Exceptional iron concentrations in larval lampreys (*Geotria australis*) and the activities of superoxide radical detoxifying enzymes

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This study aimed to elucidate the way in which larvae of the lamprey *Geotria australis* counteract the potential problems of the very high concentrations of non-haem iron they contain and thereby avoid the deleterious effects associated with iron overload in other vertebrates. Particular attention has been paid to ascertaining whether increasing concentrations of iron are accompanied by (i) a change to a less readily available form of iron and (ii) an increase in the activity of those detoxifying enzymes responsible for minimizing the production of harmful hydroxyl radicals via the Haber–Weiss reaction. The mean concentrations of haemosiderin and ferritin in larval *G. australis* were each far higher in the nephric fold than in either the liver or intestine, but all these concentrations were much greater than those in rat liver. Since haemosiderin releases iron far more slowly than ferritin, the iron it contains is much less readily available to catalyse the Haber–Weiss reaction. It is thus relevant that (i) non-haem iron in the nephric fold occurred to a greater extent as large dense haemosiderin granules than as ferritin molecules and (ii) the proportion of iron in the form of haemosiderin rose with increasing concentration of total non-haem iron. A strong correlation was also recorded between the activity of superoxide dismutase in the nephric fold and the concentrations of total non-haem iron and its haemosiderin and ferritin components. This demonstrates that enzymic detoxification of $O_2^{\cdot-}$ rises with increasing amounts of iron. The exceptional iron concentrations in the nephric fold were not reflected by a greater measured activity of superoxide dismutase than that found in other tissues. However, the nephric fold was shown to contain an augmentation factor which is presumed to enhance the activity of this enzyme *in vivo*. The activity of catalase and glutathione peroxidase, which catalyse the breakdown of H_2O_2 to O_2 and water, were each significantly correlated with the concentration of ferritin.

INTRODUCTION

Larval lampreys (ammocoetes) live for a number of years in burrows in the soft deposits of streams and rivers, feeding on detritus, diatoms and other micro-organisms in the overlying water [1,2]. Unlike the ammocoetes of other lampreys, those of *Geotria australis* are characterized by possessing exceptionally high concentrations of non-haem iron. This feature, which does not have any obvious deleterious effect on the animal, is due in part to the large amounts of haemosiderin and ferritin found in the nephric fold [3–6]. Appreciable deposits of iron are also present in the liver and columnar cells of the posterior intestine. The high concentrations of non-haem iron in larval *G. australis* would appear to be related to the unusually high haemoglobin concentrations found in this animal [6].

Normal aerobic metabolism inside the cell produces potentially toxic O_2 derivatives such as the superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\cdot}$) [7,8]. Since these products can lead to deleterious effects, including lipid peroxidation, enzyme oxidation and further free radical formation, they pose a potential threat to the chemical integrity of the living cell [9,10]. It has been suggested that, in the presence of high iron concentrations, $O_2^{\cdot-}$ and H_2O_2 generate the highly reactive hydroxyl radical via the

iron-catalysed Haber–Weiss reaction [8–11]. This conclusion is of considerable interest to medical research workers since the formation of free radicals could be the basis for the harmful effects observed in iron-related diseases such as idiopathic haemochromatosis and transfusional siderosis [9,12].

A number of enzyme systems are responsible for the complete detoxification of $O_2^{\cdot-}$ within the cell. The major group are the superoxide dismutases (SOD) (EC 1.15.1.1), which contain manganese or iron or copper and zinc at their active centre. These catalyse the reaction

Two enzyme systems, namely catalase (EC 1.11.1.6) and glutathione peroxidase (GSH-Px) (EC 1.11.1.9), are responsible for eliminating the intracellular H_2O_2 formed by this reaction [7].

The present study describes the form and location of non-haem iron within the cells of the very heavily iron-loaded nephric fold of larval *G. australis*. The activities of SOD, catalase and GSH-Px and the concentration of total non-haem iron and its haemosiderin and ferritin components were each measured in the nephric fold to ascertain whether there was a correlation between the above enzyme activities and iron concentrations. The mean enzyme activities recorded for the nephric fold were then compared with those obtained for the liver and

Abbreviations used: SOD, superoxide dismutase; GSH-Px, glutathione peroxidase.

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intestine to determine whether the far higher iron concentrations in the nephric fold were reflected by much greater enzyme activities. Finally comparisons were made between enzyme activities and iron concentrations in larval *G. australis* and those measured by the same techniques in rat liver, a representative of an iron-containing organ in a mammal.

MATERIALS AND METHODS

Chemicals

Glutathione (reduced form), glutathione reductase, *o*-dianisidine dihydrochloride, NADPH (tetrasodium salt), bovine erythrocyte SOD and bathophenanthroline disulphonic acid were obtained from Sigma (St. Louis, MO, U.S.A.). H_2O_2 and 4-nitrophenol were purchased from BDH (Poole, Dorset, U.K.). Riboflavin was supplied by Hopkin and Williams (Essex, U.K.) and thioglycolic acid was from Merck (Darmstadt, Germany).

Methods

Larval *G. australis* were collected from the Donnelly River in south-western Australia. They were immediately transported to the laboratory, where they were placed in well-aerated, filtered, stream water for a further 60 h to ensure that no food was left in the intestine [2]. The larger animals (> 60 mm and > 0.4 g), most of which would have been between 2 and 4 years old [13], were then anaesthetized in 0.01% benzocaine. Each nephric fold, liver and intestine was removed and weighed to the nearest 0.01 mg. These lamprey tissues and adult rat liver were homogenized in distilled water using glass tissue grinders. While analyses could be performed on homogenates of an individual lamprey nephric fold and segments of rat liver, sufficient material for comparable analyses on the liver and intestine of larval lampreys each required the pooling of organs from three separate animals.

Total non-haem iron and its ferritin and haemosiderin components were determined using a modification of the orthophenanthroline method of Kaldor [14]. Protein was measured by the modified Lowry technique of Hess *et al.* [15]. SOD activity was estimated using the photochemical augmentation method of Misra & Fridovich [16]. A series of dilutions of bovine erythrocyte SOD were included as standards in each set of analyses to enable the results to be expressed as μg of SOD/mg of protein. Catalase activity was determined using the Clark oxygen method described by Goldstein [17], but with a smaller reaction chamber (2.0 ml) and high purity N_2 to establish the zero baseline. The definition of 1 enzyme unit is the amount required to release $1 \mu\text{mol}$ of O_2 from $H_2O_2 \cdot \text{min}^{-1}$ at 30°C . Estimations of catalase activity using commercially available catalase yielded a value equivalent to 96.3% of that quoted by the supplier (Sigma). Since the catalase of larval lampreys was found to be extremely labile, its activity was measured within 5 min of homogenization. The activity of GSH-Px was determined using a slight modification of the method of Sheppard & Millar [18] with H_2O_2 as substrate. The definition of 1 unit of enzyme activity is that it is equivalent to the oxidation of $1 \mu\text{mol}$ of NADPH $\cdot \text{min}^{-1}$ at 20°C .

A one-way analysis of variance (ANOVA) was used to ascertain whether the mean concentration of each of

total non-haem iron, ferritin and haemosiderin and the mean activity of each of SOD, catalase and GSH-Px, differed significantly amongst the different tissues. If a significant difference was found in the variances using Cochran's test, the data were $\log_{10}$ transformed before ANOVA. When ANOVA showed significant differences between means, a Student-Newman Keuls (SNK) test *a posteriori* was used to determine which means were significantly different at $P < 0.05$.

For electron microscopy, pieces of nephric fold were immersed in 3% glutaraldehyde buffered with 0.1 M-sodium cacodylate (pH 7.4) for 1 h at 4°C . They were then washed in the corresponding buffer containing 7% sucrose before post-fixation in 1% osmium tetroxide for a further 1 h. After dehydration in ethanol and propylene oxide and embedding in Epon-Araldite, thin sections were cut using a Reichert microtome. Some sections were stained with uranyl acetate and lead citrate while others were left unstained. Sections were examined using a Philips 301 Transmission Electron Microscope. Unstained sections were also examined using an energy dispersive X-ray analysis system (EDAX) on a Philips 410LS Electron Microscope.

RESULTS

Iron concentration in lamprey tissues and rat liver

The mean concentrations of total non-haem iron in the three groups of nephric fold tissues used for measurement of enzyme activities in larval lampreys ranged from 34.5 to $43.7 \mu\text{g}/\text{mg}$ of protein (Table 1). These values were significantly higher than the mean concentrations of total non-haem iron in the larval lamprey liver (range $3.2\text{--}5.1 \mu\text{g}/\text{mg}$ of protein) and intestine ($7.7\text{--}10.9 \mu\text{g}/\text{mg}$ of protein), thereby paralleling the trends shown by values calculated previously on a wet wt. basis [4]. The mean concentrations of total non-haem iron in the nephric fold, liver and intestine of larval lampreys were always at least an order of magnitude greater than the $0.32 \mu\text{g}/\text{mg}$ of protein recorded for rat liver (Table 1).

As with total non-haem iron, the mean concentrations of both haemosiderin and ferritin in the nephric fold also differed significantly from those in the liver and intestine of larval lampreys (Table 1). While the mean concentrations of haemosiderin and ferritin were all far higher in lamprey tissues than in rat liver, the magnitude of the difference was greater with haemosiderin.

Although haemosiderin contributed only 3.1% to the total concentration of non-haem iron in the rat liver, the minimum mean contribution made by haemosiderin to the non-haem iron in any lamprey tissue was the 51.9% recorded for the intestines used for SOD analyses. The mean contribution of haemosiderin in the three groups of nephric fold material ranged from 75.1 to 83.5%, i.e. the non-haem iron in the nephric fold contained on average less than 25% ferritin.

The relationship between the percentage contribution of haemosiderin (%HS) and the concentration of total non-haem iron ([Fe]) in the samples of nephric fold used for SOD analyses is described by the following equation:

$$\%HS = 60.55 + 0.3494[\text{Fe}] \quad (r = 0.72, \quad P < 0.001, \quad n = 22) \quad (1)$$

The significant correlation between the two variables and the presence of a positive slope shows that the proportion of non-haem iron present as haemosiderin

Table 1. Iron concentrations and enzyme activities in lamprey tissues and rat liver

The concentration of total non-haem iron and its ferritin and haemosiderin components, and the activities of superoxide dismutase, catalase and glutathione peroxidase were determined in lamprey nephric fold, liver and intestine and in rat liver. Mean ($\bar{x}$), standard error (S.E.) and sample size (n) are shown.

Enzyme	Nephric fold			Liver			Intestine			Rat liver		
	$\bar{x}$	S.E.	n	$\bar{x}$	S.E.	n	$\bar{x}$	S.E.	n	$\bar{x}$	S.E.	n
Superoxide dismutase												
Total iron (μg of Fe/mg of protein)	34.5	3.51	22	5.1	1.62	14	7.7	0.81	14	0.32	0.05	10
Ferritin iron (μg of Fe/mg of protein)	8.6	0.53	22	1.7	0.23	14	3.7	0.44	14	0.31	0.05	10
Haemosiderin iron (μg of Fe/mg of protein)	25.9	3.19	22	3.4	1.47	14	4.0	0.78	14	0.01	0.01	10
Haemosiderin (%)	75.1	—	—	66.7	—	—	51.9	—	—	3.1	—	—
Superoxide dismutase (μg /mg of protein)	4.1	0.33	22	5.1	0.35	14	2.5	0.14	14	3.5	0.28	10
Catalase												
Total iron (μg of Fe/mg of protein)	41.4	3.14	38	4.8	1.20	19	9.0	1.02	21	0.32	0.05	10
Ferritin iron (μg of Fe/mg of protein)	8.3	0.45	38	1.8	0.30	19	3.4	0.30	21	0.31	0.05	10
Haemosiderin iron (μg of Fe/mg of protein)	33.1	3.01	38	3.0	1.07	19	5.6	0.99	21	0.01	0.01	10
Haemosiderin (%)	80.0	—	—	62.5	—	—	62.2	—	—	3.1	—	—
Catalase (units/mg of protein)	16.6	0.95	38	670.5	63.34	19	97.7	10.79	21	249.6	10.98	10
Glutathione peroxidase												
Total iron (μg of Fe/mg of protein)	43.7	2.46	15	3.2	0.48	13	10.9	1.05	15	0.32	0.05	10
Ferritin iron (μg of Fe/mg of protein)	7.2	0.85	15	1.3	0.40	13	3.5	0.35	15	0.31	0.05	10
Haemosiderin iron (μg of Fe/mg of protein)	36.5	2.28	15	1.9	0.30	13	7.4	0.99	15	0.01	0.01	10
Haemosiderin (%)	83.5	—	—	59.4	—	—	67.9	—	—	3.1	—	—
Glutathione peroxidase (units/mg of protein)	0.018	0.003	15	0.062	0.010	13	0.025	0.003	15	0.309	0.011	10

Fig. 1. Electron photomicrographs of sections of adipocytes of the nephric fold of larval *Geotria australis*

Note deposits of haemosiderin (H), ferritin (F) and iron spicules (S) and the absence of iron in mitochondria (M). (a) was stained with uranyl acetate and lead citrate while (b) was unstained. The bar on the electron micrographs corresponds to 1 μm (a) and 0.2 μm (b).

increases with the concentration of total non-haem iron.

Distribution of forms of iron in nephric fold

The large dense bodies in the cytoplasm of nephric fold adipocytes of larval *G. australis* (Fig. 1a) are very similar to those described as haemosiderin in mammals [19]. Spicules, which were shown by EDAX to contain iron, were found in the fatty area of some adipocytes (Fig. 1a). At a higher level of magnification, numerous small granules could be clearly observed within the cytoplasm of the adipocytes (Fig. 1b). These granules have an appearance and diameter (approx. 7 nm) similar to those which in mammals were considered by Harrison *et al.* [20] to represent the cores of ferritin molecules. Iron was not observed in mitochondria (Fig. 1b).

Assay of SOD and detection of an augmentation factor

A plot of SOD activity against the volume of rat liver homogenate used in the assay yielded a straight line which passed through zero (Fig. 2). Comparable plots using material from larval *G. australis*, which had been prepared in precisely the same way, yielded curved lines in the case of liver (Fig. 2) and intestine and to a lesser extent nephric fold. Moreover, these lines did not pass through zero and the activity increment relative to the high blank absorbance was small. The lamprey material was therefore centrifuged in a Beckman Airfuge at

Fig. 2. Plot of SOD activity against amount of rat and lamprey liver extracts

Superoxide dismutase activity was determined in the indicated volumes of rat liver homogenate (▲), lamprey liver homogenate (●) and dialysed cytosol (■).

Fig. 3. Superoxide dismutase activity assayed in the absence and presence of lamprey nephric fold extracts

The indicated amounts of bovine erythrocyte SOD were assayed in the absence (●) and presence of 50 μl (☆) and 100 μl (★) portions of dialysed cytosol from lamprey nephric fold. The activity plotted is that attributed only to bovine erythrocyte SOD.

154000 *g* for 12 min and, on the suggestion of I. Fridovich (personal communication), it was dialysed overnight against 50 mM- K_2HPO_4 , pH 7.8, with 0.1 mM-EDTA in order to remove any interfering low-molecular-mass components. Although this procedure produced a straight line, this line still did not pass through zero (Fig. 2). The assay was therefore performed on 50, 100, 200

and 300 μ l portions of the dialysed cytosol to estimate the activity increment of measured additions of the cytosol.

Since lamprey extracts behaved unusually in the above experiments, their characteristics were examined further. When bovine erythrocyte SOD was added to portions of lamprey liver and intestinal cytosol, the activity of the added SOD was the same as when assayed alone. However, this was not the case with lamprey nephric fold where the tissue extracts enhanced the activity of bovine erythrocyte SOD *in vitro* (Fig. 3). We therefore conclude that the lamprey nephric fold contains a factor which augments the activity of the enzyme, even at a final dilution of 1:1000 as used in the assay. This conclusion was substantiated by the fact that we obtained precisely the same augmentation effect using the totally different xanthine oxidase method [21] for estimating SOD activity.

Relationship between SOD activity and iron concentrations in nephric fold

The relationship between SOD activity and the concentrations of each of total non-haem iron ([Fe]), ferritin ([Ftn]), and haemosiderin ([Hs]), in the nephric fold (Fig. 4) are described by the following equations:

$$\begin{aligned} \text{SOD} &= 1.3130 + 0.0816[\text{Fe}] \quad (r = 0.87, \quad P < 0.001, \quad n = 22) \\ \text{SOD} &= 0.9073 + 0.3752[\text{Ftn}] \quad (r = 0.60, \quad P < 0.001, \quad n = 22) \\ \text{SOD} &= 1.8384 + 0.0884[\text{Hs}] \quad (r = 0.85, \quad P < 0.001, \quad n = 22) \end{aligned} \quad (2)$$

As can be seen from the above equations, SOD activity is highly correlated with total non-haem iron and both its ferritin and haemosiderin components.

Since the manganese form of SOD is restricted to the mitochondria of eukaryotes [8], and the mitochondria were removed in the present study by centrifugation, it follows that we must have been dealing with the copper/zinc form of SOD which is typically found in the cytosol of eukaryotes [8]. The restriction of conspicuous deposits of non-haem iron to the cytoplasm of nephric fold adipocytes thus shows that the SOD extracted in this study came from the regions of the cell in which the non-haem iron had been shown to be located.

Enzyme activity in lamprey tissues and rat liver

The activity of SOD was significantly greater in the lamprey liver than in lamprey nephric fold and intestine and in rat liver. The activity of this enzyme was also significantly greater in the nephric fold than in the intestine of larval lampreys. The activities of SOD and GSH-Px in rat liver recorded in this study were very similar to those given by Marklund [22] and Lee *et al.* [23] respectively.

The activity of catalase in lamprey liver was significantly greater than in the lamprey intestine, which in turn was significantly greater than in the nephric fold. The activity of catalase was also significantly greater in the lamprey liver than in the rat liver (Table 1). While the activity of GSH-Px was significantly greater in the lamprey liver than in either the lamprey nephric fold or intestine, it was significantly lower than in the rat liver (Table 1).

Within the nephric fold, the activities of both catalase and GSH-Px were significantly correlated with ferritin ($P < 0.05$ and $P < 0.001$ respectively), but not with either total non-haem iron or haemosiderin ($P > 0.05$).

Fig. 4. Plot of SOD activity against the concentration of total non-haem iron and its components

Extracts were prepared from the nephric fold of 22 individual *G. australis* larvae. The activity of SOD was determined and plotted against (a) the concentration of total non-haem iron (■) and (b) the concentrations of ferritin (●) and haemosiderin (☆) in these tissue extracts.

DISCUSSION

The present study has shown that the proportion of haemosiderin in the nephric fold of larval *G. australis* increases with increasing iron concentration. Since over 40% of all non-haem iron is located in the nephric fold [5], 'excess' iron is thus converted into haemosiderin by this tissue. Because iron is released much more slowly by haemosiderin than by ferritin [24], the storage of iron as haemosiderin renders it less available for catalysing the Haber-Weiss reaction. It has therefore been suggested that the storage of iron as haemosiderin represents a cytoprotective mechanism against iron toxicity [10,25,26].

While the conversion of large amounts of non-haem iron into haemosiderin apparently represents an adaptation to reduce the potentially deleterious effects of carrying an exceptionally high iron load, it must also be relevant that the activity of SOD in the tissue with the greatest iron load is very strongly correlated with the concentrations of total non-haem iron, ferritin and haemosiderin. This finding implies that an enhancement of SOD activity is crucial for overcoming the problems posed by normal levels of the superoxide radical in the presence of high concentrations of iron. Without such a compensating mechanism, the iron-catalysed Haber-Weiss reaction, which is promoted by superoxide radicals, would lead to greatly enhanced and potentially harmful lipid peroxidation [7-11]. The observation that the rate of lipid peroxidation is directly proportional to the amount of ferritin iron [11,27] could account for the relatively steep slope relating SOD activity to ferritin concentration.

Although the concentrations of total non-haem iron and both of its components were much higher in nephric

fold than in both the liver and intestine of larval *G. australis* and rat liver, this was not matched by a correspondingly far higher level of SOD activity. This apparent anomaly may be partly explained by the observation that, in contrast with the situation in lamprey liver and intestine and rat liver, a factor was present in nephric fold which augmented SOD activity, even in a final dilution of 1:1000 (Fig. 2). However, because of the high dilution required for the assay, this augmentation effect was minimized and would thus have yielded values for SOD activity which must be underestimates of the situation *in vivo*. The presence of an augmentation factor in the nephric fold presumably reflects a mechanism to increase the efficiency of SOD in catalysing the dismutation of superoxide radicals in this very heavily iron-loaded structure. Future studies will be directed towards characterizing in detail the chemical nature and properties of this augmentation factor.

The rise in the activity of SOD with increased concentrations of iron must lead to elevated rates of H_2O_2 production. Since H_2O_2 is potentially toxic, both on its own or through facilitating the production of $OH^\cdot$, the activities of enzymes such as catalase and GSH-Px would be expected subsequently to rise in order to enhance the conversion of H_2O_2 to O_2 and water. Such a view is consistent with the significant correlation found between the activities of both of these enzymes and the concentration of ferritin. However, the correlation with ferritin concentration was lower for catalase than for SOD activity and, unlike the situation with SOD, the activity of neither catalase nor GSH-Px was correlated with haemosiderin concentration. The less comprehensive overall relationship between catalase and GSH-Px and the concentrations of non-haem iron components presumably reflects the fact that the elevation of these enzymes is a subsequent response to the primary iron-induced rise in SOD activity. The absence of a correlation between the activities of catalase and GSH-Px and the concentration of haemosiderin may be a consequence of haemosiderin being less capable of promoting lipid peroxidation than ferritin [27].

Comparisons of the relationships of catalase and GSH-Px to iron concentration in different tissues produces rather confusing results. Thus while the greater concentration of non-haem iron and its ferritin component in lamprey liver compared with rat liver were matched by greater concentrations of catalase, the reverse situation pertained with GSH-Px. Absence of a general correlation between the activities of these enzymes and iron concentration across tissues is probably accounted for by the numerous and diverse metabolic activities of the structures from which they come. In this context, it is important to recognize that catalase and GSH-Px are responsible for the detoxification of all H_2O_2 , irrespective of its metabolic origin [7]. The presence of a vastly greater activity of catalase than of GSH-Px in each lamprey tissue parallels that found with rat liver both in this study and in that of Lee *et al.* [23].

The strong correlation between SOD activity and iron concentration shown to exist within the very heavily iron-loaded nephric fold of larval *G. australis* parallels the situation found in dog liver [28,29]. The establishment of this correlation, and the demonstration of a relationship between the proportion of haemosiderin and

the concentration of iron, demonstrates that larval *G. australis* provides an excellent model for exploring aspects of the biochemistry and physiology of iron overload in vertebrates.

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