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EFFECT OF LIPID PEROXIDATION ON *p*-AMINOHIPPURATE TRANSPORT BY RAT KIDNEY CORTICAL SLICES

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1 The effect of lipid peroxidation on *p*-aminohippurate transport by rat kidney slices was examined.

2 Ascorbic acid and Fe^{2+} promoted lipid peroxidation of rat renal cortical slices in a dose-related manner.

3 Ascorbic acid (1.0 mM) and Fe^{2+} (0.4 mM) increased tissue water and decreased the accumulation of *p*-aminohippurate.

4 The addition of N,N'-diphenyl-*p*-phenylenediamine (antioxidant), at a concentration of 1×10^{-6} M, completely inhibited the peroxidation and recovered the accumulation of *p*-aminohippurate.

5 The apparent *K*m of *p*-aminohippurate uptake was increased by ascorbic acid and Fe^{2+} with no change in the apparent V.

6 These data suggest that ascorbic acid and Fe^{2+} can cause a significant alteration in *p*-aminohippurate and water transport of renal cortical slices and that these effects can be correlated with lipid peroxidation.

Introduction

It is well known that biomembranes and subcellular organelles are susceptible to lipid peroxidation (Tappel, 1965; 1973; Demopoulos, 1973). There is a steadily increasing amount of evidence indicating that lipid peroxidation is involved in basic deteriorative mechanisms e.g. membrane damage, enzyme damage, and nucleic acids mutagenicity (Kappus & Sies, 1981).

Most studies have been directed at defining the effects of lipid peroxidation on liver (Recknagel, 1967; Recknagel & Glende, 1973; Di Luzio, 1973), whereas relatively little information is available concerning the influence of lipid peroxidation on renal cell function. Accordingly, in the present experiments we sought to define the effect of the lipid peroxidation induced by ascorbic acid and ferrous ions (Hunter, Scott, Hoffsten, Gebicki, Weinstein & Schneider, 1964; Kitabchi & Williams, 1968; Hanstein & Hatefi, 1970), on a transport system characteristic of renal proximal tubular cells, namely *p*-aminohippurate transport.

Methods

Wistar strain male rats, weighing 150–200 g, kept on a standard laboratory diet (Oriental Yeast Co., Tokyo), were used for the experiments. After decapitation, both kidneys were immediately removed, cooled and decapsulated in ice-cold saline. The kidney cortex was cut into slices (about 0.5 mm in thickness) with a razor blade on an ice-cold petri dish.

Estimation of lipid peroxides

Rat kidney cortical slices (120 mg/10 ml medium) were incubated in 0.15 M KCl – 0.02 M Tris HCl buffer (pH 7.4) at 37°C for 10 min. Ascorbic acid and $FeSO_4$ were added to the medium at the indicated concentrations. At the end of the incubation period, the slices were quickly removed from the medium, blotted lightly on filter paper, reweighed and homogenized in 5 ml of the Tris-HCl buffer. Five ml aliquots were mixed with 1.25 ml of 40% trichloroacetic acid and assayed for malonaldehyde by the thiobarbituric acid method (Tappel & Zalkin, 1959), and expressed as thiobarbituric acid values (absorbance at 532 nm/g tissue).

Effect of lipid peroxidation on the accumulation of p-*aminohippurate*

Experiments concerned with the actions of ascorbic acid and Fe^{2+} on *p*-aminohippurate accumulation system of renal cortical slices employed two sequential incubations. For the first, fresh slices were incubated for 10 min at 37°C in 0.15 M KCl – 0.02 M Tris HCl buffer with and without ascorbic acid and Fe^{2+}.

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After the primary incubation, slices were washed and then further incubated for 60 min and 120 min at 25°C in Cross & Taggart buffer.

Determination of p-*aminohippurate accumulation*

Slices (approx. 200 mg/10 ml medium) were incubated in conical flasks containing Cross & Taggart buffer (Cross & Taggart, 1950) of the following composition (mmol/l): sodium chloride 86, potassium chloride 40, sodium phosphate buffer (pH 7.3) 7, sodium acetate 11, calcium chloride 0.7, sodium *p*-aminohippurate 0.074. The incubation medium also contained inulin, 1 g/100 ml, to allow estimation of inulin space. Incubations were carried out at 25°C under a gas phase of 95% O_2 plus 5% CO_2 for 60 min and 120 min (final pH 7.0). At the end of the incubation period, the slices were quickly removed from the medium, blotted lightly on filter paper, weighed and homogenized in 2.5 ml of 10% trichloroacetic acid; 0.3 ml of incubation medium was treated similarly. After centrifugation the supernatant was assayed for the concentrations of *p*-aminohippurate and inulin. The concentrations of *p*-aminohippurate and inulin of the medium and tissue homogenate were determined by the methods of Bratton & Marshall (1939), and Roe, Epstein & Goldstein (1949), respectively. The remaining slices were weighed before and after 24 h of dehydration at 100°C to determine total tissue water. The accumulation of *p*-aminohippurate was, in most cases, expressed as the slice/medium (S/M) ratio (concentration of *p*-aminohippurate/g of tissue divided by the concentration of *p*-aminohippurate/ml of medium). In some experiments, the value of S/M ratio was converted to ICF/M ratio (ICF; concentration of *p*-aminohippurate/ml of intracellular fluid). Inulin space, calculated from tissue inulin content and inulin concentration of the medium, was used as an estimate of tissue extracellular water. Intracellular water was calculated by subtracting extracellular water from total tissue water.

Determination of p-*aminohippurate uptake: kinetic studies*

Uptake of *p*-aminohippurate was determined in rat renal cortical slices (about 200 mg). The concentrations of *p*-aminohippurate in the medium were 1.0, 2.0 and 4.0×10^{-4}M. Incubations were performed in conical flasks at 25°C under 95% O_2 plus 5% CO_2 for 30 min. The rate of transport was expressed as mmol *p*-aminohippurate kg^{-1} cell water $30\,min^{-1}$ of incubation.

Statistics

The values were represented as means ± s.e. Statistical significance was determined by Student's *t* test.

Results

Ascorbic acid and ferrous ions are important initiators of non-enzymatic tissue peroxidation. Therefore, we first examined the influence of ascorbic acid and Fe^{2+} on the lipid peroxidation of rat renal cortical slices.

As shown in Table 1, ascorbic acid alone (0.1, 1.0, 10 mM) in the absence of Fe^{2+} showed little or no effect on lipid peroxidation. On the other hand, Fe^{2+} (0.04, 0.4, 4 mM) was able to promote lipid peroxidation, but these effects were unstable. When ascorbic acid (0.1 mM) plus Fe^{2+} (0.04 mM) were added to the incubation medium, the values of thiobarbituric acid of slices increased about 2 fold compared with the control (Table 1). The effects of ascorbic acid plus

Table 1 Effects of ascorbic acid (AsA) and Fe^{2+} on lipid peroxidation of rat renal cortical slices.

Additions	*Thiobarbituric acid value (absorbance at 532 nm/g tissue*
None (control)	0.47 ± 0.05
AsA (0.1 mM)	0.45 ± 0.05
AsA (1.0 mM)	0.47 ± 0.13
AsA (10 mM)	0.57 ± 0.16
Fe^{2+} (0.04 mM)	0.80 ± 0.17*
Fe^{2+} (0.4 mM)	2.43 ± 0.32**
Fe^{2+} (4.0 mM)	7.98 ± 0.40**
AsA (0.1 mM) + Fe^{2+} (0.04 mM)	1.00 ± 0.02**
AsA (1.0 mM) + Fe^{2+} (0.4 mM)	2.77 ± 0.04**
AsA (10 mM) + Fe^{2+} (4.0 mM)	6.51 ± 0.05**

Slices were incubated as described in the text with ascorbic acid and Fe^{2+} for 10 min at 37°C. All values are mean ± s.e. of 8 experiments. Significantly different from control: * $0.01 < P < 0.02$; ** $P < 0.01$.

Fe^{2+} appeared to be concentration-dependent. Increasing the concentration from 0.1 to 10 mM (ascorbic acid) and from 0.04 to 4 mM (Fe^{2+}) produced a progessive increase in the values of thiobarbituric acid of slices (from 1.0 ± 0.02 to 6.5 ± 0.05). These results suggest that Fe^{2+} is the active peroxidant species, maintained in the reduced form by ascorbic acid.

Furthermore, we found that the lipid peroxidation induced by ascorbic acid plus Fe^{2+} reached the maximum in about 10 min (data not shown). In the following experiments, the concentrations of 1 mM ascorbic acid and 0.4 mM $FeSO_4$ and 10 min preincubation period were selected as optimal for lipid peroxidation of rat renal cortical slices.

To clarify how lipid peroxidation has an effect on the function of membrane transport, we examined the effect of lipid peroxidation on *p*-aminohippurate transport by rat kidney cortical slices.

Ascorbic acid and Fe^{2+}-induced lipid peroxidation was associated with compromised *p*-aminohippurate accumulation capacity of rat renal cortical slices (Figure 1). *p*-Aminohippurate accumulation by unperoxidized slices increased progessively in Cross & Taggart buffer with prolonged incubation time. Lipid peroxidation induced by ascorbic acid and Fe^{2+} coincided with a 45% or 80% decrease in *p*-aminohippurate accumulation after 60 min or 120 min of incubation.

Unperoxidized slices in Cross & Taggart buffer showed a small increase in the values of thiobarbituric acid; however, after 120 min of incubation there was no significant difference in the values in comparison to zero time (Figure 1). On the other hand, within the first 10 min, there was a 50% decrease in the values of thiobarbituric acid of peroxidized slices and this was maintained until the end of the 120 min incubation and kept the high level as compared with the control.

Table 2 summarizes the effect of ascorbic acid and Fe^{2+} on tissue water. Total tissue water was significantly increased when rat kidney slices were preincubated in the Tris-HCl buffer containing ascorbic acid and Fe^{2+} for 10 min, washed and incubated in Cross & Taggart buffer for 60 min. The increase in tissue water was related to changes in intracellular and extracellular water, which rose significantly during the preincubation with ascorbic acid and Fe^{2+}.

It is generally accepted that the antioxidant, N,N'-diphenyl-*p*-phenylenediamine (DPPD) is a potent inhibitor of lipid peroxidation (Ernster & Nordenbrand, 1967; Castro, Sasame, Sussman & Gillette, 1968). We found (unpublished) that DPPD inhibited lipid peroxidation at extremely low concentrations as compared with other antioxidants (e.g. α-tocopherol or tinoridine). Moreover, the ability of DPPD to modify the functional and structural hepatic lesions induced by the chronic administration of carbon tet-

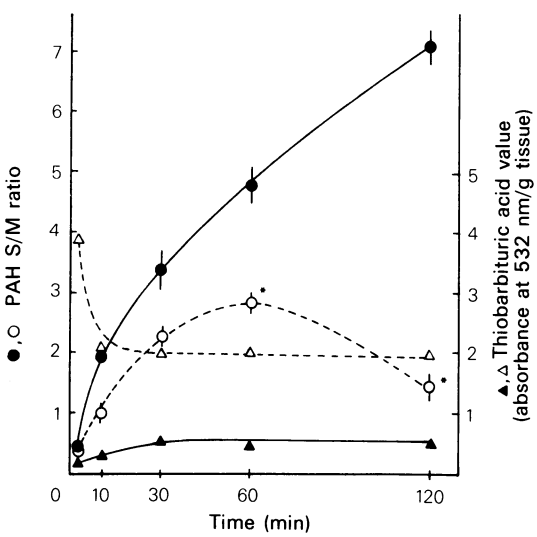

Figure 1 *p*-Aminohippurate (PAH) accumulation in rat kidney cortical slices previously incubated with and without ascorbic acid plus Fe^{2+}. Slices were incubated at 25°C in Cross & Taggart buffer after preincubation for 10 min at 37°C in 0.15M KCl-0.02M Tris HCl buffer in the absence (continuous line) and presence (dashed line) of ascorbic acid (1.0 mM) and Fe^{2+} (0.4 mM). (●, ○) *p*-Aminohippurate S/M ratio; (▲, △) thiobarbituric acid value. Each point indicates mean of 8 experiments; vertical lines show s.e. Significantly different from control: $*P < 0.01$.

Table 2 Effect of ascorbic acid plus Fe^{2+} on tissue water

	Tissue H_2O (% wet wt.)	*ECW* (μl/100 mg dry wt.)	*ICW* (μl/100 mg dry wt.)
Control	74.25 ± 0.65	127.18 ± 4.59	163.69 ± 7.70
Ascorbic acid + Fe^{2+}	80.83 ± 0.34*	194.39 ± 9.11*	228.07 ± 4.82*

ECW = extracellular water; ICW = intracellular water.
Slices were preincubated for 10 min at 37°C in 0.15M KCl-0.02M Tris HCl buffer with and without ascorbic acid (1.0 mM) + Fe^{2+} (0.4 mM), and then further incubated for 60 min at 25°C in Cross & Taggart buffer. All values are mean ± s.e. of 4 experiments.
Significantly different from control: * $P < 0.01$.

Table 3 Effect of N,N'-diphenyl-*p*-phenylenediamine (DPPD) on the decrease of *p*-aminohippurate (PAH) accumulation by ascorbic acid + Fe^{2+}

	Thiobarbituric acid value	*PAH S/M ratio*	*PAH ICF/M ratio*
Control	0.53 ± 0.07	4.75 ± 0.28	9.37 ± 0.87
DPPD (10^{-6} M)	0.41 ± 0.01	4.30 ± 0.25	8.71 ± 0.71
Ascorbic acid + Fe^{2+}	2.03 ± 0.07	2.64 ± 0.26	5.30 ± 0.45
+ DPPD (10^{-6} M)	0.65 ± 0.01*	4.34 ± 0.09*	8.90 ± 0.59*

Slices were incubated for 60 min at 25°C in Cross & Taggart buffer after preincubation for 10 min at 37°C in 0.15M KCl-0.02M Tris HCl buffer with and without ascorbic acid (1.0 mM) + Fe^{2+} (0.4 mM). All values are mean ± s.e. of 4 experiments. Significantly different from ascorbic acid, Fe^{2+}-treated values: * $P < 0.01$.

rachloride was also studied (Hartman, Di Luzio & Trumbull, 1968). Therefore, the effect of DPPD on the decrease in the accumulation of *p*-aminohippurate by ascorbic acid and Fe^{2+} was studied (Table 3). Ascorbic acid and Fe^{2+}-induced lipid peroxidation was markedly inhibited by the addition of DPPD at a concentration of 1×10^{-6} M to the preincubation medium, and *p*-aminohippurate S/M ratio and ICF/M ratio recovered completely.

Since *p*-aminohippurate uptake by renal cortical cells is a saturable process exhibiting Michaelis-Menten kinetics (Huang & Lin, 1965), we decided to investigate effects of ascorbic acid and Fe^{2+} on *p*-aminohippurate uptake by kinetic analysis. The linear increase observed up to 30 min in the present preparations suggests that the efflux from the cells is negligible at least up to 30 min (Figure 1). Thus, *p*-aminohippurate uptake during the initial 30 min could be regarded as initial influx. Therefore, a 30 min incubation period was chosen for the determination of the rate of *p*-aminohippurate uptake.

Figure 2 shows a double-reciprocal plot of effects of ascorbic acid and Fe^{2+} on *p*-aminohippurate uptake. Ascorbic acid and Fe^{2+} did not alter the maximal rate of transport, but rather, affected the apparent affinity of this transport mechanism for *p*-aminohippurate. The effects of ascorbic acid and Fe^{2+} were to increase the apparent *K*m of transport from 0.17 to 0.25 mM. The kinetic constants were calculated from the plot of Figure 2.

Discussion

Lipid peroxidation in isolated tissue homogenates, cells, and organelles is well known to involve both enzymatic and non-enzymatic catalytic mechanisms. In biological systems, polyunsaturated fatty acids in phospholipids of membranes are utilized as substrates. The polyunsaturated fatty acids form free radical intermediates during lipid peroxidation. These free radicals react with proteins, and with sulfydryl and nonsulfhydryl enzymes (Lewis & Wills, 1962; Chio & Tappel, 1969; Tappel, 1973). It may be considered that if such a reaction takes place in living cells, lipid peroxidation exerts complex effects on cell membranes including alteration in membrane swelling, membrane permeability, enzyme activity, and solute transport.

Furthermore, Hruszkewycz, Glende & Recknagel (1978) have shown that altered membrane lipids from peroxidized microsomes are capable of inhibiting cytochrome p-450 and glucose-6-phosphatase; Willis & Recknagel (1979) have reported on the

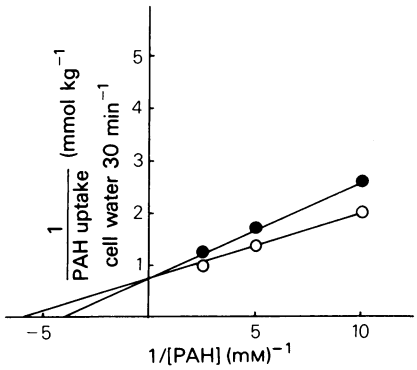

Figure 2 Double-reciprocal plot of effects of ascorbic acid plus Fe^{2+} on *p*-aminohippurate (PAH) uptake by rat kidney cortical slices. Slices were incubated for 30 min at 25°C in Cross & Taggart buffer after preincubation for 10 min at 37°C in 0.15M KCl-0.02M Tris HCl buffer with and without ascorbic acid (1.0 mM) and Fe^{2+} (0.4 mM). Each point is a mean of duplicate. (○) Control; (●) in presence of ascorbic acid plus Fe^{2+}.

isolation of these altered lipids with reference to haemolytic activity. Although these results have demonstrated the toxic nature of peroxidative metabolites, the effect of lipid peroxidation on the function of membrane transport has not been ascertained.

The present study showed that ascorbic acid plus Fe^{2+} increased lipid peroxidation of rat renal cortical slices in a dose-related manner (Table 1). *p*-Aminohippurate accumulation by rat kidney slices was significantly decreased by the presence of ascorbic acid (1 mM) and Fe^{2+} (0.4 mM) during a preincubation (Figure 1). In addition to decreasing *p*-aminohippurate accumulation, the presence of ascorbic acid and Fe^{2+} during preincubation caused changes in cell water (Table 2). This suggests that the peroxidation impaired renal tubular cells and altered the permeability of cell membranes.

According to the model described by Tune, Burg & Patlak (1969), the accumulation of *p*-aminohippurate by kidney slices is mediated by an active transport process localized at the peritubular cell membrane. Net accumulation of *p*-aminohippurate by slices reflects the rate of active transport from medium to cell as well as passive efflux from cell to medium. Therefore, the decrease of *p*-aminohippurate accumulation observed in our experiments might be due to: (1) a decrease in the active transport of *p*-aminohippurate, (2) an increase in the passive efflux of *p*-aminohippurate secondary to increased permeability of peritubular membrane, or (3) a combination of the two.

As shown in Table 3, the addition of DPPD (1×10^{-6} M) to the preincubation medium completely inhibited the lipid peroxidation and returned the accumulation of *p*-aminohippurate to the control level. These results imply that the decline of the accumulation of *p*-aminohippurate by ascorbic acid and Fe^{2+} could be mainly responsible for lipid peroxidation.

In contrast to the above observations, Stacey & Klaassen (1981) have reported that lipid peroxidation induced by sodium iodoacetamide, diethyl maleate or sodium vanadate is essentially abolished by DPPD in isolated liver cells, but cellular toxicity is only delayed and not consistently inhibited. It is possible that the differences between the present work and that of Stacey & Klaassen (1981) may be related to experimental protocol (i.e. preincubation system), preparation of tissues (i.e. the use of slices), or the nature of the injurious agent employed. However, no data are available to clarify this point, and further experiments are necessary to understand this discrepancy.

To elucidate how lipid peroxidation has an effect on active transport of *p*-aminohippurate, we examined the effect of ascorbic acid and Fe^{2+} on *p*-aminohippurate uptake kinetically.

It has been reported that the specific role of lipid in transport and catalytic activity is generally linked to the stabilization of active protein conformation (Fourcans & Jain, 1974). Lipids also seem to modify the cooperative behaviour of multicomponent complexes. These aspects of lipid-protein interaction are best reflected in a modification of the kinetic properties of various catalytic proteins. Generally, delipidation leads to an increase in *Km*, and a decrease in *Vmax* (Cunningham & Hager, 1971 a, b).

The kinetic data in Figure 2 indicate that the change produced by ascorbic acid and Fe^{2+} is an increased *Km* with the apparent *Vmax* remaining constant. This suggests that ascorbic acid and Fe^{2+} caused reduction of polyunsaturated fatty acids in phospholipids and affected the carrier affinity of this transport mechanism for *p*-aminohippurate.

Furthermore, *p*-aminohippurate uptake by renal proximal tubular cells is dependent on oxidative metabolism (Forster & Taggart, 1950). On the other hand, lipid peroxidation in membranes and subcellular organelles causes an inhibitory effect on the coupling of phosphorylation with oxidation in rat liver mitochondria (Tappel, 1973). Therefore, the effects of ascorbic acid and Fe^{2+} on the intracellular *p*-aminohippurate content of rat kidney cortex slices may be a consequence of depressed cellular respiration. If the effects of lipid peroxidation on membrane permeability, active *p*-aminohippurate transport, and oxidative metabolism observed in other cell systems also apply to renal cells, then it seems likely that alteration in the accumulation of *p*-aminohippurate reflects the combined influence of increased membrane permeability and decreased active transport.

The results of the present work strengthen the possibility that lipid peroxidation induces an appropriate functional or structural impairment of the renal cell, which in turn affects the membrane transport.

Studies on the effect of lipid peroxidation on renal cell function are now under way in this laboratory.

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