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MEASUREMENT BY HPLC OF DESFERRIOXAMINE-AVAILABLE IRON IN RABBIT KIDNEYS TO ASSESS THE EFFECT OF ISCHAEMIA ON THE DISTRIBUTION OF IRON WITHIN THE TOTAL POOL

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A method for the determination of desferrioxamine-available iron in tissue fractions is described which involves incubation with desferrioxamine, extraction of desferrioxamine and its iron-bound form, ferrioxamine, and quantitation of these two forms of the drug by reversed-phase hplc analysis. Chelatable iron levels in the 1-10 μMolar region could be accurately and reproducibly measured using this technique.

The desferrioxamine-available iron levels in both the cortex and medulla of rabbit kidneys were significantly elevated (up to 2-fold) after the organs had been subjected to 2 hours warm ischaemia or 24 hours cold storage at 0°C in hypertonic citrate solution. There was no change in the total iron content of the tissues under these circumstances and thus a redistribution of intracellular iron to more available pools had presumably taken place as a result of ischaemia. This redistribution of iron may be an important factor in the initiation of peroxidative damage to cell membranes upon reperfusion of the organ with oxygen.

KEY WORDS: Desferrioxamine, iron, ischaemia, rabbit kidney.

INTRODUCTION

There is considerable interest in the possibility that oxygen-derived free radicals may be involved in the process of cell damage which occurs in a variety of pathological conditions such as the necrosis of tissues following ischaemia and subsequent re-oxygenation. One such damaging process is the peroxidation of membrane polyunsaturated fatty acids which is a chain reaction requiring transition metals, particularly iron, for its initiation.[1,2] This may be the consequence of the autoxidation of Fe^{2+} which leads to the formation of superoxide anions (O_2^-), H_2O_2 and the highly reactive $OH\cdot$ radical.[3,4] It is also hypothesised that iron complexes with oxygen and possibly citrate can directly initiate lipid peroxidation.[5,6] Another important reaction involving iron is the decomposition of lipid hydroperoxides (LOOH) to alkoxy ($LO\cdot$) or peroxy ($LOO\cdot$) radicals which will stimulate the chain reaction of lipid peroxidation.[4]

The physiological processing of iron is designed to minimise the existence of free metal ions presumably to prevent catalysis of the aforementioned reactions. Iron is transported in transferrin and stored intracellularly in ferritin (MW 44,000) as a hydrated complex of Fe^{3+}-oxide and phosphate.[7] However, for synthetic processes, the cell must have access to free iron and there is evidence that a small pool of

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intracellular iron is available in the form of low molecular weight chelates with ATP, GTP, citrate, glycine and cysteine.[8,9]

Measurement of loosely-bound iron complexes in biological fluids has been carried out by exploiting bleomycin's specific requirement for iron in order to degrade DNA.[4] Oxygen and a reducing agent (ascorbate) need to be present and DNA-degradation products are measured by formation of coloured complexes with thiobarbituric acid. This method has been applied to serum, synovial and cerebrospinal fluids. The exact source of the iron responsible for catalysing this reaction under these conditions has not been fully elucidated. Other methods involve either spectrophotometric determination of iron in ultrafiltrated tissue homogenates after complexing with phenanthroline[10] or a bioassay which equates the level of lipid peroxidation in liver microsomes with the amount of catalytic iron present in tissue ultrafiltrates.

In this study, we have measured the level of iron which is available for chelation by desferrioxamine (DFX) in 10,000 g_{AV} supernatants of tissues. This sideramine binds one atom of Fe^{3+} with a very high stability constant (10^{31}) but has very little affinity for other metal ions.[11] The method involves incubation with DFX, extraction of DFX and its iron-bound form ferrioxamine (FX) using solid-phase cartridges, and quantitation of these two forms of the drug by reversed-phase hplc analysis based on a previously described method.[12]

We have measured DFX-available iron in rabbit kidneys subjected to periods of warm and cold ischaemia. Previous work in this laboratory has shown that homogenates of this organ exhibit higher rates of lipid peroxidation following these ischaemic insults and that periods of reperfusion *in vivo* result in further damage.[13,14] The formation of markers of lipid peroxidation under these conditions could be reduced by administration of free-radical scavengers and by desferrioxamine which suggested the involvement of chelatable iron in the mechanism of this damage.[14-16] The present study was therefore carried out in order to investigate further the possibility that redistribution of iron to more available and catalytic forms during ischaemia may be responsible, at least in part, for the initiation of peroxidative damage upon reoxygenation of the kidney.

MATERIALS AND METHODS

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 was supplied via an open face mask at a rate of 2 l/min. For warm ischaemia, both kidneys were exposed through a mid-line abdominal incision and the renal vessels ligated and cut. One kidney was removed, flushed with hypertonic citrate solution (30 ml) and assayed immediately (control); the remaining kidney was flushed as above and left *in situ* for 2 hours before being analysed. In the cold ischaemic model, the organs were removed, the renal arteries cannulated and both kidneys were flushed with 30 ml of hypertonic citrate solution.[17] One organ was assayed immediately (control) and the other was stored in a sterile beaker of hypertonic citrate solution at 0°C for 24 hours.

Trace iron contamination from buffers and distilled water was removed by the method of Gutteridge using a dialysis sac containing conalbumin.[18] All glassware, tubes and pipette tips were washed with EDTA (4 mM) followed by distilled water.

Kidneys were divided into cortex and medulla, suspended in 0.1 M Tris-HCl buffer,

pH 7.4 (25% w/v) and homogenised using a Potter–Elvehjem homogeniser. The samples were centrifuged at 10,000 g_{AV} for 15 minutes at room temperature and the supernatants collected for analysis. For the determination of desferrioxamine-chelatable iron, triplicate aliquots (1 ml) of supernatant were mixed with 100 μl of desferrioxamine (22 mM; final concentration 2 mM) and incubated at 37°C for 60 minutes to allow the reaction of chelatable iron with desferrioxamine. The parent compound (desferrioxamine; DFX) and the iron-bound form (ferrioxamine; FX) were extracted from the incubation mixture using Bond-Elut C18 cartridges (6 ml) (Anachem, Luton, Beds). The columns were first wetted with methanol (5 ml) and conditioned with distilled water (5 ml). The sample was applied to the column which was then washed with water (1 ml) and DFX and FX were eluted with methanol (2 ml). After filtration (Acrodisc, 0.45 μm; Gelman Sciences), the methanol extracts were dried under vacuum in a rotary evaporator (Uniscience, London) and stored at −70°C while awaiting analysis.

Analysis of DFX and FX was performed on an LKB model 2150 hplc fitted with a variable wavelength u.v. detector (2151) linked to a Trio Computing Integrator (Trivector, Sandy, Beds). An Ultropac Spherisorb 3 μm C18 column (4.6 mm i.d × 100 mm) was used and the mobile phase (0.5 ml/min) consisted of 0.02 M sodium phosphate buffer (pH 6.6) containing 12.8% (v/v) acetonitrile, 4 mM EDTA and 1 M ammonium acetate. Samples were redissolved in mobile phase (100 μl), and after repeated washing of the syringe and injection loop with mobile phase, 20 μl was injected onto the column. After elution of FX, which was detected at 430 nm, the wavelength was changed to 229 nm for detection of DFX.

The (area of FX)/(area of DFX) ratio was calculated and the amount of chelatable iron in each sample determined from a standard curve produced the same day from triplicate standards which contained 0, 10 and 25 nmoles Fe^{3+}/ml and were subjected to the same procedure.

The total iron content of samples was determined, after suitable dilution, by atomic absorption at 249 nm on a Perkin Elmer 2380 spectrometer.

Filtered cytosolic fractions of the cortex were prepared by centrifugation of homogenates at 100,000 g_{AV} for 1 hour at 4°C followed by filtration of the supernatant through 50,000 and 25,000 MW cut-off filters (Amicon). The protein content of the samples was determined by the method of Lowry[19] using bovine serum albumin as the standard.

Ferritin, Type 1 from horse spleen was obtained from Sigma Chemical Co., Poole, Dorset.

RESULTS AND DISCUSSION

The method described was found to be a rapid and reliable procedure for the recovery and quantitation of DFX and its iron-bound form, FX, in biological materials. Each Bond-Elut cartridge could be used for at least 10 extractions with methanol and water washes between samples. Figure 1 shows the hplc analysis of a standard solution containing DFX and 25 μM iron. The addition of EDTA to the mobile phase was necessary to remove iron originating from the stainless-steel hplc equipment.[12] As EDTA has a high u.v. absorbance at 229 nm, the u.v. detector had to be 'backed-off' when used at this wavelength. The use of a non-ferrous hplc system would circumvent the necessity for the presence of EDTA. Ammonium acetate was added to the mobile phase to reduce the tailing of the DFX peak.

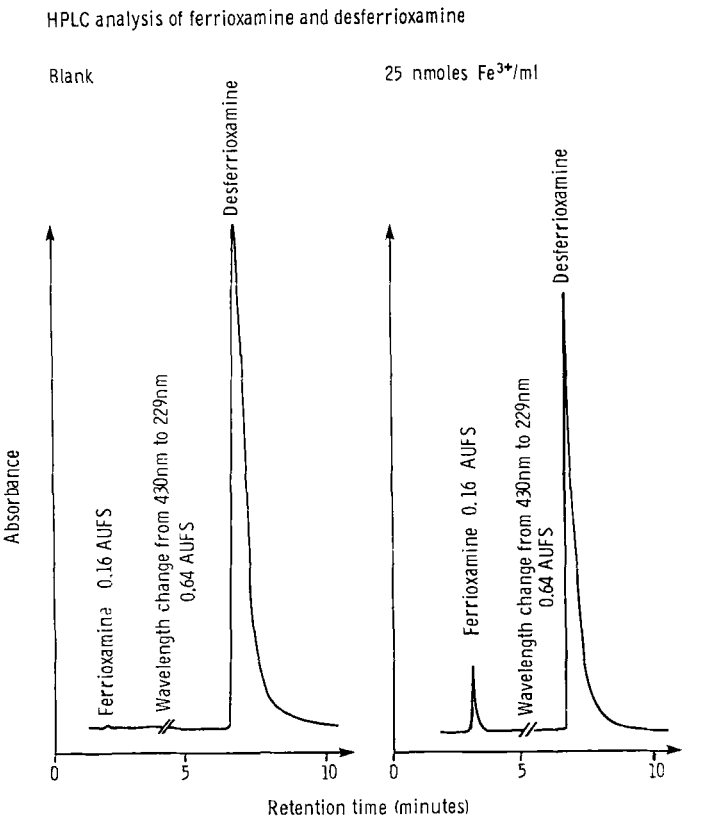

FIGURE 1 HPLC analysis of standard solutions containing 2 mM desferrioxamine and 0 or 25 μM ferric ions.

By determining the FX/DFX ratio, levels of chelatable iron in the 1–10 μMolar region could be accurately and reproducibly determined as shown by the standard curve (Figure 2). The background level of FX was consistently near to 1 μMolar when iron contamination was dialysed from the buffers prior to use[18] and the glassware was cleaned and kept dust-free (Figure 2).

Desferrioxamine chelates iron only in the ferric form.[11] It does however exert a powerful ferroxidase activity on ferrous ions. Incubation of DFX with ferrous ions resulted in the rapid formation of FX and a FX/DFX ratio which was equal to that of an equimolar amount of ferric ions. Thus, total oxidation to the ferric state had occurred during incubation with DFX and this was also the case when ascorbate was present (data not shown).

We have used this method to investigate the pool of DFX-chelatable iron in rabbit kidney homogenates. The linear relationship between the amount of tissue assayed and the FX/DFX ratio (Figure 3) demonstrated the existence of a pool of chelatable iron in this tissue and the applicability of this method for these studies. The level of chelatable iron in supernatants of control (HCA-flushed) kidneys in comparison to the total iron content of the supernatants and the tissue homogenates is shown in

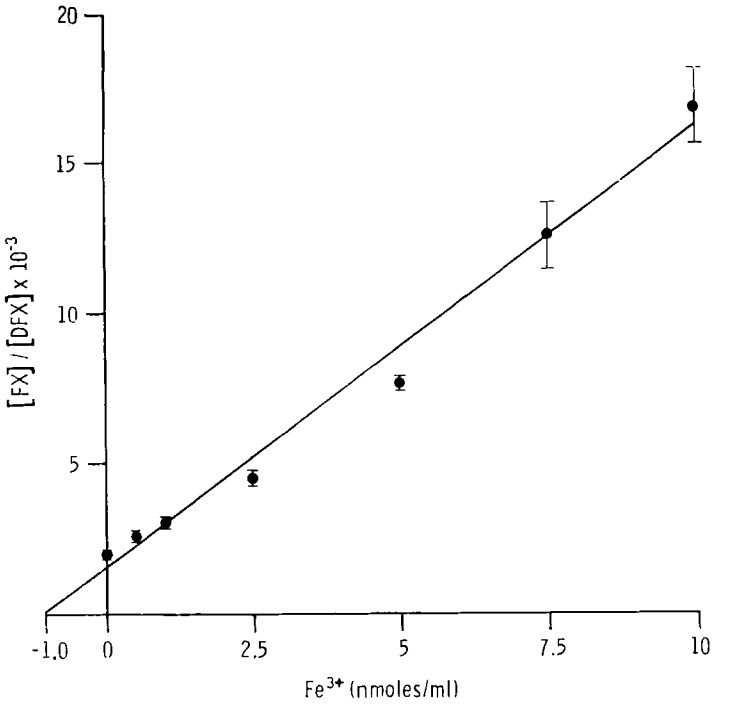

FIGURE 2 Standard curve of the relationship between the concentration of ferric ions and the ferrioxamine/desferrioxamine ratio determined by hplc. Values represent the mean ± S.E.M. of three determinations.

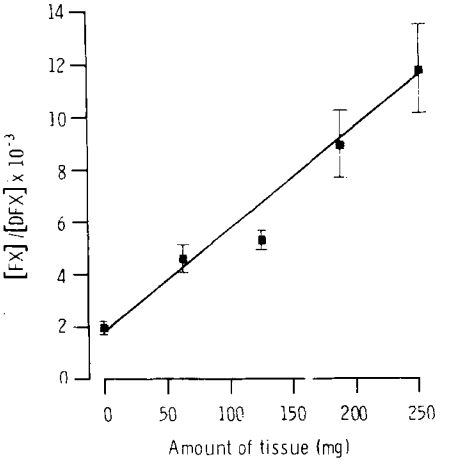

FIGURE 3 The relationship between the amount of renal cortex analysed and the ferrioxamine/desferrioxamine ratio determined by hplc following incubation of 10,000 g_{AV} supernatants of the tissue with desferrioxamine (2 mM) for 1 h at 37°C.

TABLE 1
Total iron and desferrioxamine-available iron in subcellular fractions of the rabbit kidney cortex and medulla

	Whole tissue Homogenate	10,000 g_{AV} Supernatant		Filtered cytosol	
	Total Iron+	Total Iron	DFX-A Iron*	Total Iron	DFX-A Iron
Cortex	1060 ± 220	347.5 ± 48	15.9 ± 1.3	1.6 ± 1.6	2.7 ± 2.0
Medulla	626 ± 110	189.5 ± 38	12.1 ± 1.6	ND°	ND

+ Total iron measured by atomic absorption spectroscopy (nmol/g tissue)
* Desferrioxamine-available iron measured by hplc (nmol/g tissue)
° ND – Not determined
Values represent the mean ± S.D of 6 determinations. See Methods and Materials for details of preparative procedures.

Table 1. The iron available for chelation represented 4–6% of the total iron in the supernatant fraction and 2–3% of the total iron content of the tissue.

Determination of the level of low molecular weight (LMW) iron chelates (MW < 10,000) present in filtered cytosolic fractions of the cortex yielded similar results when analysed by atomic absorption spectroscopy and DFX-chelation (Table 1). Thus all the iron in this fraction was chelatable. This LMW chelatable pool was significantly lower than the DFX-chelatable pool in the 10,000 g_{AV} supernatant indicating that DFX was scavenging iron from high molecular weight sources in this fraction. The major intracellular iron storage protein is ferritin which can bind up to 4,500 molecules of iron.[7] Previous studies have demonstrated that iron in this protein is available for chelation by DFX[11] and a time course of the release of ferritin iron during incubation with DFX using hplc analysis of FX and DFX is shown in Figure 4. After 1 hour of incubation at 37°C, 1–2% of the iron had been released and was in the form of ferrioxamine. DFX has been reported elsewhere as able to remove iron from the iron-transporting protein transferrin[11] and this has also been demonstrated in our laboratory using the method described in this study (data not shown). However, as the kidneys were thoroughly flushed with HCA via the renal artery before homogenisation, it is unlikely that transferrin iron contributed significantly to the pool of DFX-chelatable iron measured in these tissues. It is also possible that a small amount of iron may have been released from iron proteins by proteolysis during the homogenisation and centrifugation.

Measurement of the FX/DFX ratio in kidney homogenates incubated with DFX showed that periods of both warm ischaemia and cold ischaemia resulted in significant increases in the DFX-chelatable iron pool in both the medulla and cortex (Table 2). There was no change in the total iron content of the tissues (Table 2) hence the percentage of total iron available to DFX must have increased as a result of either the 2 hours warm ischaemia or 24 hours cold ischaemia. The likely source of this extra available iron is ferritin which stores iron in the ferric form. The release of ferritin iron involves its reduction to the ferrous state by a mechanism which has not been fully elucidated.[20] The increase in the level of reducing species in ischaemic tissues, including a decreased pH, may stimulate iron release from this protein or make ferritin iron more susceptible to mobilisation. It is hypothesised that superoxide anions may be formed during the reperfusion of ischaemic tissues as a result of increased leakage from the mitochondrial electron transport chain or the conversion of xanthine dehydrogenase to xanthine oxidase which utilises hypoxanthine and produces O_2^- from O_2.[21] Superoxide is known to release iron from ferritin[22] and may therefore have been

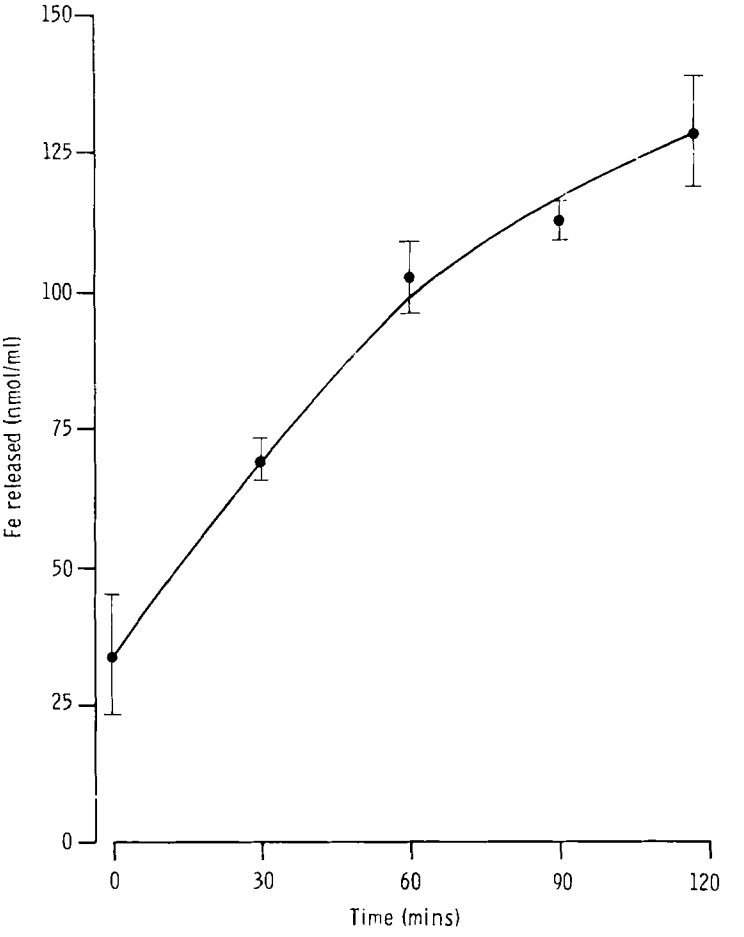

FIGURE 4 Time course of the removal of iron from ferritin (1 mg/ml) by desferrioxamine (2 mM) at 37°C as determined by hplc analysis of the ferrioxamine/desferrioxamine ratio. Values represent the mean ± S.E.M. of three determinations.

responsible for chelatable iron release during the *in vitro* incubation of the ischaemic tissue homogenates with DFX.

The level of LMW-chelatable iron in canine brain has been reported to increase three-fold following 15 minutes of cardiac arrest.[23] Iron levels were determined by ultrafiltration of the homogenates followed by spectroscopic measurement of iron-phenanthroline complexes. 123 nmol LMWC iron/g tissue was reported for control animals and this is considerably greater than the levels of DFX-chelatable iron in rabbit kidneys reported here which in addition includes iron released from high molecular weight sources. In another study, a small rise in perfusate DFX-Fe levels (measured by EPR spectroscopy) was found following 30 minutes warm ischaemia of an isolated rabbit preparation.[24]

It cannot be stated with certainty that the iron measured in this study is in a form which is able to catalyse either the formation of the highly damaging OH· radical

TABLE 2
The effect of cold ischaemia and warm ischaemia on the level of total iron and desferrioxamine-available iron in the rabbit kidney

	Cortex		Medulla	
	Total Iron[+]	DFX-A Iron[°]	Total Iron	DFX-A Iron
Control (HCA-Flush)	6.63 ± 0.95	296 ± 100	5.61 ± 1.45	345 ± 177
24 h cold ischaemia	5.41 ± 0.91	604 ± 118**	4.40 ± 0.56	620 ± 139**
2 h warm ischaemia	5.42 ± 0.27	488 ± 94*	5.59 ± 1.10	599 ± 160*

[+] Total iron measured by atomic absorption spectroscopy (nmol/mg protein)
[°] Desferrioxamine-available iron measured by hplc (pmol/mg protein)
* Significantly different ($P < 0.05$) from control
** Significantly different ($P < 0.005$) from control
Values represent the mean ± S.D. of 6 determinations

from $O_2^{\cdot-}$ or the initiation of lipid peroxidation by converting lipid hydroperoxides to peroxy radicals. However, the availability of iron for chelation by DFX is highly suggestive of its availability to catalyse these damaging reactions. Indeed it has been shown that ferritin can donate the necessary iron for microsomal lipid peroxidation induced by NADPH or xanthine oxidase[25] and can stimulate the peroxidation of lipids.[26,27]

In conclusion, the method reported here allows determination of DFX and FX in biological tissues and hence the quantitation of DFX-available iron levels. We are currently applying this procedure to investigate levels of FX and DFX in ischaemic tissues following administration of DFX *in vivo*. The redistribution of iron to more available pools as a result of ischaemia, as shown in this study, may be an important factor in the underlying mechanism which results in increased levels of lipid peroxidation under these conditions.[12,13] These results in conjunction with our previous findings provide yet more evidence for a role of iron in the pathogenesis of ischaemic tissue damage and the possible therapeutic value of iron-chelators.

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