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Prevention by antioxidants of oxidative damage to rabbit kidneys subjected to cold ischaemia

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Cold storage of kidneys while awaiting transplantation into a suitable recipient involves deprivation of the blood supply to the organ and concomitant starvation of oxygen. This period of ischaemia results in gradual deterioration of the organ and eventually to irreversible loss of renal function. That the damage becomes more manifest when the organ is transplanted and reperfused with oxygenated blood has led to the hypothesis that oxygen-derived free radicals (reactive species containing an unpaired electron) may be involved in the pathogenesis of this condition [1].

One consequence of an unchecked burst of free radical activity is the peroxidation of membrane-bound polyunsaturated fatty acids which can result in the loss of membrane integrity and dysfunction of intracellular organelles. We have previously shown that storage of rabbit kidneys at 0° results in significant increases in the rate of lipid peroxidation in both the medulla and cortex [2, 3] and that further substantial increases are observed when the organs are replanted and reperfused *in vivo* [2]. The levels of markers of lipid peroxidation were inversely correlated to the known physiological function of the kidney transplants [2] and could be reduced by administration of free radical scavengers and the chelation by desferrioxamine of iron [4] which catalyses the conversion of the superoxide anion ($O_2^{\cdot-}$) to the highly reactive hydroxyl radical ($OH^{\cdot}$).

Other studies have suggested that altered cell calcium homeostasis leading to increased cytosolic free calcium levels during storage of kidneys plays a role in mediating oxidative membrane damage [5]. This may be the result of stimulation of calcium-activated phospholipases which remove fatty acids, in particular arachidonic acid, from the membrane. Subsequent enzymic peroxidation of free arachidonic acid by cyclooxygenase results in the formation of prostaglandins including thromboxane (a vasoconstrictor) and prostacyclin (a vasodilator). Ischaemia has been reported to upset the delicate balance between these two compounds (e.g. [6]) which results in vasoconstriction and may lead to blockage of the vascular bed on reperfusion. In addition, oxidation of arachidonic acid by lipoxygenase leads to the production of leukotrienes some of which cause vasoconstriction, are chemotactic and enhance capillary permeability and have been implicated in ischaemic/reperfusion damage [7].

In view of the possibility that several mechanisms may be working in consort to cause membrane damage, cell necrosis and vascular injury in stored organs, we have investigated the effect of a number of agents which inhibit both non-specific and enzyme-catalysed peroxidation of polyunsaturated fatty acids, on the production of markers of lipid peroxidation during *in vitro* incubation of homogenates of stored kidneys.

Materials and methods

Rabbit kidneys were harvested, flushed with and stored in isotonic saline solution for 24 hr at 0° as previously described [3]. After division into cortex and medulla, homogenates (10% w/v) were prepared in phosphate-buffered saline (40 mM KH_2PO_4: K_2HPO_4; pH 7.4) and incubated, with shaking, at 37° in the presence of various test compounds. Aliquots were taken at 0 and 60 min incubation and the rate of lipid peroxidation quantitated by measuring the formation of Schiff bases and thiobarbituric acid (TBA)-reactive material, as previously described [3]. The amount of TBA-reactive material was quantitated using malonaldehyde tetraethylacetal and the results were corrected for the protein content of the homogenates determined by the method of Lowry *et al.* [8].

The compounds tested were silymarin (Legalon®, a gift from Dr. Madaus & Co., Koln, F.R.G.) a flavonoid isolated from the milk thistle which is a mixture of three isomers including silibinin (Fig. 1); chlorpromazine, a psychotrophic drug; propyl gallate (Fig. 1) a water soluble antioxidant; quercetin (Fig. 1) a flavonoid; and the antioxidant nordihydroguaiaretic acid (NDGA) (Fig. 1).

Statistical analysis was performed using a paired *t*-test modified according to the method of Bonferroni for simultaneous multiple comparisons as described by Wallenstein *et al.* [9].

Results and discussion

We have previously shown that homogenates of kidneys undergo lipid peroxidation when incubated at 37° in an aerobic environment and that the rate of this process is very significantly greater when the kidney has undergone a period of cold storage [2, 3]. Addition of compounds with antioxidant properties to the incubation medium allows the investigation of the mechanisms of lipid peroxidation in homogenates of stored kidneys and provides a rapid indication of whether a compound may have possible therapeutic value in preventing free radical induced damage to ischaemic kidneys *in vivo*. Two markers of lipid peroxidation were measured: TBA-reactive material includes

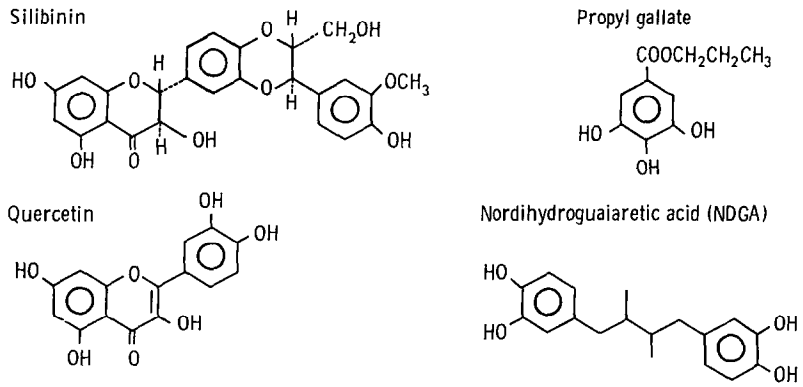

Fig. 1. Molecular structures of some of the test substances used in this study.

short-chain aldehydes such as malondialdehyde formed from non-specific lipid peroxidation and prostaglandin synthesis and several of the intermediates of prostaglandin synthesis also have a positive TBA-reactivity [10]; Schiff bases are products of the reaction of aldehydes with amino groups and are a good index of non-specific lipid peroxidation pathways [3].

In all cases, the initial values of TBA-reactive material and Schiff bases in the homogenates prior to incubation were not significantly affected by addition of the test substances (data not shown). This clearly indicated that the compounds did not interfere with the assays for lipid peroxidation products by quenching fluorescence or changing the conditions of the TBA-test. Thus decreases in these parameters following incubation with the test compounds could be assigned to the inhibition of lipid peroxidation in the tissue homogenates.

Silymarin which has an antihepatotoxic effect *in vivo* and inhibits lipid peroxidation, cyclooxygenase and lipoxygenase activity *in vitro* [11] reduced TBA-reactive material and Schiff base formation in the cortex to around 50% of control levels at a concentration of 100 μM (Table 1). At this concentration silymarin had a similar effect on Schiff base formation in the medulla but little effect on the production of TBA-reactive material. At a concentration of 1 mM, this compound was considerably more effective at inhibiting lipid peroxidation in both regions of the kidney (Table 1).

Chlorpromazine had a similar effect on the rate of production of TBA-reactive material and Schiff bases as silymarin at the same concentration (Table 1). This compound inhibits lipid peroxidation, cyclooxygenation at low arachidonic acid concentrations *in vitro* but not *in vivo* and lipoxygenase activity [12] and has been shown to afford some protection against the ischaemic death of liver cells [13].

Propyl gallate was very effective at inhibiting lipid peroxidation in the cortex but had less effect on the production of TBA-reactive material in the medulla. This compound has been shown to exert a protective effect in myocardial ischaemia which was thought to be due to its ability to inhibit lipoxygenase [14].

Both quercetin and NDGA abolished Schiff base production in the medulla almost completely and were the most effective agents along with propyl gallate at inhibiting Schiff base production in the cortex (Table 1). These compounds also reduced the levels of TBA-reactive material in the cortex to low levels and halved the rate of production of this marker of lipid peroxidation in the medulla (Table 1). In addition to having antioxidant properties and inhibiting lipoxygenation [15], quercetin reduces platelet aggregation and may therefore benefit the microcirculation of a transplanted organ. NDGA is an antioxidant which inhibits lipoxygenase effectively and cyclooxygenase weakly [12, 16].

Using identical methodology, we have previously shown

Table 1. The effect of test substances on the rate of lipid peroxidation in homogenates of rabbit kidneys subjected to 24 hr cold ischaemia

	Cortex		Medulla	
	TBA-RM production	SB production	TBA-RM production	SB production
None	100 ± 32	100 ± 23	100 ± 24	100 ± 26
Silymarin (100 μM)	51 ± 16*	58 ± 18*	90 ± 26	53 ± 13*
Silymarin (1 mM)	13 ± 15*	34 ± 16*	39 ± 17*	22 ± 7*
Chlorpromazine (100 μM)	53 ± 18*	69 ± 17*	88 ± 33	65 ± 13*
Propyl gallate (100 μM)	33 ± 19*	28 ± 12*	70 ± 21	21 ± 21*
Quercetin (30 μM)	26 ± 22*	23 ± 18*	52 ± 28*	7 ± 7*
NDGA (100 μM)	20 ± 28*	32 ± 12*	62 ± 25*	6 ± 7*

The rates are expressed as percentages of control (i.e. no additions) values. Values represent the mean ± SD of 6 determinations performed in duplicate.

TBA-RM: TBA-reactive material; SB: Schiff bases.

* $P < 0.05$, $t > 3.69$, 5 degrees of freedom, according to the method of Bonferroni [9].

Control values: Cortex: TBA-RM = 11.6 ± 3.7 pmol MDA/mg protein/min; SB: 5.6 ± 1.3 fluorescent units/mg protein/hr. Medulla: TBA-RM = 22.4 ± 5.5 pmol MDA/mg protein/min; SB: 8.4 ± 2.2 fluorescent units/mg protein/hr.

that lipid peroxidation in the cortex of stored kidneys is predominantly non-specific and iron-catalysed (desferrioxamine-inhibitable) with no contribution from cyclooxygenase (unaffected by indomethacin) [3]. However, desferrioxamine (5 mM) only reduced Schiff base production to 47 ± 9% of control levels which suggested the existence of an iron-independent pathway of peroxidation in this part of the kidney [3]. In the present study, low levels of Schiff base formation were observed when cortical homogenates were incubated in the presence of NDGA, propyl gallate or quercetin, all of which inhibit lipoxygenase. It is therefore possible that the lipoxygenase pathway, which is localised predominantly in the cortex of the kidney [17], is activated as a result of storage and produces peroxides which subsequently form Schiff bases. In view of the properties of some lipoxygenase products [7], increased oxidation of polyunsaturated fatty acids by this route is likely to have adverse effects on the vasculature of the stored kidney on replantation.

Studies have demonstrated the existence of two separate pathways of lipid peroxidation in the medulla of stored kidneys; an iron-dependent process inhibited by desferrioxamine and a cyclooxygenase catalysed reaction inhibited by indomethacin [3]. Thus, in the presence of these two compounds, the formation of both markers of lipid peroxidation were abolished [3]. In the present study, quercetin and NDGA were effective at inhibiting Schiff base production in this region of the kidney but only reduced TBA-reactivity to about 50% of control levels (Table 1). As half of the TBA-reactive material in the medulla originates from cyclooxygenase-mediated processes [3], it would appear that these compounds, at the concentration given, were ineffective at inhibiting this pathway of lipid peroxidation.

It is clear from this and previous studies that the mechanism of lipid peroxidation during reoxygenation of ischaemic organs is a complex multifactorial process. In addition, the effects of oxidation products of polyunsaturated fatty acids on the vasculature need to be considered. Thus improved viability of stored organs has been reported following administration of free radical scavengers [18], calcium-antagonists [19] and prostacyclin analogues [20]. It is likely that iron-catalysed free radical processes along with imbalances in enzyme-linked peroxidative reactions are involved in the pathogenesis of cold ischaemic damage to the kidney. Thus pharmacological intervention by agents, such as those described in this study, which protect cell membranes from free radical attack and prevent the formation of products which have adverse effects on the vasculature of the organ, may result in significant improvement in the viability of stored organs.

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