUDIES IN ISCHAEMIC RABBIT KIDNEYS

C.J. GREEN, J.D. GOWER, G. HEALING, L.A. COTTERILL, B.J. FULLER and S. SIMPKIN

*Section of Surgical Research, MRC Clinical Research Centre, Northwick Park Hospital, Harrow, Middlesex HA1 3UJ*



An overview of a series of experiments attempting to link iron and calcium redistribution and release of free fatty acids with falls in pH and adenine nucleotide levels during cold storage of rabbit kidneys is presented. The data reviewed strongly suggest that these events are inextricably linked to subsequent reperfusion injury. Circumstantial evidence incriminating iron was provided by experiments showing that iron chelation decreased reperfusion injury after warm (WI) and cold ischaemia (CI) in rat skin flap and rabbit kidney models. Evidence for a role for calcium was provided when it was found that a calcium channel blocking agent added to the saline flush solution before storage inhibited lipid peroxidation, whereas chemicals which caused release or influx of calcium into the cell exacerbated oxidative damage. Additional involvement of breakdown products of adenine nucleotides was suggested by the protection from lipid peroxidation afforded by allopurinol. Involvement of calcium-activated phospholipase $A_2$ was strongly suggested by increases in free fatty acids during cold storage and both this increase and lipid peroxidation were inhibited by addition of dibucaine to the storage solution.

KEY WORDS: Iron, calcium, reperfusion injury, ischaemia, kidney.

Retrieval of donor kidneys in optimum condition is one important aspect of clinical renal transplantation. The organs are usually subjected to a short period of warm ischamia (WI) between cessation of the blood supply and harvesting, followed by various periods of cold ischamea (CI) whilst they are stored and transported to the scheduled recipient. They are then rapidly reperfused with fully oxygenated whole blood as soon as the vascular pedicle is reconstructed. Typically, the preservation time in renal transplantation is less than 24 hours but may be as long as 72 hours: other organs are more sensitive to ischaemic damage, and it is considered inadvisable to store liver, heart or lung for longer than 4–12 hours. There is no clear CI time beyond which subsequent function can be predicted to fail, but it is an indictment of our current harvesting and storage techniques that in some centres as many as 50% of transplanted kidneys fail to function immediately, resulting in patients requiring dialysis until the organs recover sufficiently to support life.

The causes of deterioration during prolonged periods of *ex vivo* storage remain unresolved. Several pathological events have been confirmed including depletion of high-energy adenine nucleotides,[1] accumulation of metabolites, particularly $H^+$ ions,[2] autolysis by release of lysosomal enzymes,[3] damage to cellular membranes with loss of constituent phospholipids[4] and vascular injury that results in oedema, loss of circulating proteins, loss of erythrocyte deformibility, sludging and leakage of blood

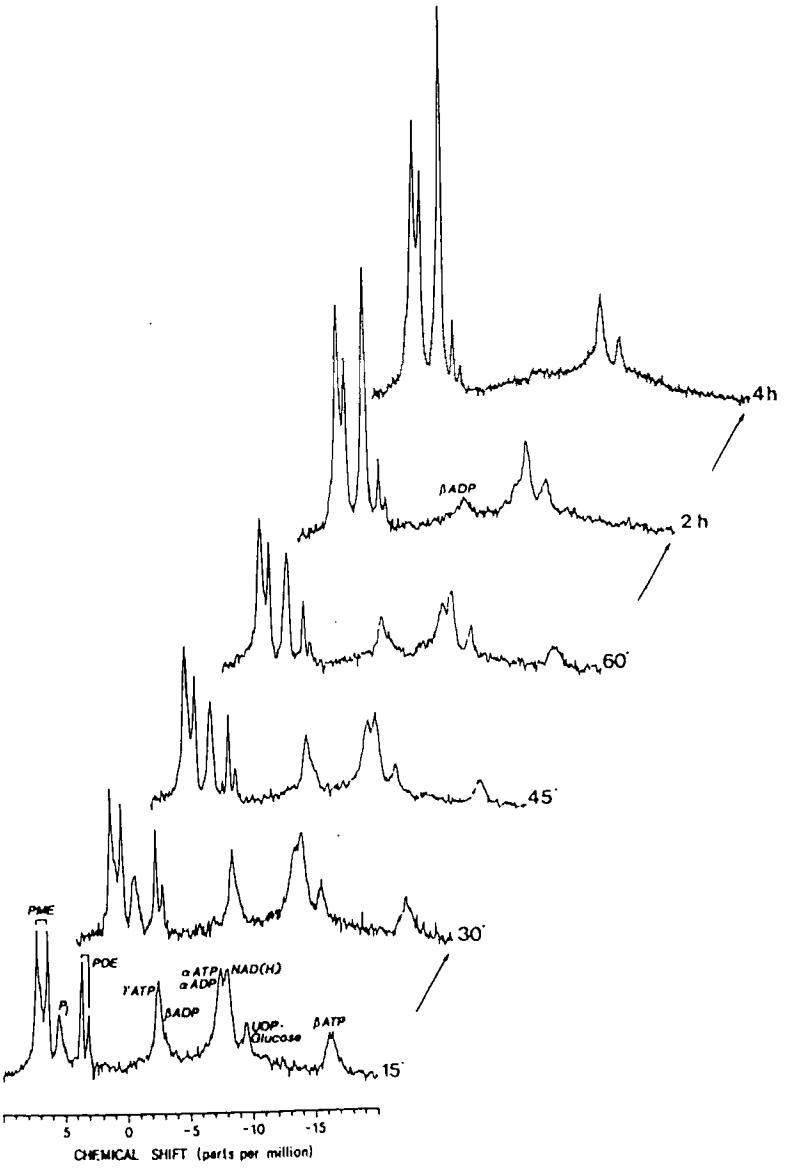


FIGURE 1 $^{31}$Phosphorus nuclear magnetic resonance spectra for a rat liver stored at 4–8°C after vascular flush with hypertonic citrate solution. Time of cold storage increases from the first spectrum (bottom) at 15 min up to 4 hr. Signals for $\alpha$, $\beta$, and $\gamma$ phosphates of ATP, $\alpha$ and $\beta$ phosphates of ADP, inorganic ($P_i$), phospho-mono and di-ester — PME, PDE are identified. It can be seen that during cold ischaemia, there is a decrease in ATP and ADP, with an increase in PME (including AMP) and $P_i$. There is also a fall in pH (not depicted) (after Fuller *et al.*, 1987). Reproduced with permission of the Society for Experimental Biology Symposium XXXX1.

cells into the extravascular compartment. Severely damaged kidneys are slow to perfuse when revascularised, become mottled in appearance and develop a micro-coagulopathy which results within minutes in an outflow block and venous stasis. The spectrum of perservation damage is similar if not identical to that seen in simple normothermic renal ischaemia although hypothermia undoubtedly slows the rate of deterioration. For example, from our own studies using $^{31}P$ NMR spectroscopy with cold preserved rat liver and kidneys, ATP and ADP peaks virtually disappear after 2–4 hr CI and this is accompanied by a rise in inorganic phosphate levels (Figure 1). Measured pH falls to around 6.9 in 6–8 hr of simple refrigeration after flush with either Euro-Collins, hypertonic citrate or the recently formulated lactobionate-hydroxy ethyl starch (UW) solutions.[5] In contrast, in rabbit kidneys, such changes can be anticipated within 10–30 mins of WI and these organs are irreversibly damaged within 2 hours.[6]

It has become fashionable to talk about 'reperfusion injury' almost as if this occurs independently of the ischaemic period itself or as if the damage incurred during ischaemia was incidental rather than essential in predisposing the organ to failure. Mere commonsense dictates that this cannot be so. Nevertheless, it is true that at a visual level (both microscopic and macroscopic) many pathological changes only become evident after restoration of blood flow through the organ. For example, several workers have shown that most myocardial damage occurs during the early phases of recirculation and reoxygenation after normothermic ischaemia.[7,8] In some of our earlier experiments with rabbit kidneys attempting to correlate increases in lipid peroxidation either with warm or cold ischaemia *per se* or with additional effects attributable to reperfusion after transplantation, we noted significant increases in oxidative stress associated with reperfusion; in some cases, reperfusion injury was superimposed on increased lipid peroxidation attributable to ischaemia alone, whilst in others it was the only significant damage demonstrable.[6,9]

From that evidence and the work of others which provided circumstantial evidence incriminating oxygen-derived free radicals in the 'storage-damage' syndrome[10–12], we made two base line assumptions around which to design a series of experiments. The first was that lowered adenine nucleotide levels were central to a number of events *during* ischaemia; the second was that damage to the endothelium and its subsequent interraction with incoming blood would inevitably be linked to reperfusion injury. Accumulation of reducing equivalents and fall in pH could be expected to facilitate release of iron from ferritin stores and could therefore increase the susceptibility of the organ to lipid peroxidation on reperfusion. Decreased ATP levels upset $Ca^{++}$ homeostasis as the differential between high extracellular ($10^{-3}$M) and low cytosolic ($10^{-7}$M) concentration is maintained by membrane-bound ATP-dependent pumps. Increased cytosolic $Ca^{++}$ in turn would stimulate several $Ca^{++}$-dependent processes. For example, concentrations of free fatty acids (particularly polyunsaturated acids) would increase under the influence of $Ca^{++}$-dependent phospholipase $A_2$; $Ca^{++}$-dependent protein kinases would be activated; leukotrienes are produced as a consequence of enhanced lipoxygenase activity; and increased hypoxanthine from breakdown of adenine nucleotides would provide a substrate for xanthine oxidase (converted by $Ca^{++}$-dependent proteolysis from xanthine dehydrogenase) to produce superoxide radical anions and thence hydroxy radicals. Some of these events might be most important during ischaemia in predisposing the organ to reperfusion injury but then have indirect consequences during reperfusion. Increased free $Ca^{++}$ and arachidonic acid released from membranes could, for example, upset the delicate

balance in prostaglandin turnover such that vasoconstriction and platelet aggregation was favoured in the vascular bed during reperfusion with blood. Likewise, the vasoconstrictor effects of leukotrienes would be important during reperfusion. This scenario is supported by evidence in which initial infusion with a prostacyclin analogue has been shown to protect rat livers after cold storage,[13] and continuous infusion of $PGI_2$ prevented microcoagulopathy during xenograft hyperacute rejection.[14] Furthermore in rats, subsequent development of acute tubular necrosis can be prevented if ischaemic kidneys are initially reperfused with diluted blood instead of whole blood.[15] After considering these possibilities, we decided to direct our studies toward direct and circumstantial evidence for the interrelationship between iron, $Ca^{++}$ and free fatty acids in cold ischaemic damage using rabbit kidneys as one model and to seek pharmacological means of alleviating this damage in clinical renal transplantation.

## THE ROLE OF IRON

We investigated whether iron is important in ischaemia and reperfusion injury by assessing the value of deferrioxamine (DFX) in a model of warm ischaemia (WI) in which we had previously demonstrated that significant rises in markers of lipid peroxidation Schiff bases (SB) and TBA-reactive material (TBARs) were proportional to 60 or 120 min WI and still further significantly increased after 60 min of reperfusion.[6] DFX, administered to rabbits at 15 or 50 mg/kg i.v. 15 min before reperfusion of kidneys which had been subjected to 60 or 120 min of WI, was found to inhibit detrimental deviations in each marker of lipid peroxidation including glutathione redox activity.[16] This was considered likely to reflect an effect of DFX *in vivo* rather than an *in vitro* effect during incubation of homogenates since relatively high concentrations of DFX had to be added directly to homogenates to achieve similar alterations in lipid peroxidation *in vitro*.[17] DFX also inhibited peroxidation when added to the saline solution used to flush rabbit kidneys prior to 24 hr storage at O°C but, in these experiments, the kidneys were not transplanted and reperfused.[18]

In another model of warm ischaemia and reperfusion injury[19], intravenous injection of DFX to rats 10 min before engraftment of vascularised skin flaps which had been totally ischaemic at 37°C for 14 hours, not only stopped the rise in markers of lipid peroxidation observed in untreated animals, but also prevented the flaps from becoming necrotic (100% necrosis in untreated rats, 8% in DFX treated rats).

In further experiments in which DFX was compared with other agents added to homogenates of cold-stored rabbit kidneys, it was found that DFX effectively inhibited lipid peroxidation in the renal cortex but less so in the medulla whereas the cyclooxygenase inhibitor indomethacin inhibited oxidative stress in the medulla rather than the cortex.[20] Based on this information, experiments were conducted to assess these two agents *in vivo* administered either separately or concurrently.[21] To mimic the clinical situation more closely, rabbit kidneys were flushed with hypertonic citrate solution (HCA) and stored at 0°C for 48 or 72 hours when they were either immediately homogenised and assayed for lipid peroxidation or were autotransplanted and then examined after 60 min reperfusion. There was a significant rise in SB and TBAR levels in both cortex and medulla after 48 and 72 hr storage compared with fresh unstored kidneys. These levels were further elevated when the kidneys were

replanted and reperfused for 60 min. Administration of DFX (50 mg/kg) 15 min before harvesting and before reperfusion reduced the production of SB and TBARs in the cortex after storage to significantly ($p < 0.001$) lower levels, indeed near to control levels. A significant ($p < 0.02$) reductiom was also observed in medulla. Indomethacin (3 mg/kg) alone had no effect on cortical levels of lipid peroxidation but significantly reduced SB and TBARs in the medulla ($p < 0.01$) following storage for 72 hrs. Administration of DFX (50 mg/kg) and indomethacin (3 mg/kg) together prior to storage effectively inhibited lipid peroxidation in both cortex and medulla when compared with untreated stored controls ($p < 0.001$) The combination of the two agents also effectively inhibited the observed rises in SB after 48 hr and 72 hr of CI with 60 min reperfusion.[21] Taken together with the data in which the agents were added to homogenates, these results provided some evidence for the existence of two separate pathways of lipid peroxidation in medulla of ischaemic kidneys — one iron-catalysed and the other cyclo-oxygenase catalysed.

Although these studies provided some circumstantial evidence of a role for iron in storage damage, we now wanted more direct evidence that iron was involved in its initiation. We therefore set out to develop a method for measuring levels of loosely bound iron available for chelation by DFX in tissue homogenates by reversed-phase HPLC and then used this to measure chelatable iron in the stored organs.[22] Rabbit kidneys were divided into cortex and medulla, homogenised in 0.1 M *Tris*-HCl buffer, pH 7.4, and centrifuged at 10,000 gav for 15 mins. Triplicate aliquots of supernatants were incubated with DFX (2 mM) for 1 hour, and the parent drug and its iron-bound form ferrioxamine (FX) were extracted using Bond-Elut $c_{18}$ cartridges. Quantitation of the two forms of drug was achieved using reversed-phase HPLC with u.v. detection and the ratio of FX:DFX was calculated. The amount of chelatable iron in each sample was determined from a standard curve obtained from triplicate standards containing 0, 10 or 25 nmoles iron/ml subjected to the same procedure. The total iron content of the samples was determined by atomic absorption spectroscopy.[22]

Measurement of the FX:DFX ratio in kidney homogenates revealed significant increases in the DFX-chelatable iron pool in both the medulla and cortex after warm and cold ischaemia. In fact, it was virtually doubled after 2 hr of WI and after 24 hr of cold storage at 0°C, whereas the total iron pool remained essentially unchanged.[22] Hence, it appears that iron had been redistributed to more available forms as a result of ischaemia. Since the release of ferritin-bound iron involves reduction of ferric iron to the ferrous state, and we know that pH falls and reducing species accumulate as a result of ischaemia, it is likely that the extra available iron measured was derived from ferritin. It cannot, of course, be stated with certainty that the iron measured in this study is in a form which is available to catalyse the initiation of lipid peroxidation. However, the availability of iron for chelation with DFX strongly suggests that it is available for catalysis of free radical reactions. Redistribution of iron to more accessible pools as a result of ischaemia may be an important factor underlying the increased levels of lipid peroxidation under these circumstances, and provides a biochemical explanation for the effectiveness of DFX.

These studies were extended still further[23] by taking rabbit kidneys which had been subjected to 2 hr WI or 24 hr CI and reperfusing them *ex vivo* at 37°C on a suitable circuit using an oxygenated ($95\%O_2$:$5\%CO_2$) asanguinous crystalloid solution to which had been added haemaccel to provide colloid osmotic pressure. After perfusion for 5, 10, 15, 30 or 60 min the kidneys were removed from the circuit and assayed for

DFX-chelatable iron as above. It was found that although iron levels were raised initially, they returned to normal within 15 min of reperfusion. This period, although short, would be sufficient for initiation of free radical reactions and reperfusion injury.

## THE ROLE OF CALCIUM

Raised concentrations of cytoplasmic $Ca^{++}$ have been linked to irreversible cell injury in many systems[24] but whether this is the common denominator *initiating* cell death or is merely a post-mortem *result* of cell death is difficult to determine. Some studies do suggest that $Ca^{++}$ must be a prime suspect as the trigger for a catastrophic cascade of events. For example, the presence of $Ca^{++}$ has previously been reported to induce damage to isolated renal mitochondria *in vitro*[25] and potentiated oxygen free radical-mediated damage to plasma membranes in isolated hepatocytes.[26] As the differential between high extracellular ($10^{-3}$ M) and low cytosolic ($10^{-7}$ M) concentrations is normally maintained by membrane-bound ATP-dependent pumps which extrude $Ca^{++}$ from the cell, and sequester excess cytosolic $Ca^{++}$ in mitochondria and endoplasmic reticulum, it would not be surprising if this mechanism failed as ATP levels fall during ischaemia. This idea is supported by some studies which demonstrated that $Ca^{++}$ homeostasis is deranged in tissues after WI[27] and that cytosolic $Ca^{++}$ levels rise in isolated cells exposed to anoxia.[28]

We investigated these possibilities by studying rabbit kidneys which had been flushed before cold storage with either a poor preservation solution (isotonic saline) or with hypertonic citrate solution (HCA) which is used routinely in clinical renal transplantation. To these solutions were added various agents which either affect $Ca^{++}$movements or interfere with enzymes which are thought to require $Ca^{++}$ for their activation. Assessment was again based on alterations in lipid peroxidation following *in vitro* incubation of homogenates of stored organs. The agents used were verapamil (100 $\mu$M), a $Ca^{++}$-channel blocker; A23187 (10 $\mu$M), an ionophore which renders membranes permeable to $Ca^{++}$; calcium chloride (1 mM $CaCl_2$) to increase extracellular concentrations of $Ca^{++}$; ruthenium red (5 $\mu$M) which inhibits mitochondrial $Ca^{++}$ uptake; dibucaine (250 $\mu$M) which inhibits $Ca^{++}$-activated phospholipases by competing with $Ca^{++}$-binding sites; and allopurinol (5 mM), a xanthine oxidase inhibitor.

Addition of the $Ca^{++}$-channel blocking agent verapamil to the isotonic saline solution used to flush and store kidneys for 24 hours, resulted in significant inhibition in formation of lipid peroxidative products encountered in untreated stored control kidneys both in cortex (Figure 2) and medulla (figure 3); indeed, the markers for peroxidation were close to levels measured in fresh unstored kidneys. Since the addition of verapamil directly to kidney homogenates had no effect on lipid peroxidation, it appears likely that the protective effect of verapamil was due to its ability to prevent the influx of extracellular $Ca^{++}$ into the cytosol of intact cells *in vivo* during the storage period.[29]

The importance of extracellular $Ca^{++}$ in mediating oxidative damage to cold stored kidneys was also suggested by the observation that addition of $CaCl_2$ (1 mM) to the saline storage medium resulted in a significant increase in peroxidative markers. Furthermore, permeabilisation of the cell membrane to $Ca^{++}$ by addition of the ionophore A23187 also significantly increased these indices (Figures 2 and 3). Inhibi-

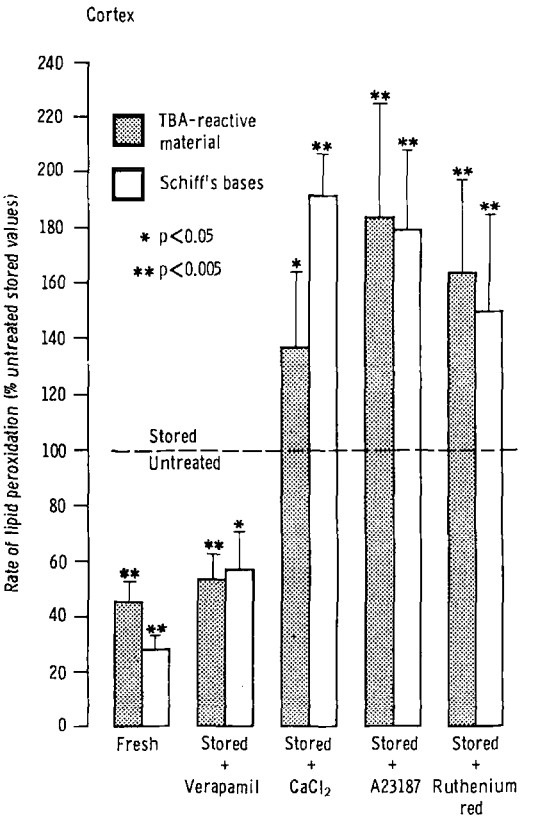


FIGURE 2 The rate of formation of two markers of lipid peroxidation (TBA-reactive material and Schiff's bases) in homogenates of renal CORTEX from kidneys flushed and stored for 24 hours at O°C in isotonic saline containing verapamil (100 $\mu$M), $CaCl_2$ (1 mM), A23187 (10 $\mu$M) and ruthenium red (5 $\mu$M). Values are mean $\pm$ S.E.M (n = 6) expressed as % of control (stored in saline only). 100% values: TBA: 0.81 $\pm$ 0.17 nmol malonaldehyde/hr/mg protein; Schiff's base: 4.67 $\pm$ 1.04 Fluorescence units/hr/mg/protein.

tion of mitochondrial $Ca^{++}$ uptake by ruthenium red during cold storage also resulted in a significant increase in membrane peroxidation, suggesting an important role for mitochondrial sequestration of $Ca^{++}$.

Addition of verapamil or $CaCl_2$ to HCA, however, did not affect the rise in lipid peroxidation observed after 72 hr cold storage. This was thought likely to be due to the ability of the large excess of citrate (55 mM) in this medium to itself chelate $Ca^{++}$ during the storage period. Measurement of the free $Ca^{++}$ concentration when various amounts of calcium were added to the citrate solution demonstrated the very effective $Ca^{++}$ buffering capacity of this medium and supported this conclusion — for example, the free $Ca^{++}$ concentration was only 1 $\mu$M in the presence of 100 $\mu$M total calcium and 10 $\mu$M when 1 mM calcium was added. This property of citrate may therefore contribute to the well known efficacy of HCA, although the increased rate of lipid peroxidation observed after 72 hr storage suggests that significant amounts of

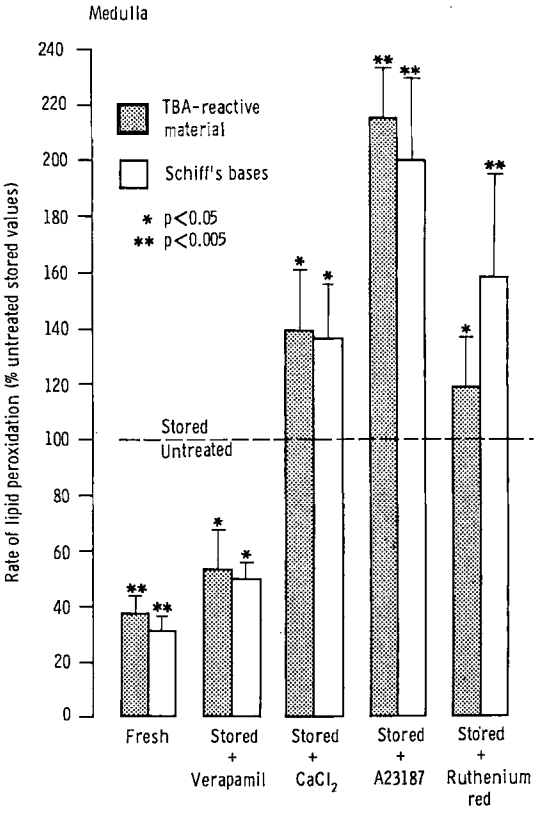


FIGURE 3 The rate of formation of two markers of lipid peroxidation (TBA-reactive material and Schiff's bases) in homogenates of renal MEDULLA from kidneys flushed and stored for 24 hours at 0°C in isotonic saline containing verapamil (100 $\mu$M), $CaCl_2$ (1 mM), A23187 (10 $\mu$M) and ruthenium red (5 $\mu$M). Values are means $\pm$ S.E.M. (n = 6) expressed as % of control (stored in saline only). 100% values: TBA: 1.35 $\pm$ 0.26 nmol malonaldehyde/hr/mg protein; Schiff's base 5.70 $\pm$ 1.48 fluorescence units/hr/mg protein.

$Ca^{++}$ may still enter the cell over an extended period or that other mechanisms may be responsible.

There are several mechanisms by which increased cytosolic $Ca^{++}$ levels could potentiate free radical damage to cell membranes. Phospholipase $A_2$ is activated by $Ca^{++}$ and removes fatty acids from membranes by a process which does not require oxygen or energy and could function during the storage period. However, both fatty acid catabolism and reincorporation of fatty acids back into phospholipids do require ATP and will therefore be slowed during anoxia. Hence, free fatty acids are likely to accumulate, and configurational disturbances in membranes due to accumulation of lysophosphatides could, as a result, render them more susceptible to free radical attack and peroxidation. Consistent with this hypothesis is the demonstration that dibucaine, an inhibitor of phospholipase $A_2$, decreased the formation of TBARs when added to the saline flush. Furthermore, addition of dibucaine together with A23187, inhibited the rises described above when A23187 was added alone.

Increased cytosolic $Ca^{++}$ may also be important in enhancing free radical production by activating proteases which convert the enzyme xanthine dehydrogenase to xanthine oxidase.[30] This would be particularly important during reperfusion of compromised cells presented with molecular oxygen; the oxidase utilizes hypoxanthine which has accumulated from adenine nucleotide degradation during ischaemia, to yield xanthine, superoxide anion and, in the presence of transition metal catalysts, hydroxyl and iron-complexed free radicals which are highly reactive. In our experiments, addition of the xanthine oxidase inhibitor allopurinol alone to the saline flush certainly inhibited the rate of peroxidation. When it was added together with A23187 it prevented the increased peroxidation encountered when A23187 was added alone.

The data obtained in this group of experiments strongly suggest that $Ca^{++}$ redistribution takes place during cold storage of kidneys resulting in elevated cytosolic $Ca^{++}$ levels. Several reasons are likely. Influx of extracellular $Ca^{++}$ may result from the inability of anoxic cells to maintain a $Ca^{++}$ gradient across the membrane as this is dependent on ATP-pumps. In addition, low temperatures depress membrane fluidity and this could also influence $Ca^{++}$ movements across cell membranes. Lipid peroxidation of the cell membrane may render it permeable to $Ca^{++}$ which would therefore leak in from the extracellular space. Intracellular $Ca^{++}$ redistribution could also contribute to elevated cytosolic levels during ischaemia as energy-dependent sequestration by mitochondria and endoplasmic reticulum is depressed.

To investigate the possible activation of phospholipase $A_2$ as a result of raised cytosolic $Ca^{++}$ levels, we then carried out a series of experiments, in which free fatty acids (FFA) were measured directly by gas liquid chromatography. Again using the rabbit kidney model with the organ flushed with saline and stored for 48 hr, or with HCA and stored for 72 hr, we found that increasing concentrations of FFA correlated with increases in TBA reactive markers of lipid peroxidation. Addition of dibucaine (250 $\mu$M) to the flush solution inhibited these rises in both FFA and TBARs whereas addition of the $Ca^{++}$ ionophore A23187 alone lead to significant increases in FFA and TBARs over and above the increases detected in the stored untreated kidneys. Perhaps of greatest importance, the increases in polyunsaturated fatty acids were significantly greater than saturated fatty acids and the release of arachidonic acid was most marked when A23187 was added to the solution.

## CONCLUSIONS

Though far from resolving the spatial and temporal sequence of these events, we conclude from these data that an early fall in adenine nucleotide levels accompanied by increased $H^+$ is associated *during* cold ischaemia with increased cytosolic $Ca^{++}$ levels, a redistribution of iron from ferritin stores and activation of phospholipases which release fatty acids from the confines of phospholipid bi-layers. The organ is thus rendered highly susceptible to reperfusion injury in two possible ways. First, exposure to high concentrations of molecular oxygen on reperfusion allows free radical reactions to overwhelm any remaining scavenging capacity and may proceed to irreversible peroxidative damage. Second, raised arachidonic acid metabolism via cyclo-oxygenase pathways may upset the balance in the vascular bed to favour constriction and platelet aggregation. It is unlikely that the two processes are mutually exclusive.

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