# Increase in bleomycin-detectable iron in ischaemia/reperfusion injury to rat kidneys

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Iron has been shown to be important in ischaemic, immune and toxic forms of tissue injury in various organs. Although it is generally accepted that iron participates in the generation of powerful oxidant species (e.g. hydroxyl radicals) there has not been any direct evidence that iron capable of catalysing free-radical reactions is increased in tissues in these models of injury. In the present study we demonstrate that ischaemia/reperfusion injury to the kidney results in no significant change in total, non-haem or ferritin iron levels, but there is a marked and specific increase in bleomycin-detectable iron (capable of catalysing free-radical reactions) in the kidney. The increase in bleomycin-detectable iron is observed only after reperfusion but not during the ischaemic period. In a separate study we demonstrate that despite a drastic reduction in the iron content in the kidney, as a result of feeding an iron-deficient diet, there is a similar and a marked increase in the bleomycin-detectable iron in kidneys accompanied by a lack of protection against ischaemia/reperfusion injury.

## INTRODUCTION

Iron, an essential element in all living cells, participates in numerous metabolic pathways. A transition metal, iron can serve as a carrier for oxygen and electrons and as a catalyst for oxygenation, hydroxylation and other critical metabolic processes, in part because of its ability to reversibly and readily cycle between the ferrous and the ferric oxidation states. The ease with which iron is reversibly oxidized and reduced, while essential for its metabolic functions, also makes iron potentially hazardous because of its ability to participate in the generation of powerful oxidant species, such as the hydroxyl radical, [1] (the metal catalysed Haber–Weiss reaction) and/or in the generation of the highly reactive iron–oxygen complexes such as ferryl or perferryl ions [1–5]. Indeed, a large body of evidence indicates an important role for iron in tissue injury [1]. Thus, iron chelators, including desferrioxamine and 2,3-dihydroxybenzoic acid, have been shown to be protective in several forms of tissue injury, including ischaemia/reperfusion injury to the heart [1,6], the kidney [1,7,8] and the gut [1,9]; and toxic and immune injury to the kidney [10] and lung [1,11]. In addition, in several *in vitro* models of oxidant-induced cell injury, iron chelators have been shown to be protective [1,12–14].

Despite compelling evidence for the role of iron in various forms of tissue injury, there has not been any direct evidence that levels of iron capable of catalysing free-radical reactions are increased in tissues in these models of injury. *In vivo* most of the iron is bound to haem and non-haem proteins, and does not directly catalyse the generation of hydroxyl radicals or a similar oxidant [1]. Gutteridge et al. have described an assay, based on the use of the antibiotic bleomycin, to detect iron complexes capable of catalysing free-radical reactions in biological samples [1,15–17].

The major purpose of our study was to examine whether an increase in bleomycin-detectable iron could be demonstrated in the kidney in a model of ischaemia/reperfusion-induced acute renal failure using the bleomycin-detectable-iron assay. In a separate study we also examined the effect of an iron-deficient diet on renal function and levels of bleomycin-detectable iron in kidneys in response to ischaemia/reperfusion injury.

## MATERIALS AND METHODS

### Ischaemia/reperfusion injury in rats fed normal Purina rodent chow

Animal studies were performed in accordance with the National Institutes of Health guidelines for animal care and with the approval of the institutional animal care and use subcommittees. Acute renal failure was induced in adult male Sprague–Dawley rats fed a normal Purina rodent chow. Bilateral flank incisions were made under pentobarbital anaesthesia and the renal pedicles were occluded for 40 min by non-traumatic clamps followed by reperfusion for several time periods. In the control group bilateral flank incisions were made without clamping renal pedicles. Incisions were closed, animals were allowed to recover, returned to the metabolic cages and then killed. Blood and kidneys and a piece of liver were obtained for the various measurements.

### Ischaemia/reperfusion injury in rats fed either an iron-deficient diet or an iron-replete diet

Weanling male Sprague–Dawley rats (21 days old) were housed in rust-free cages and fed a diet containing 50 mg of iron/kg of diet for 10 days (minimum requirement of iron, for a rat, to prevent anaemia) to establish baseline iron stores. These rats were then divided into two equal groups by weight and placed on their respective isocaloric iron diets for 21 days (Teklad, RD80396) $<6$ mg (deficient); and 200 mg (normal) iron/kg of diet, a diet corresponding to the iron present in the rodent chow. Only the iron content of the diets was varied and the iron was added as ferric citrate. At the end of this period, acute renal failure was induced as previously described by occlusion of the renal pedicles for 40 min. Blood and kidneys were obtained 24 h later at the time of killing.

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### Analytical methods

Haematocrit readings were determined by centrifugation of blood in heparinized glass capillary tubes. Serum iron was measured from serum separated from the cells within 2 h of blood drawing and then stored. Analyses were performed using Sigma diagnostic kit procedure no. 565. Plasma urea nitrogen and creatinine concentrations were measured using a Beckman SUN Analyser 2 and a Beckman Creatinine Analyser.

For tissue iron determinations all buffers and reagents were made in Chelex-treated pyrogen-free water in iron-free plastic containers or acid-washed glass containers. The tissues were minced and homogenized in 25% (w/v) of Chelex-treated pyrogen-free water as described in the bleomycin assay and total iron was determined as described by Doeg and Ziegler [18]. Ferritin iron was measured by ammonium sulphate precipitation as described by Avol et al. [19] and non-haem iron was determined by acid hydrolysis as described by Torrance and Bothwell [20].

### Bleomycin assay [1,15–17]

At the time of killing the kidneys were removed and decapsulated. The kidney tissues or liver tissues were immediately kept on ice. The kidney tissues were weighed after the medulla was removed. Tissues were minced in 10% (w/v) Chelex-treated pyrogen-free water in specially cleaned glassware, and homogenized by 6–10 cycles at 600 rev./min using a Potter–Elvehjem homogenizer at 4 °C. The mean weights of tissues used for homogenization were 0.66±0.04 g for kidney and 1.57±0.12 g for liver. The tissue homogenates, prepared with Chelex-treated pyrogen-free water, were loaded into a Centriprep-30, which has a cut-off value of 30000 Da, and centrifuged at 4 °C for 30 min at 1500 *g*. The clear ultrafiltrate was collected and used for bleomycin-detectable iron determinations. All reagents, except for the sample under test, were made up in Chelex-treated pyrogen-free water in iron-free plastic containers and shaken with Chelex-100 to remove as much contaminating iron as possible. The reaction mixture contained 0.5 ml of calf thymus DNA (1 mg/ml), 0.05 ml of bleomycin sulphate (1 mg/ml), 0.1 ml of $MgCl_2$ (50 mM), 0.1 ml of sample or iron standard, 0.05 ml of HCl (10 mM), 0.1 ml of Chelex-treated pyrogen-free water and 0.1 ml of ascorbic acid solution. Sample blanks were identical except that bleomycin was omitted. Tubes were mixed before and after addition of ascorbate and then incubated at 37 °C for 2 h with shaking. The reaction was stopped by adding 0.1 M EDTA, the contents transferred to glass tubes and mixed with 1 ml of thiobarbituric acid [1% (w/v) in 50 mM NaOH] and 1 ml of HCl (25%, v/v). This reaction mixture was then heated at 100 °C for 15 min, cooled and the resulting chromogen measured using a spectrophotometer by its absorbance at 532 nm.

A standard curve was prepared using various concentrations of $FeCl_3$ (0, 3.125, 6.25, 12.5, 25, and 50 nmol per 0.1 ml) in Chelex-treated pyrogen-free water. Each standard also had a corresponding blank that was identical except that bleomycin was omitted. The absorbance of the zero standard (which contained only Chelex-treated pyrogen-free water), reflecting iron contamination in the reagents etc., was subtracted from all standards to construct the standard curve. The amounts of bleomycin-detectable iron in test samples were calculated from the standard curve obtained in each experiment. We confirmed that various amounts of filtrates showed linearity in bleomycin-detectable iron content, and when each amount of filtrate was spiked with a constant amount of $FeCl_3$, the resultant curve was linear and parallel to the original unspiked curve (results not shown). These findings further validate the assay for bleomycin-detectable iron used in our study. The amount of bleomycin-detectable iron in the total amount of filtrate recovered was calculated and the results expressed as nmol/wet wt. of tissue.

**Table 1 Results from rats with ischaemia/reperfusion-induced acute renal failure compared with control rats**

Ischaemia was induced by clamping renal pedicles for 40 min, followed by reperfusion for 24 h. Results are given as means±S.E.M. and derived from three separate experiments with 4–7 animals in the control group or the experimental group in each of the studies. The number of rats used is given in parentheses. Ferritin, non-haem and total iron levels are expressed in μmol/g wet wt. of kidney. The groups were compared using analysis of variance and *post hoc* with Fischer's least-significant-difference (LSD) procedure. * $P < 0.01$ comparing ischaemia/reperfusion with controls.

| | Control rats | (*n*) | Ischaemia/reperfusion-induced rats | (*n*) |
|---|---|---|---|---|
| Weight (g) at death | 314±4 | (16) | 319±5 | (18) |
| Haematocrit reading (%) | 45±1 | (4) | 47±2 | (6) |
| Serum iron content (μmol/l) | 23.1±2.5 | (4) | 20.0±2.7 | (8) |
| Plasma urea nitrogen concn. (mg/dl) | 17±1 | (16) | 64±8* | (18) |
| Plasma creatinine concn. (mg/dl) | 0.4±0.0 | (16) | 2.0±0.3* | (18) |
| Kidney | | | | |
| Total iron (μmol/g) | 0.79±0.04 | (4) | 0.70±0.04 | (6) |
| Non-haem iron (μmol/g) | 0.66±0.04 | (9) | 0.59±0.25 | (14) |
| Ferritin (μmol/g) | 0.45±0.05 | (5) | 0.52±0.04 | (8) |
| Liver | | | | |
| Total iron (μmol/g) | 1.15±0.11 | (4) | 1.45±0.09 | (6) |
| Non-haem iron (μmol/g) | 1.18±0.11 | (9) | 1.20±0.05 | (14) |

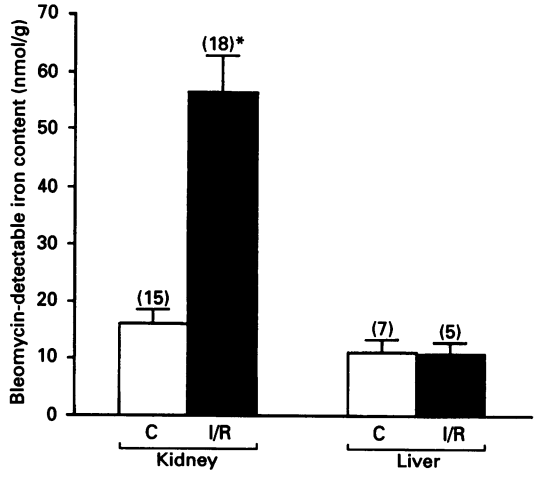


**Figure 1 Bleomycin-detectable iron in the kidney and liver from rats subjected to ischaemia/reperfusion injury**

Results are expressed as nmol/g wet wt. of tissue. Results for kidneys are means±S.E.M. derived from three separate experiments with 4–7 animals in the control group or the experimental group in each of these studies. Results for liver are from the same animals in one of these studies. The number of rats used is given in parentheses. The level of bleomycin-detectable iron was significantly higher in kidneys obtained from rats subjected to 40 min ischaemia followed by 24 h reperfusion in each of the three separate experiments. The groups were compared using analysis of variance and *post hoc* with Fischer's least-significant-difference (LSD) procedure. * $P < 0.01$ comparing ischaemia/reperfusion-injured animals with controls. Abbreviations: C, control; I/R, ischaemia/reperfusion.

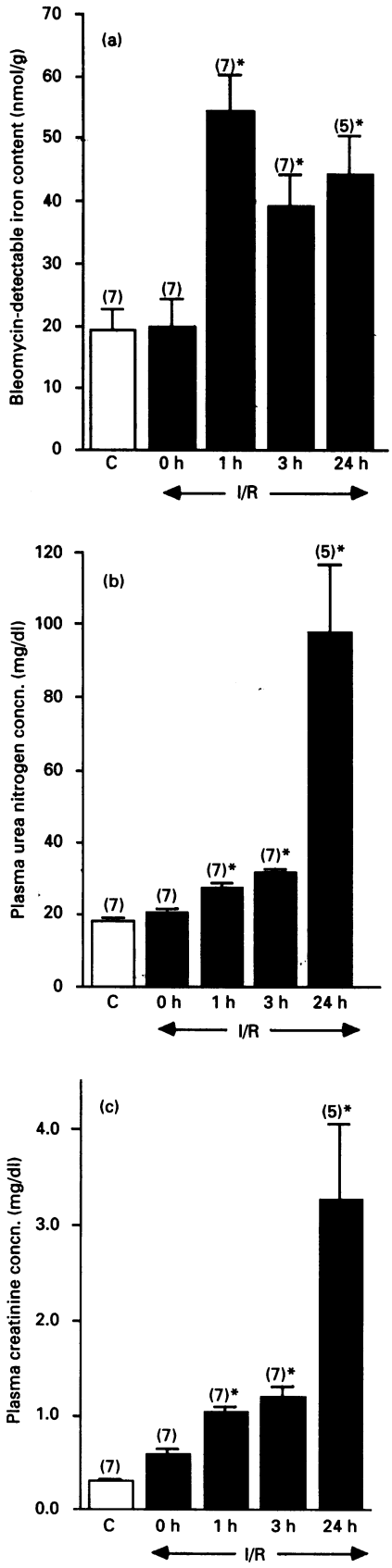


**Figure 2 Bleomycin-detectable iron (a), plasma urea nitrogen (b), and plasma creatinine (c) in the kidneys of rats after ischaemia (40 min) and various periods of reperfusion (0, 1, 3, 24 h)**

* $P < 0.01$ as compared with controls. Abbreviations: C, control; I/R, ischaemia/reperfusion.

## RESULTS

In rats that underwent 40 min ischaemia, followed by 24 h reperfusion, there were no significant differences in body weight, haematocrit reading or serum iron compared with the control group (Table 1). As expected, renal function (as measured by plasma urea nitrogen and plasma creatinine) was significantly worse in rats that underwent ischaemia/reperfusion compared with controls (Table 1). There were no significant differences in the total iron, non-haem iron, or ferritin levels in either the kidney or liver between control rats and rats with acute renal failure (Table 1). In contrast, using the bleomycin assay to detect iron complexes capable of catalysing free-radical reactions, we found a significant increase in the level of bleomycin-detectable iron in the kidney (Figure 1). There were no differences in the levels of bleomycin-detectable iron in the liver. These results indicate a marked and specific increase in bleomycin-detectable iron in ischaemia/reperfusion injury to the kidney.

We next examined the change of bleomycin-detectable iron in the kidney at various time points, just after 40 min ischaemia as well as after 1 h, 3 h and 24 h of reperfusion. As illustrated in Figures 2(b) and 2(c), there was a significant increase in plasma urea nitrogen and creatinine at 1 h after reperfusion, with marked increases at 24 h. The bleomycin-detectable iron in the kidney just after ischaemia was similar to sham-operated controls, but increased significantly after reperfusion (Figure 2a).

In separate experiments, we examined the effect which feeding rats an iron-deficient (< 6 mg/kg) isocaloric diet had on bleomycin-detectable iron in the kidney and on renal function in ischaemic acute renal failure compared with rats fed a normal (200 mg/kg) iron diet. As shown in Table 2 rats fed an iron-deficient diet had lower body weights compared with rats on a normal iron diet. As expected rats fed an iron-deficient diet had significant and marked reduction in haematocrit readings and serum iron levels, thus documenting that these rats were indeed markedly iron deficient. In addition, rats fed an iron-deficient diet had significantly lower levels of non-haem iron in the kidney (Figure 3a). As in the previous study, there was no significant difference in the non-haem iron content of the kidneys from rats with ischaemic/reperfusion injury compared with their corresponding controls (Figure 3a).

**Table 2 Results from rats fed either an iron-deficient diet or an iron-replete diet and subjected to ischaemia/reperfusion injury**

| Diet... | Normal iron diet (200 mg of iron/kg) | | Iron-deficient diet (< 6 mg of iron/kg) | |
|---|---|---|---|---|
| | Control ($n = 5$) | I/R ($n = 6$) | Control ($n = 5$) | I/R ($n = 6$) |
| Weight at death (g) | 330 ± 14 | 322 ± 12 | 293 ± 9 | 307 ± 11 |
| Haematocrit reading (%) | 47 ± 1 | 47 ± 2 | 26 ± 1* | 28 ± 2* |
| Serum iron content (μmol/l) | 22.9 ± 3.4 | 19.2 ± 2.1 | 3.2 ± 0.7* | 3.8 ± 0.8* |
| Plasma urea nitrogen concn. (mg/dl) | 17 ± 2 | 79 ± 13† | 19 ± 1 | 95 ± 9† |
| Plasma creatinine concn. (mg/dl) | 0.3 ± 0.01 | 2.2 ± 0.2‡ | 0.3 ± 0.02 | 2.7 ± 0.5† |

* $P < 0.001$, iron-deficient versus its corresponding group on normal iron diet.
† $P < 0.001$, control versus ischaemia/reperfusion-treated group (I/R).
‡ $P < 0.01$, control versus ischaemia/reperfusion-treated group.

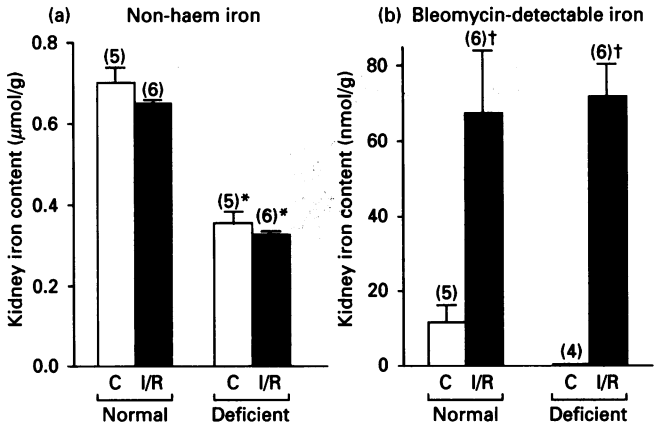


**Figure 3 Non-haem iron (a) and bleomycin-detectable iron (b) in kidneys of rats fed either an iron-deficient diet or normal iron diet and subjected to ischaemia/reperfusion injury to the kidney**

*$P < 0.001$ comparing non-haem iron in kidney from rats fed an iron-deficient diet (< 6 mg/kg) to rats fed a normal iron diet (200 mg/kg). †$P < 0.01$ comparing ischaemia/reperfusion group to its corresponding controls. Abbreviations: C, control; I/R, ischaemia/reperfusion.

As shown in Figure 3(b), rats fed a normal iron diet exhibited a marked increase in the bleomycin-detectable iron in the kidneys obtained from rats with ischaemic acute renal failure. In rats fed an iron-deficient diet, despite the marked reduction in non-haem iron, there was a marked increase in the bleomycin-detectable iron in kidneys obtained from rats with ischaemic acute renal failure. This increase was similar to the increase observed in rats on a normal iron diet (Figure 3b). Feeding an iron-deficient diet did not protect against ischaemia/reperfusion injury as measured by plasma urea nitrogen and creatinine (Table 2). Thus, despite the markedly lower level of non-haem iron in the kidney as a result of feeding an iron-deficient diet, there was a marked increase in the bleomycin-detectable iron in kidneys, accompanied by a lack of protection against ischaemia/reperfusion injury.

## DISCUSSION

The major purpose of our study was to examine whether an increase in bleomycin-detectable iron could be demonstrated in the kidney in a model of ischaemia/reperfusion-induced acute renal failure using the bleomycin-detectable iron assay [1,15–17]. The assay is based on the observation that the anti-tumour antibiotic bleomycin, in the presence of an iron salt and a suitable reducing agent, binds to and degrades DNA with the formation of a product that reacts with thiobarbituric acid to form a chromogen. The binding of the bleomycin–iron complex to the DNA makes the reaction site specific and antioxidants rarely interfere. Iron detected in this assay is, thus, a measure of iron available from the biological sample to bleomycin. Although other metal ions can bind to bleomycin, they do not result in DNA degradation. The assay conditions have been designed to prevent any interference from iron-containing proteins [15]. Iron bound to transferrin, lactoferrin, ferritin or iron-containing enzymes does not register in the bleomycin assay [15].

In the present study, we have shown that ischaemia/reperfusion injury to the kidney results in no significant change in total, non-haem or ferritin iron levels but causes a marked increase in bleomycin-detectable iron in the kidney after reperfusion. In addition, this increase in bleomycin-detectable iron is injury-specific, as no similar increase was observed in the liver. In contrast with our study, no obvious differences were noted in tissue levels of bleomycin-detectable iron in the brain following ischaemia and reperfusion [17], although similar to our results an increase in desferrioxamine-chelatable iron (ferric iron) has been reported in rabbit kidneys exposed to ischaemia [21].

It has been suggested that the iron status may be important in understanding differences in susceptibility to tissue injury [22,23]. For example, Sullivan has suggested that iron status may be the key to understanding gender difference in death rates from ischaemic heart disease [22] and the beneficial effect of nutritional iron restriction in a model of acute lung injury [24] has been reported. The rat has been widely accepted as a model of iron deficiency because it mirrors iron metabolism in man in most respects [25]. Feeding rats an iron-deficient diet to reduce the iron content in the kidney failed to protect against either the marked increase of the bleomycin-detectable iron in the kidneys or to protect against acute renal failure in rats subjected to ischaemia/reperfusion.

The source of the bleomycin-detectable iron is not known. *In vivo* most of the iron is bound to haem and non-haem proteins, and does not directly catalyse the generation of hydroxyl radicals or a similar oxidant [1]. However, based on the ability of superoxide [26–28] to release iron from ferritin *in vitro*, the iron-storage protein ferritin, which otherwise provides a secure means of storing iron in an inert form, has been suggested as a possible source of iron to participate in free-radical reactions. While this is a possibility, in an *in vitro* study by Kvietys et al. it was shown that ferritin was not the source of iron [29]. This observation, together with our results demonstrating a marked increase in the bleomycin-detectable iron in kidneys from rats fed an iron-deficient diet, makes ferritin a less likely source of catalytic iron. Hydrogen peroxide and organic hydroperoxides can oxidatively degrade haemoglobin and promote the release of iron from haem chelate [1,30]. Thus, haemoglobin [31], cytochrome *P*-450 enzymes [32], or in special circumstances myoglobin [33] (which are basically haem proteins with an iron–porphyrin prosthetic group) are other possible sources of iron. Another potential source of iron within the cell is iron-rich mitochondria, although currently there is no evidence to suggest that the mitochondrial pool of iron participates in tissue injury. Regardless of the source of iron, our results demonstrate a marked and a specific increase in bleomycin-detectable iron with no significant change in total, non-haem or ferritin iron, and that despite a drastic reduction in the iron content in the kidney as a result of feeding an iron-deficient diet, there is a similar and marked increase in the bleomycin-detectable iron in the kidneys.

## REFERENCES

1 Halliwell, B. and Gutteridge, J. M. C. (1991) Methods Enzymol. **186**, 1–85
2 Aust, S. D., Morehouse, L. A. and Thomas, C. E. (1985) J. Free Radical Biol. Med. **1**, 3–25
3 Hochstein, P., Nordenbrand, K. and Ernster, L. (1964) Biochem. Biophys. Res. Commun. **14**, 323–328
4 Imlay, J. A., Chin, S. M. and Linn, S. (1988) Science **240**, 640–642
5 Gutteridge, J. M. C., Maidt, L. and Poyer, L. (1990) Biochem. J. **269**, 169–174
6 Williams, R. E., Zweier, J. L. and Flaherty, J. T. (1991) Circulation **83**, 1006–1014
7 Ratych, R. E. and Bulkley, G. B. (1986) J. Free Radical Biol. Med. **2**, 311–319
8 Paller, M. S. and Hedlund, B. E. (1988) Kidney Int. **34**, 474–480
9 Smith, J. K., Carden, D. L., Grisham, M. B., Granger, D. N. and Korthuis, R. J. (1989) Am. J. Physiol. **256**, H1472–H1477
10 Shah, S. V. (1989) Kidney Int. **35**, 1093–1106
11 Johnson, K. J., Ward, P. A., Kunkel, R. G. and Wilson, B. S. (1986) Lab. Invest. **54**, 499–506
12 Starke, P. E. and Farber, J. L. (1985) J. Biol. Chem. **260**, 10099–10104

13 Hiraishi, H., Terano, A., Ota, S., Mutoh, H., Razandi, M., Sugimoto, T. and Ivey, K. J. (1991) Am. J. Physiol. **260**, G556–G563
14 Walker, P. D. and Shah, S. V. (1991) Kidney Int. **40**, 891–898
15 Gutteridge, J. M. C., Rowley, D. A. and Halliwell, B. (1981) Biochem. J. **199**, 263–265
16 Gutteridge, J. M. C., Rowley, D. A. and Halliwell, B. (1982) Biochem. J. **206**, 605–609
17 Gutteridge, J. M. C., Wu, C. and Chevion, M. (1991) Free Radical Res. Commun. **11**, 317–320
18 Doeg, K. A. and Ziegler, D. M. (1962) Arch. Biochem. Biophys. **97**, 37–40
19 Avol, E., Carmichael, D., Hegenaner, J. and Saltman, P. (1973) Prep. Biochem. **3**, 279–290
20 Torrance, J. D. and Bothwell, T. H. (1968) S. African J. Med. Sci. **33**, 9–11
21 Gower, J., Healing, G. and Green, C. (1989) Free Radical Res. Commun. **5**, 291–299
22 Sullivan, J. L. (1989) Am. Heart J. **117**, 1177–1188
23 McCord, J. M. (1991) Circulation **83**, 1112–1114
24 Sullivan, J. L., Till, G. O., Ward, P. A. and Newton, R. B. (1989) Nutrition Res. **9**, 625–634
25 Dallman, P. R. (1986) Annu. Rev. Nutr. **6**, 13–40
26 Biemond, P., Swaak, A. J. G., Beindorff, C. M. and Koster, J. F. (1986) Biochem. J. **239**, 169–173
27 Bolann, B. J. and Ulvik, R. J. (1987) Biochem. J. **243**, 55–59
28 Biemond, P., van Eijk, H. G., Swaak, A. J. G. and Koster, J. F. (1984) J. Clin. Invest. **73**, 1576–1579
29 Kvietys, P. R., Inauen, W., Bacon, B. R. and Grisham, M. B. (1989) Am. J. Physiol. **257**, H1640–H1646
30 Gutteridge, J. M. C. (1986) FEBS Lett. **201**, 291–295
31 Sadrzadeh, S. M. H., Anderson, D. K., Panter, S. S., Hallaway, P. E. and Eaton, J. W. (1987) J. Clin. Invest. **79**, 662–664
32 Bysani, G. K., Kennedy, T. P., Ky, N., Rao, N. V., Blaze, C. A. and Hoidal, J. R. (1990) J. Clin. Invest. **86**, 1434–1441
33 Puppo, A. and Halliwell, B. (1988) Free Radical Res. Commun. **4**, 415–422

Received 10 August 1992/18 November 1992; accepted 24 November 1992