Free Rad. Res. Comms., Vol. 3, No. 1–5, pp. 107–115
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Printed in Great Britain

LIPID PEROXIDATION IN THE CORTEX AND MEDULLA OF RABBIT KIDNEYS SUBJECTED TO COLD ISCHAEMIA AND THE VALUE OF PROTECTIVE AGENTS

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(*Received July 21st 1986*)

The storage of rabbit kidneys for 24 hr at 0 C in isotonic saline resulted in significantly increased rates of lipid peroxidation, as measured by the formation of thiobarbituric acid-reactive material and Schiff bases during *in vitro* incubation of homogenates prepared from the cortex and medulla. In addition, the content of thiobarbituric acid-reactive material in the medulla was also significantly elevated as a result of cold storage for 24 hr.

The effects of antioxidants (vitamin E), iron-chelation (desferrioxamine) and inhibitors of arachidonic acid oxidation (indomethacin and dazmegrel) on the rate of lipid peroxidation in homogenates prepared from ischaemic kidneys were studied. This demonstrated that lipid peroxidation in the cortex was predominantly non-specific and iron-catalysed whereas in the medulla approximately 50% of the TBA-reactive material was formed enzymically from arachidonic acid by cyclooxygenase.

KEY WORDS: Lipid peroxidation; cold ischaemia; rabbit kidney.

INTRODUCTION

During the *ex vivo* storage of kidneys for transplantation, the organ gradually deteriorates and this eventually leads to the irreversible loss of renal function. The biochemical changes observed when kidneys are subjected to ischaemia include the depletion of high energy adenine nucleotides,[1] a rapid fall in intracellular pH[2] and the functional impairment of intracellular organelles such as the mitochondria[3] and endoplasmic reticulum.[4] In addition, vascular injury takes place which results in oedema.[3] Upon reoxygenation of stored organs after replantation, this damage is further exacerbated and other pathological changes become apparent which include vasoconstriction and the eventual blockage of the vascular bed (the no-reflow phenomenon).[5]

There is evidence to suggest that oxygen-derived free radicals, which have been implicated in many forms of cell death[6] and in vascular injury,[7,8] may be at least partially responsible for the irreversible damage which occurs in a number of ischaemic and reperfusion situations[7,9–12] and during the hypothermic storage of rabbit and pig kidneys.[13–15] Storage of rabbit kidneys at 0°C results in significant increases in the rate of lipid peroxidation in whole kidney homogenates measured *in vitro*.[13] Furthermore,

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replantation followed by reperfusion *in vivo* results in further substantial increases in the rate of lipid peroxidation and this correlates well with the known physiological function of the kidney transplants.[13]

The peroxidation of membrane-bound polyunsaturated fatty acids can occur as a result of attack by hydroxyl radicals ($OH^{\cdot}$), whose formation from the superoxide anion ($O_2^{\cdot-}$) is dependent on iron, or by specific enzyme-catalysed reactions. In kidneys the enzyme-catalysed oxidation of arachidonic acid differs between medulla and cortex; the rate of prostaglandin synthesis is considerably greater in the medulla than the cortex[16] whereas in the cortex and particularly the glomeruli lipoxygenase activity predominates.[17,18] Thus, in the present investigation, previous studies have been extended by investigating lipid peroxidation in homogenates of cortex and medulla prepared from kidneys subjected to 24 hours hypothermic storage.

A number of compounds were added to homogenates of the stored kidneys prior to *in vitro* incubation in order to estimate the possible contributions of different peroxidation pathways of membrane-bound polyunsaturated fatty acids. In addition, these *in vitro* determinations were used to assess any possible protection which these test compounds might provide against damage resulting from organ storage and other *in vivo* ischaemic situations. The test compounds investigated were vitamin E, a potent natural antioxidant;[19] desferrioxamine, a clinically approved iron chelating agent;[20] indomethacin, an inhibitor of cyclooxygenase[21] and dazmegrel, a thromboxane synthetase inhibitor.[22]

MATERIALS AND METHODS

Adult male New Zealand white rabbits (average weight 3 kg) were anaesthetised by i.m. injection of fentanyl-fluanisone (Hypnorm; 0.2 ml/kg) followed by slow i.v. injection of diazepam (1.0 mg/kg). Oxygen (2 l/min) was supplied via an open face mask. Both kidneys were exposed through a mid-line abdominal incision and removed after careful dissection and ligation of the vessels and ureter. The warm ischaemic period was less than 2.0 min. The renal artery of each kidney was cannulated and the organs were flushed with sterile isotonic 0.9% sodium chloride solution (30 ml). The kidneys were either examined immediately (control) or placed in sterile beakers containing flush solution and stored for 24 hours surrounded by ice within a closed polystyrene container held in a refrigerator.

Kidneys were divided into cortex and medulla and these samples were suspended in phosphate-saline buffer (40 mM KH_2PO_4:K_2HPO_4; pH 7.4) (10% w/v) and homogenised using a Potter–Elvehjem homogeniser. The protein content of the homogenates was determined by the method of Lowry *et al.*[23] using bovine serum albumin as the standard.

The lipid peroxide content and rate of lipid peroxidation in homogenates of cortex and medulla were determined using two methods. Samples (0.5 ml) were taken before and following incubation of the homogenate for 60 min at 37°C in a shaking water bath, lipid soluble Schiffs bases were extracted into chloroform:methanol (2:1) (4 ml) and quantitated by fluorescence (excitation wavelength 360 nm, emission maximum between 400–450 nm). Initial levels and the rates of formation of thiobarbituric acid (TBA)-reactive material during incubation of the homogenates for 60 min were determined by the method of Yagi.[24] Aliquots of homogenate (0.5 ml) were mixed with 7% (w/v) sodium dodecyl sulphate (100 μl), 0.1 M HCl (1 ml), 10% (w/v)

phosphotungstic acid (150 μl) and 0.67% (w/v) thiobarbituric acid (0.5 ml). After heating at 95°C for 1 hour, the coloured TBA complex was extracted into butan-1-ol (2.5 ml) and quantitated by fluorescence (excitation wavelength 515 nm, emission maximum 553 nm). This assay was calibrated using malonaldehyde (MDA) tetraethylacetal.

In experiments where the effect of test compounds were studied, the cortices and medullae of four kidneys were pooled and 9% (w/v) homogenates prepared. Test substances were dissolved in phosphate-saline buffer at 10-times the required final concentration and 1 ml added to 9 ml of homogenate prior to sampling and incubation.

The compounds investigated were: indomethacin and α-tocopherol (50 mg/ml in absolute alcohol) (Sigma Chemical Co., Poole, Dorset); desferrioxamine (Desferal®, Ciba Geigy Ltd., Horsham, Surrey); α-tocopherol acetate suspension (Ephynal®, a gift from Roche Products Ltd., Welwyn Garden City, Herts) and dazmegrel (UK-38,485, a gift from Pfizer Ltd., Sandwich, Kent).

RESULTS

The effect of cold ischaemia on the rate of lipid peroxidation in rabbit kidney homogenates

The rate of lipid peroxidation, measured as Schiff bases and TBA-reactive material, in incubated homogenates of control (unstored) kidneys was found to be greater in the medulla than the cortex (Figures 1 and 2). The storage of kidneys at 0°C for 24 hours resulted in a dramatic (5-fold) increase in the rate of Schiff base production in the cortex, measured *in vitro*, whereas an increase to only 147% of control values was observed in the medulla (Figure 1). The production of TBA-reactive material in homogenates of both the cortex and medulla, when incubated at 37°C, was also significantly elevated as a result of storage but to a lesser extent than that found with the Schiff base determinations (Figure 2). In addition, the content of TBA-reactive material in the medulla increased significantly ($p < 0.05$) when kidneys were stored for 24 hours although there was no change in the cortices from the same kidneys (Figure 3).

The effect of test substances on the rate of lipid peroxidation in homogenates of rabbit kidneys subjected to 24 hours cold ischaemia

Addition of vitamin E to the homogenates prior to incubation resulted in a significant decrease in the rate of lipid peroxidation in the cortex but had no effect on production of TBA-reactive material in the medulla (Table I). Ephynal (a solubilised preparation of α-tocopherol acetate) was more effective than vitamin E, although the formation of TBA-reactive material in the medulla was only reduced to 82% of control values compared to 34% in the cortex (Table I).

Desferrioxamine inhibited the production of TBA-reactive material in the cortex almost completely although a significant amount of TBA-reactive material was produced in the medulla in the presence of 5 mM desferrioxamine (Table I). Conversely, desferrioxamine was very effective at preventing Schiff base formation in the medulla but reduced this index of lipid peroxidation in the cortex to only half of the control value (Table I).

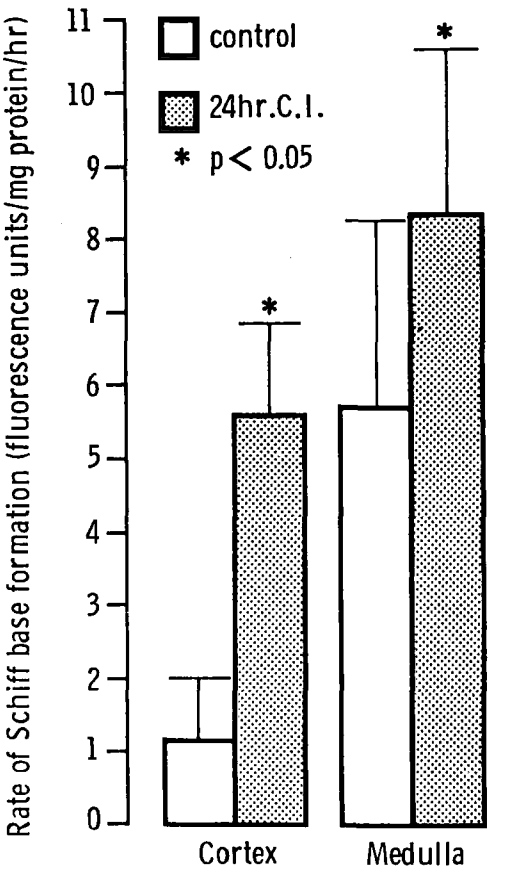

FIGURE 1 The rate of Schiff base formation in homogenates of rabbit kidneys subjected to 24 hours cold ischaemia. Values represent the means ± S.D. of 10 determinations performed in duplicate.

Malonaldehyde is produced as a side product during prostaglandin and thromboxane synthesis and several intermediates of these pathways give a positive TBA-reactivity.[25] The cyclooxygenase inhibitor indomethacin was added to the homogenates in order to estimate the relative extents of enzymic arachidonic acid oxidation in the two regions of the ischaemic kidney. The results showed that no TBA-reactive material was produced via cyclooxygenation of arachidonic acid in the cortex but that approximately half the TBA-reactive material produced in the medulla was formed by this mechanism (Table I). Schiff base production in the medulla was only minimally affected by indomethacin (Table I). When desferrioxamine and indomethacin were added together, the production of both Schiff bases and TBA-reactive material in the medulla was completely abolished (Table I). Dazmegrel had no effect on the rate of lipid peroxidation in the homogenates suggesting that the TBA-reactive material was not produced as a result of thromboxane synthesis.

DISCUSSION

By incubating homogenates of stored kidneys, which simulates the reoxygenation of the organ *in vivo*, the present experiments demonstrate that the rate of lipid peroxida-

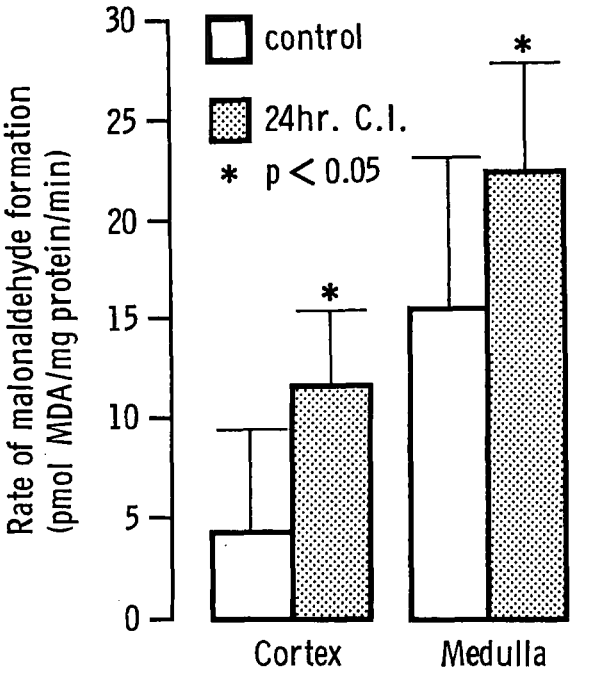

FIGURE 2 The rate of formation of TBA-reactive material in homogenates of rabbit kidneys subjected to 24 hours cold ischaemia. Values represent the means ± S.D. of 10 determinations performed in duplicate.

tion, a measure of oxidative stress, increases significantly in both the cortex and medulla as a result of hypothermic storage for 24 hours. The results of this investigation therefore support the hypothesis that oxygen derived free radicals may at least be partially responsible for the damage resulting from the storage and subsequent reperfusion of kidneys. This conclusion is also supported by other studies elsewhere which have shown protective effects of superoxide dismutase and allopurinal.[15]

It seems from the present data that almost all the TBA-reactive material produced in the cortex after hypothermic storage is dependent upon the presence of iron whereas in the medulla only half the TBA-reactive material is produced by this mechanism, the rest being formed as a result of the oxidation of arachidonic acid by cyclooxygenase (inhibitable by indomethacin at a concentration (10 μM) which does not result in any general antioxidant activity). The product of this reaction is the prostaglandin PGH_2 which can be further metabolised to other prostaglandins, prostacyclin (a vasodilator) or thromboxane (a vasoconstrictor). The ineffectiveness of dazmegrel at a concentration known to inhibit thromboxane synthesis *in vitro*, strongly suggested that prostaglandin formation was responsible for the production of the TBA-reactive material, although, the formation of thromboxanes under these conditions cannot be ruled out. The kidney is capable of synthesizing PGE_2 and $PGF_{2\alpha}$ (particularly in the medulla)[16] and hypoxia has been shown to stimulate the production of these metabolites in renal mesangial cell cultures.[26] $PGF_{2\alpha}$ is a universal vasoconstrictor[27] and there is evidence to suggest that prostaglandins are involved in the development of oedema following cerebral ischaemia.[28] Thus the increased capacity of the medulla to synthesise these metabolites of arachidonic acid following hypothermic storage, as suggested by these results, is likely to have an adverse effect when the organ is transplanted.

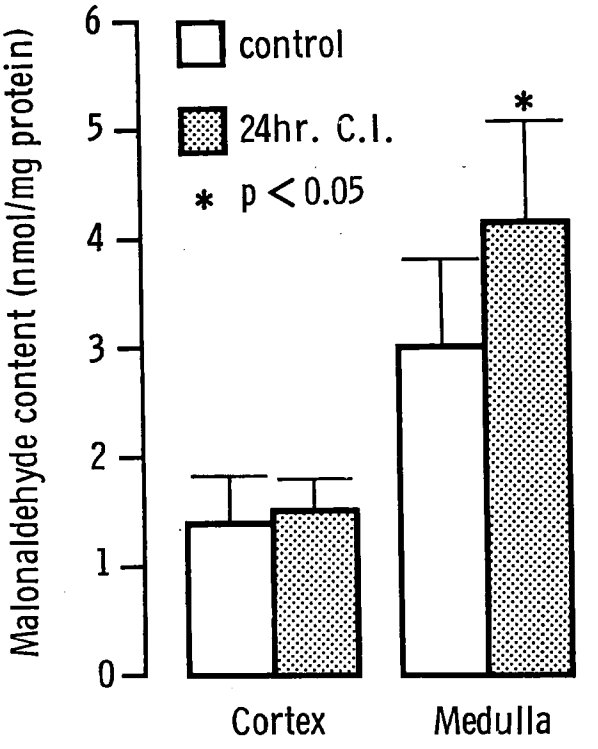

FIGURE 3 The content of TBA-reactive material in homogenates of rabbit kidneys subjected to 24 hours cold ischaemia. Values represent the means ± S.D. of 10 determinations performed in duplicate.

TABLE I
The effect of test substances on the rate of lipid peroxidation in homogenates of rabbit kidneys subjected to 24 hours cold ischaemia

	CORTEX		MEDULLA	
Compound	TBA-RM† Production	SB‡ Production	TBA-RM Production	SB Production
None	100 ± 32	100 ± 23	100 ± 24	100 ± 26
Vitamin E (100 μM)	63 ± 29	63 ± 18	108 ± 19	74 ± 24
Ephynal (100 μM)	34 ± 22	51 ± 25	82 ± 26	69 ± 15
Desferrioxamine (5 mM)	16 ± 19	47 ± 9	45 ± 19	22 ± 12
Indomethacin (10 μM)	99 ± 12	86 ± 22	43 ± 15	79 ± 28
Desferrioxamine + Indomethacin	27 ± 16	52 ± 18	−12 ± 18	8 ± 18
Dazmegrel (100 μM)	124 ± 36	94 ± 11	109 ± 26	95 ± 11

† TBA-reactive material.
‡ SB – Schiff bases.
These rates are expressed as percentages of control (i.e. no additions) values. Values represent the mean ± S.D. of 6 determinations performed in duplicate.

Indomethacin had little effect on Schiff base formation in the medulla whereas desferrioxamine reduced it to 22% of the control value. Thus, it appears that Schiff base formation was a good index of non-specific iron-catalysed lipid peroxidation whereas the majority of the products of cyclooxygenase which gave a positive TBA-reaction did not react with amino groups to form these fluorescent derivatives.

Lipid peroxidation in the medulla was completely inhibited by the presence of desferrioxamine and indomethacin whereas in the cortex, addition of these agents reduced Schiff base formation by only 48%. Thus, in the cortex of stored kidneys, it appears that lipid peroxidation involves both an iron-dependent pathway and a mechanism which is independent of iron or cyclooxygenase. Lipoxygenation of arachidonic acid is one possibility which could not be confirmed in this investigation due to the lack of specific lipoxygenase inhibitors. However, an increase in lipoxygenation of arachidonic acid as a result of transient cerebral ischaemia has been demonstrated.[27] Lipoxygenation of arachidonic acid results in the formation of leukotrienes and it may be significant that the leukotrienes B_4, C_4 and D_4 are known to be important in the shock syndrome[27] and cause potent vasoconstriction of the renal vascular beds.[30]

Vitamin E, although known to be a potent antioxidant, was relatively ineffective at inhibiting lipid peroxidation and this may have been due to its poor solubility in this *in vitro* system. Lipid peroxidation in the cortex was more effectively inhibited by Ephynal which is a solubilised preparation of α-tocopherol acetate. As α-tocopherol acetate is not an effective antioxidant due to the esterification of the phenolic group, its inhibitory properties suggest that it may have been hydrolysed to α-tocopherol by the kidney homogenates during the incubation or that it exerted some type of membrane stabilising effect. Administration of vitamin E or Ephynal *in vivo* may result in a better accumulation of α-tocopherol into the cell membranes which would protect against lipid peroxidation but which would not affect the enzymic oxidations of arachidonic acid which take place under these conditions.

It is likely that several mechanisms are responsible for the increased rate of peroxidation during the *in vitro* reoxygenation of kidneys subjected to cold ischaemia. It has been reported that during ischaemia there is a Ca^{2+}-dependent proteolysis of xanthine dehydrogenase to xanthine oxidase which utilizes hypoxanthine generated from the catabolism of ATP during ischaemia. On reperfusion, this enzyme catalyses the production of the superoxide anion from oxygen.[31] In addition, the conversion of $O_2^{\cdot -}$ to $OH^{\cdot}$ may also be stimulated under these conditions due to an increase in low molecular weight iron.[12] During ischaemia, the impairment of intracellular organelles and the depletion of ATP stores needed for energy-dependent pumps are likely to result in an increase in cytosolic Ca^{2+} levels and this has been demonstrated in cultured kidney cells.[32] As well as being directly implicated in cell death,[33] an increased level of cytosolic Ca^{2+} activates the phospholipases which remove arachidonic acid from the membrane making it available for prostaglandin biosynthesis and lipoxygenation.[34] A role for Ca^{2+} in cold storage injury is supported by experimental data suggesting that Ca^{2+} antagonists such as verapamil afford some protection to the kidney.[35]

In conclusion, acute renal failure following transplantation continues to be a clinical problem and it is likely that damage occurring during the storage period is a contributing factor to this primary graft failure. The results of this investigation suggest that changes occur during the storage of kidneys which result in an increased level of oxidative stress and a stimulation of enzymic arachidonic acid oxidation upon

reoxygenation. This leads to the disruption of cellular membranes and the formation of products which have known adverse effects on the vasculature of the organ. These biochemical events may therefore be an underlying cause of ischaemic damage to kidneys and further work is underway to positively identify the products of these peroxidative reactions by HPLC and radioimmunoassay. In addition, we are also evaluating a number of compounds, administered singly or in combinations, for their ability to inhibit both non-specific and enzyme-catalysed lipid peroxidation and increase the viability of rabbit kidneys following periods of cold and warm ischaemia.

Acknowledgements

The authors express their gratitude to Roche Products Ltd., for a sample of Ephynal and Pfizer Ltd., for a sample of dazmegrel. We also thank Sue Marley, Colin Shurey and Guy Healing for valuable technical assistance.

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Accepted by Prof. H. Sies and Drs. B. Halliwell and J.M.C. Gutteridge