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CuZn-superoxide dismutase, Mn-superoxide dismutase, catalase and glutathione peroxidase in pancreatic islets and other tissues in the mouse

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Exogenous superoxide dismutase, catalase and scavengers of the hydroxyl radical protect pancreatic-islet cells against the toxic actions of alloxan *in vitro* [Grankvist *et al.* (1979) *Biochem. J.* **182**, 17–25]. To test whether the extraordinary sensitivity of islet cells to alloxan is due to a deficiency of endogenous enzymes protecting against oxygen-reduction products, we assayed CuZn-superoxide dismutase, Mn-superoxide dismutase, catalase and glutathione peroxidase in mouse islets and other tissues. To correct for any blood contamination, haemoglobin was also measured in the tissue samples. Pancreatic islets were found to belong to tissues with relatively little activity of the protective enzymes. However, the deviation from other tissues in this respect is probably not large enough to explain the especially great susceptibility of islet cells to alloxan.

Alloxan [pyridine-2,4,5,6(1*H*,3*H*)-tetraone] has been used to induce experimental diabetes since 1943 (Rerup, 1970). It destroys the insulin-producing β-cells in the pancreatic islets.

When exposed to reducing substances and molecular oxygen *in vitro*, alloxan and its reduced analogue, dialuric acid, form a reduction–oxidation cycle in which superoxide anion radicals ($O_2^{-\cdot}$) and H_2O_2 arise (Deamer *et al.*, 1971; Cohen & Heikkila, 1974). A number of alcohols and other substances with high reactivity toward the hydroxyl radical ($OH^\cdot$) protect against the diabetogenic action of alloxan *in vivo* (Heikkila *et al.*, 1976; Tibaldi *et al.*, 1979). Therefore the hypothesis was advanced (Heikkila *et al.*, 1976) that alloxan action is mediated by the very toxic $OH^\cdot$, and that such radicals are produced by a (metal-ion-catalysed) reaction (the 'Haber–Weiss reaction'):

$$H_2O_2 + O_2^{-\cdot} \rightarrow OH^\cdot + OH^- + O_2.$$

Although the toxicity of $O_2^{-\cdot}$ *in vivo* has been questioned (Fee, 1980), we recently verified the hypothesis *in vitro* with isolated mouse islets and islet cells. Alloxan-induced inhibition of $^{86}Rb^+$ accumulation and failure to exclude Trypan Blue were counteracted by superoxide dismutase, catalase and $OH^\cdot$ scavengers (Grankvist *et al.*, 1979*a*). Diethylenetriaminepenta-acetic acid ('DETAPAC'), which binds iron ions and prevents catalysis of the Haber–Weiss reaction (Halliwell, 1978), was also protective (Grankvist *et al.*, 1979*b*). Similar results have been obtained in independent studies on alloxan-induced inhibition of insulin release from isolated rat islets (Fischer & Hamburger, 1980*a*,*b*).

It is not quite clear why the insulin-secreting β-cells are particularly vulnerable to alloxan. Attention has been paid (Grankvist *et al.*, 1979*a*) to their unusually effective system for transport of D-glucose (Hellman *et al.*, 1971, 1974*a*), which might provide an entrance for the drug, and to the possible role of membrane-located redox processes in the control of insulin secretion (Hellman *et al.*, 1974*b*); perhaps β-cells are more effective than other cells in capturing and reducing alloxan.

These ideas notwithstanding, another possibility is that the β-cells could have a relative deficiency of endogenous enzymes protecting against $O_2^{-\cdot}$ and H_2O_2, and thus also against production of $OH^\cdot$. To evaluate this possibility, the activities of CuZn-superoxide dismutase, Mn-superoxide dismutase, catalase and glutathione peroxidase were assayed in mouse pancreatic islets and other tissues. The experiments were undertaken with a view to the facts that toxic products of oxygen reduction can also arise in the course of inflammation (e.g., Babior *et al.*, 1973; Johnston *et al.*, 1976, 1978; DeChatelet *et al.*, 1977; Petrone *et al.*, 1980; Sacks *et al.*, 1978;

Abbreviations used: GSSG, oxidized glutathione; GSH, reduced glutathione.

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McCord & Wong, 1979), that there is often inflammation of the pancreatic islets in the onset of human diabetes mellitus (Gepts, 1976), and that diabetes may be caused by the combined onslaught of viruses and chemical agents on the β-cells (Toniolo *et al.*, 1980).

Materials and methods

Animals and isolation of tissues

Adult obese mice homozygous for the gene *ob* were taken from the non-inbred Umeå colony (Umeå *ob/ob* mice). These animals are mildly hyperglycaemic, probably as the result of peripheral insulin resistance and hyperphagia (Stauffacher *et al.*, 1967; Stauffacher & Renold 1969). Owing to a compensatory proliferative response among the insulin-producing β-cells, the animals also have unusually large pancreatic islets with an exceptionally high proportion of β-cells (90% or more of the endocrine cells; Hellman; 1965).

There is no indication of any difference in principle between Umeå-*ob/ob*-mouse β-cells and those of normal mice as regards the mechanisms of insulin secretion; previous investigations on the Umeå-*ob/ob*-mouse islets have documented their usefulness as an experimental model for elucidating fundamental mechanisms of insulin secretion (see, e.g., Hahn *et al.*, 1974; Idahl *et al.*, 1976; Gagerman *et al.*, 1978) as well as alloxan cytotoxicity (Idahl *et al.*, 1977; Grankvist *et al.*, 1977, 1979*a*,*b*,*c*). Lean mice of normal phenotype were also taken from the same local stock. All mice were females. Islets were isolated by hand picking under a stereomicroscope. Islets as well as samples of other tissues were rapidly frozen and stored at −80°C until being assayed for enzyme activities. The wet weight of islet tissue was calculated from determinations of the native protein fluorescence in the islet homogenates (Gagerman, 1980).

Compensation for erythrocyte enzymes by specific assay of haemoglobin in tissue homogenates

Erythrocytes contain large amounts of three of the enzymes of the present investigation. Therefore, although the mice were bled to death, it was necessary to compensate for the enzyme activity contributed by erythrocytes. For this purpose, haemoglobin was specifically determined by a previously developed method (Marklund, 1979) based on the differential protective activity of serum (haptoglobin) and egg white on the peroxidative activity of the haemoprotein. This method was originally devised for human haemoglobin, but was here found to be applicable to mouse haemoglobin as well; human serum (haptoglobin) could be used together with mouse haemoglobin.

The enzyme activities in blood differed somewhat between animals. The corrections for blood contamination in the tissue homogenates were therefore made with regard to the actual enzyme activities in blood from the respective animals. All enzyme activities presented have been corrected for blood contamination; corrections ranged from less than 1% to about 50%.

Superoxide dismutase assay

Tissues were homogenized (Ultra-Turrax mechanical blender) in 100 vol. of 10 mM-potassium phosphate buffer, pH 7.4, supplemented with 30 mM-KCl. The homogenates were then sonicated (Sonifier cell disruptor; 1 min) and left for 30 min at 4°C. This procedure was found to effectively solubilize both CuZn-superoxide dismutase and Mn-superoxide dismutase from mouse tissue. After centrifugation at 20000 *g* for 15 min, superoxide dismutase in the supernatants was determined in terms of its ability to catalyse the disproportionation of $O_2^{-\bullet}$ in alkaline aqueous solution. The disproportionation was directly measured in a spectrophotometer as previously described (Marklund, 1976), except that both CuZn-superoxide dismutase and Mn-superoxide dismutase were assayed at pH 9.50. One unit of enzyme activity is defined as the activity causing $O_2^{-\bullet}$ to decay at the rate of $0.1\ s^{-1}$ in a 3 ml reaction volume. One such unit corresponds to 8.3 ng of human CuZn-superoxide dismutase, 4.1 ng of bovine CuZn-superoxide dismutase, or 65 ng of bovine Mn-superoxide dismutase.

Under the conditions of the present assay with high $O_2^{-\bullet}$ concentration and high pH, the Mn-superoxide dismutase has lower activity than the CuZn-containing enzyme. Under physiological conditions, neutral pH and low $O_2^{-\bullet}$ concentration, superoxide dismutase may be assayed with the aid of xanthine oxidase and cytochrome *c* (McCord & Fridovich, 1969). For human and bovine enzymes, one unit in the present direct assay corresponds to 0.024 unit of CuZn-superoxide dismutase or 0.24 unit of Mn-superoxide dismutase as measured by the xanthine oxidase/cytochrome *c* method. The present assay is thus about ten times more sensitive for CuZn-superoxide dismutase activity than for the Mn-superoxide dismutase activity. A corresponding comparison for the mouse enzymes has not been performed, but similar relations between the two assays are also likely to exist for these enzymes.

Catalase assay

Tissues were generally homogenized (Ultra-Turrax) in 10 vol. of ice-cold 10 mM-potassium phosphate buffer, pH 7.4, supplemented with 30 mM-KCl and, to prevent formation of catalase compound II, 1% (v/v) ethanol. The homogenates were sonicated for 1 min and, except for islets, centrifuged at 20000 *g* for 15 min. Islet homogenates were not

centrifuged after sonication. Control experiments with other tissue homogenates showed that the centrifugation step did not influence the activity of a homogenate.

Catalase is very sensitive to dilution. Therefore, when homogenates had to be diluted before assay, care was taken to perform the analysis immediately after dilution. Islet homogenates were analysed within a few minutes after sonication. In separate experiments it was found that muscle homogenates diluted to the same catalase activity as the islet homogenates did not lose much catalase activity within 5–10 min. After longer periods of time, both muscle and islet homogenates gradually lost enzyme activity.

Catalase activity was measured with a Clark oxygen electrode essentially as described by Del Rio *et al.* (1977). Homogenates were added to 3 ml of

Table 1. *Activities of superoxide dismutases, catalase and glutathione peroxidase in mouse tissues*

Values for individual mice, separated by commas, as well as pooled mean values ± S.E.M. are shown. In the case of islets, mean values ± S.E.M. refer to the numbers of experiments, not mice, indicated within parentheses; the numbers of mice were larger, as each batch of isolated islets was prepared from the pooled pancreatic glands of two to three mice. Assays are described in the text. Intra-abdominal adipose tissue, quadriceps muscle, whole liver, kidney, heart and brain were used. Protein was also measured (Bradford, 1976) in the homogenates. To obtain the results in terms of units/mg of protein, the values in the Table should be multiplied by the following factors: islets, 12; exocrine pancreas, 11; liver, 6.9; kidney, 12; erythrocytes, 2.9; heart, 19; brain, 26; muscle, 30; adipose tissue, 340.

Tissue	Obese mice	Lean mice	Mean ± S.E.M.
CuZn-superoxide dismutase (kunits/g wet wt.)			
Islets	26.6 ± 3.4 (6)	—	27 ± 3 (6)
Exocrine pancreas	40.4 ± 15.2 (5)	—	40 ± 15 (5)
Liver	94, 106	84, 90	94 ± 5 (4)
Kidney	38, 62	38, 42	45 ± 6 (4)
Erythrocytes	18, 18	17, 20	18 ± 1 (4)
Heart	13, 17	15, 18	16 ± 1 (4)
Brain	16, 16	14, 14	15 ± 1 (4)
Muscle	8, 10	9, 10	9 ± 0 (4)
Adipose tissue	2, 2	3	2 ± 0 (3)
Mn-superoxide dismutase (kunits/g wet wt.)			
Islets	0.63 ± 0.14 (4)	—	0.6 ± 0.1 (4)
Exocrine pancreas	0.64 ± 0.20	—	0.6 ± 0.2 (4)
Liver	1.0, 2.2	1.5, 2.0	1.7 ± 0.3 (4)
Kidney	2.6, 6.3	2.3, 2.8	3.5 ± 0.9 (4)
Erythrocytes	0, 0	0, 0	0 ± 0 (4)
Heart	3.8, 4.7	4.2, 5.1	4.5 ± 0.3 (4)
Brain	0.7, 0.7	0.7, 0.7	0.7 ± 0.0 (4)
Muscle	0.4, 0.5	0.4, 0.4	0.4 ± 0.0 (4)
Adipose tissue	0.01, 0.02	0.02	0.02 ± 0.00 (3)
Catalase (units/g wet wt.)			
Islets	2.26 ± 0.23 (5)	—	2.3 ± 0.2 (5)
Exocrine pancreas	4.0, 13	7.9, 9.1	8.5 ± 1.9 (4)
Liver	159, 215	181, 187	186 ± 12 (4)
Kidney	108, 119	57, 62	87 ± 16 (4)
Erythrocytes	51, 68	85, 91	74 ± 9 (4)
Heart	5.1, 5.7	5.1, 7.9	6.0 ± 0.7 (4)
Brain	1.1, 1.4	0.70, 0.79	1.0 ± 0.2 (4)
Muscle	1.2, 1.64	0.84, 1.1	1.2 ± 0.2 (4)
Adipose tissue	1.7, 2.0	2.4, 3.3	2.4 ± 0.3 (4)
Glutathione peroxidase (units/g wet wt.)			
Islets	2.9, 3.1	—	3.0
Exocrine pancreas	11, 13	9, 12	11 ± 1 (4)
Liver	115, 150	186, 203	164 ± 20 (4)
Kidney	92, 108	66, 89	89 ± 9 (4)
Erythrocytes	78, 87	95, 117	94 ± 8 (4)
Heart	2.1, 2.3	1.0, 2.5	2.0 ± 0.3 (4)
Brain	2.3, 2.5	1.5, 1.8	2.0 ± 0.2 (4)
Muscle	0.9, 1.2	0.8, 1.5	1.1 ± 0.2 (4)
Adipose tissue	1.7, 2.8	1.1, 3.6	2.3 ± 0.6 (4)

de-aerated 10mM-potassium phosphate + 0.1mM-diethylenetriaminepenta-acetic acid, pH 7.4, containing 15mM-H_2O_2. Catalase activity was calculated from the initial rate of O_2 liberation. One unit is defined as the amount that disproportionates 1% of the H_2O_2 in 1min. The method is about 50 times more sensitive than conventional spectrophotometric or titration methods.

Glutathione peroxidase assay

Tissues were treated as in the catalase assay except that no ethanol was added. Glutathione peroxidase was assayed by the method of Günzler *et al.* (1974), with some modifications. In a final volume of 500μl, homogenate was added to 50mM-potassium phosphate buffer, pH 7.0, containing 2mM-diethylenetriaminepenta-acetic acid, 0.16mM-NADPH, 2mM-GSH, 0.6mM-t-butyl hydroperoxide and 0.50 unit of glutathione reductase. t-Butyl hydroperoxide was used instead of H_2O_2 because much lower blanks, and hence a higher sensitivity, was obtained. A low concentration of t-butyl hydroperoxide, 0.6mM, was chosen to avoid co-determination of non-selenium-containing glutathione peroxidase. The non-selenium-containing enzyme has a much higher K_m for the peroxide than the selenium-containing glutathione peroxidase (Burk *et al.*, 1978); in not reacting with H_2O_2, it was of little interest in the present investigation.

When the activity of erythrocytes was determined, the haemolysates were treated with 1mM-$K_3Fe(CN)_6$ and 8.7mM-NaCN in order to inhibit the activity of haemoglobin. Haemolysates were also analysed without these additions and the results used to compensate for the blood contamination of tissue homogenates as described above.

Protein measurements

For protein analyses, Coomassie Brilliant Blue G-250 was used (Bradford, 1976), with human serum albumin employed as standard. This sensitive and convenient method was compared with the more established Folin phenol technique of Lowry *et al.* (1951). Mouse tissue homogenates (exocrine pancreas, liver, kidney, heart, brain, muscle, adipose tissue) were analysed by both methods, which were standardized in the same way. The results were very similar, the ratio between the Lowry-method and the one used in the present investigation being 1.13 (S.D. 0.07; range 1.02–1.23).

Results

The results are shown in Table 1. There was in general no obvious difference between the enzyme activities in obese as compared with lean mice. Although minor differences could perhaps be detected with larger groups of animals, it is sufficient to say that, in the present study, the variation between mice was much less than that between tissues.

Taking into account that the superoxide dismutase assay is ten times more sensitive for the CuZn-enzyme than for the Mn-enzyme, the proportions of the two major forms of superoxide dismutase were calculated. Expressed as a percentage of total activity, the CuZn-enzyme represented 100 in erythrocytes (owing to lack of mitochondria, the site of the Mn-enzyme; Weisiger & Fridovich, 1973), 92 in adipose tissue, 86 in exocrine pancreas, 85 in liver, 81 in islets, 69 in brain, 68 in muscle, 56 in kidney, and 26 in heart.

There were comparatively small differences (one order of 10, adipose tissue apart) between the tissues with respect to CuZn-superoxide dismutase and Mn-superoxide dismutase. For both these enzymes, the results for pancreatic islets lie in the middle of the series. On the other hand, there were large differences (two orders of 10) between the tissues with respect to the enzymes scavenging H_2O_2, catalase and glutathione peroxidase. In both cases the islets belong to the tissues possessing very little enzymic activity.

When the tissues are ranked from highest to lowest with regard to the activity of each investigated enzyme, the following average rank values are obtained as crude indices of how well the tissues are enzymically protected against the toxic reduction products of oxygen: liver, 1.5; kidney, 2.3; exocrine pancreas, 4.0; erythrocytes, 4.8; heart, 4.9; islets, 5.5; brain, 6.9; adipose tissue, 7.3; and muscle, 8.0. Because adipose tissue contains relatively little water and protein per unit wet weight, the values may appear misleadingly low for this tissue; if expressed per unit of protein, the results indicate that adipose tissue is relatively well equipped with protective enzymes.

Discussion

We made the present enzyme measurements because exogenous superoxide dismutase and catalase had been shown to protect β-cells against the toxic action of alloxan (Grankvist *et al.*, 1979*a*; Fischer & Hamburger, 1980*a*). If pancreatic islets were found to contain markedly lower endogenous activities of these enzymes as compared with other tissues, this relative lack of protection against oxygen-reduction products might explain why the β-cells are exceptionally vulnerable to alloxan. Superoxide dismutase catalyses the disproportionation of $O_2^{-\cdot}$ that presumably arises in the autoxidation of dialuric acid:

$$2O_2^{\cdot} + 2H^+ \rightarrow O_2 + H_2O_2$$

(McCord & Fridovich, 1969; Deamer *et al.*, 1971; Cohen & Heikkila, 1974; Grankvist *et al.*, 1979*a*).

The H_2O_2 could theoretically be removed by catalase:

$$H_2O_2 + H_2O_2 \rightarrow O_2 + 2H_2O$$

or glutathione peroxidase:

$$H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$$

In eukaryotic cells, two forms of superoxide dismutase are generally observed: one cytoplasmic and mitochondrial enzyme containing Cu and Zn, and one mitochondrial enzyme containing Mn (Weisiger & Fridovich, 1973). In primates the Mn-superoxide dismutase is possibly also found in cytoplasm (McCord *et al.*, 1977). The partly different localizations of superoxide dismutase isoenzymes could give them different roles in protecting various targets in cells against a source of $O_2^{-\bullet}$. The high proportion of Mn-superoxide dismutase in the heart and the low proportion in islets and exocrine pancreas parallel the activities of lactate dehydrogenase isoenzymes. In *ob/ob* mice the heart has a high proportion of lactate dehydrogenase subunits predominating in tissues with a high oxygen tension, whereas both islets and exocrine pancreas have a low proportion of such subunits (Hellman & Täljedal, 1967).

Although glutathione peroxidase has not been tested for protective effects against alloxan *in vitro*, there were theoretical reasons for including it in the present assays of endogenous enzymes. Glutathione peroxidase has a broader protective spectrum than catalase in catalysing the reduction of both H_2O_2 and other hydroperoxides, including lipid hydroperoxides (Christophersen, 1969). In addition to the Se-containing enzyme measured by the present assay, some tissues exhibit an Se-independent glutathione peroxidase activity, apparently due to glutathione *S*-transferases. The Se-independent enzyme accepts several organic hydroperoxides as substrates, but reacts only poorly with H_2O_2 (Burk *et al.*, 1978) and so was considered to be of less interest in the present context. The protective efficiency of glutathione peroxidase depends not only on the amount of enzyme, but also on the availability of GSH. It is therefore noteworthy that pancreatic islets of several species have been reported to contain fairly large amounts of GSH (Havu, 1969) as well as a high activity of glutathione reductase (Berne, 1975), the enzyme providing GSH from GSSG and NADPH.

When comparing the various tissues, the pancreatic islets had relatively little activity of superoxide dismutase, catalase or glutathione peroxidase, although the deviation from the other tissues apparently is not large enough to provide a full explanation for the very high, fairly specific susceptibility *in vivo* of β-cells to alloxan. But the low activities may contribute to the alloxan-sensitivity of the cells and may conceivably also make them comparatively vulnerable to inflammatory free-radical damage.

The main reason for the high susceptibility of the β-cells to alloxan is still unknown. They may possess receptors or efficient reductive mechanisms for alloxan. Another possibility, so far not explored, is that the β-cells are more sensitive than other cells to $O_2^{-\bullet} + H_2O_2$. Iron, or another transition-metal ion, catalysing the Haber–Weiss reaction, might be located at a site where the production of the very reactive and very short-lived hydroxyl radical is particularly deleterious for the cell. In accord with this proposition, addition to the medium of iron bound to transferrin or EDTA did not increase the toxic action of alloxan (Grankvist *et al.*, 1979*b*). These forms of iron have, in other systems *in vitro*, been shown to catalyse the Haber–Weiss reaction (McCord & Day, 1978). Diethylenetriaminepentaacetic acid, which binds and inactivates iron with respect to the Haber–Weiss reaction, was found to protect β-cells efficiently against alloxan (Grankvist *et al.*, 1979*b*).

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