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Diethyldithiocarbamate injection induces transient oxidative stress in goldfish tissues

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Abstract

The effects of intraperitoneal injection of diethyldithiocarbamate (DDC) on free radical processes were examined in brain, liver and kidney of goldfish (*Carassius auratus*). Levels of oxidatively modified lipids and proteins as well as the activities of antioxidant and associated enzymes were measured. Intraperitoneal injection of DDC at a concentration of 0.01 mg/g wet mass decreased SOD activities by about 30–50% after 48 and 168 h compared to corresponding sham-injected values. This treatment resulted in transient oxidative stress. Lipid peroxide content increased after DDC injection at all time points in the kidney, after 48 h in the liver and was elevated in most experimental groups in the brain. Thiobarbituric-acid reactive substances (end products of lipid peroxidation) rose within the first 48 h after injection, but returned to initial levels after 168 h. Two other indices of oxidative stress were also transiently modified: protein carbonyl levels in the brain and kidney increased 24 h post-injection, and the low-molecular mass thiol content was reduced over the same period in all tissues examined. Activities of catalase, glutathione peroxidase, glutathione-*S*-transferase, glutathione reductase, and glucose-6-phosphate dehydrogenase showed differential responses to DDC treatment that rebounded by 168 h post-injection. Glutathione peroxidase activities were reduced by 60, 45 and 65% in the brain, liver and kidney, respectively, after 24 h but rebounded thereafter. After 48 h post-injection with DDC significant decreases were also seen in liver and kidney catalase, GST activities in all three tissues, and kidney GR and G6PDH activities. In some cases, catalase, GST, GR and G6PDH activities transiently increased after 24 h. It was concluded that DDC injection depleted SOD and simultaneously stimulated lipid peroxidation, but did not require compensatory enhancement of other enzymatic defenses. Different actions of the superoxide anion in cellular metabolism and possible consequences of the impairment of superoxide dismutase are discussed.

Keywords: Goldfish; Superoxide dismutase; Diethyldithiocarbamate; Oxidative stress; Superoxide anion

1. Introduction

The goldfish *Carassius auratus* is highly tolerant of multiple environmental stresses [1]. This species endures both long-term anoxia [2–5] and hyperoxia [6] as well as a wide range of low and high temperatures [7–10]. All these stresses are accompanied by an environmen-

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tally induced elevation of reactive oxygen species (ROS) in tissues, including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$) [11,12]. The steady-state level of ROS is the balance between production and decomposition, and an imbalance in these processes in favor of the former has been termed "oxidative stress" [11–13]. Oxidative stress can disturb and damage many cellular processes, sometimes leading to cell death.

Organisms subjected to environmental stress often need to deal with at least three problems – energy provision, accumulation of metabolic end products, and emerging oxidative stress. The first two aspects of biochemical adaptation have been well studied in many species with respect to low oxygen stress. The responses to these problems include high levels of fermentable substrates, mainly glycogen, that are used for anaerobic energy production [3], and high activities of glycolytic enzymes [14,15]. The problem of end product build-up is tackled in one of several ways; in goldfish, the lactic acid produced by all organs is converted by skeletal muscle into ethanol and carbon dioxide which are then excreted into the environment via the gills [2–4,16]. The third problem, oxidative stress development, has not received enough attention and just a few years ago it was shown that goldfish possess relatively high levels of antioxidants which may be altered according to the needs of the organism. Elevation of several oxidative stress markers, particularly oxidized proteins and lipids, has been described in tissues of goldfish exposed to anoxia and recovery [5], hyperoxia [6], and heat shock [9,10]. To help clarify the involvement of specific mechanisms of antioxidant defense in the protection of goldfish tissues against ROS, we inhibited the antioxidant enzyme, catalase, using aminotriazole and found that this procedure resulted in oxidative stress in brain [17], liver and kidney [18].

The present study was designed to assess the metabolic consequences of inhibition of another key enzyme of antioxidant defense, the copper and zinc containing superoxide dismutase (Cu,Zn-SOD). To investigate this, we inhibited SOD *in vivo* using diethyldithiocarbamate (DDC). DDC inhibits Cu,Zn-SOD both *in vitro* [19] and *in vivo* [20–22] by extracting the copper ion from the enzyme active center. However, DDC may also affect other enzymes like xanthine oxidase [23] by the same mechanism. Here we evaluated the consequences of DDC treatment on the activities of other antioxidant enzymes as well as on the levels of oxidatively modified proteins and lipids in goldfish tissues. We hypothesized that SOD inhibition would result, firstly, in oxidative stress development in goldfish tissues and then, secondly, in compensatory responses by other antioxidant enzymes. We compared organs with highly intensive oxidative metabolism, namely brain, liver and kidney; the latter two organs have major involvement in the catabolism of xenobiotics, whereas brain, that is the most protected organ in animals is also highly oxygen dependent and consumes high amount of oxygen.

2. Materials and methods

2.1. Chemicals

Butylated hydroxytoluene (BHT), 1-chloro-2,4-dinitrobenzene (CDNB), cumene hydroperoxide, diethyldithiocarbamate (DDC), 2,4-dinitrophenylhydrazine (DNPH), 5,5′-dithio-*bis*(2-nitrobenzoic acid) (DTNB), ethylenediamine-tetraacetic acid (EDTA), glucose-6-phosphate (G6P), reduced glutathione (GSH), oxidized glutathione (GSSG), $NADP^+$, NADPH, phenylmethylsulfonyl fluoride (PMSF), thiobarbituric acid (TBA), sodium azide, xylenol orange, and yeast glutathione reductase (GR) were purchased from Sigma–Aldrich Chemical Co. (USA). *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), quercetin and Tris–HCl were from Reanal (Hungary), and guanidine–HCl was from Fluca. All other reagents were of analytical grade.

2.2. Animals and experimental design

Goldfish (*C. auratus* L.) of both sexes weighing 25–53 g were purchased at a local fish farm (Ivano-Frankivsk district, Ukraine). They were kept in dechlorinated tap water and fed with standard fish food. Temperature was maintained at $20 \pm 1\,^{\circ}C$ with a natural light–dark cycle with light from about 8:00 to 17:00. Goldfish were acclimated to these conditions for at least a month before experimentation.

In preliminary studies 10 specimens per group were injected intraperitoneally with DDC diluted in physiological saline (0.9%, w/v NaCl) at final concentrations of 0.01, 0.025 or 0.05 mg/gram wet body mass (gwm). The volume of injected solution was 0.3% of body mass. Concentrations of 0.025 and 0.05 mg DDC/gwm had lethal effects, and a sublethal concentration of 0.01 mg/gwm was chosen for further experiments. Two experimental groups (5–7 animals per group) were set up: (a) sham-injected animals that received only 0.9% NaCl, and (b) DDC-treated fish that were injected with 0.01 mg/gwm DDC solution. Control fish were not treated. After 24, 48 or 168 h fish injected with DDC or NaCl solutions were sampled and brain, liver and kidney tissues were

rapidly excised and processed immediately to measure the parameters of interest. In preliminary experiments no sex-specific differences in the measured parameters were found; therefore, we combined all experimental data without sex differentiation.

2.3. Indices of oxidative stress

Tissue samples were homogenized (1:10, w/v) using a Potter-Elvehjem glass homogenizer in 50 mM potassium phosphate (KPi) buffer, pH 7.0, containing 0.5 mM EDTA and a few crystals of PMSF, a protease inhibitor. A 250 μl aliquot of this homogenate was then mixed with 0.5 ml of 10% (final concentration) trichloroacetic acid (TCA) and centrifuged for 5 min at 13,000 × *g*. Protein carbonyl (CP) levels were measured in the resulting protein pellets, and thiobarbituric acid-reactive substances (TBARS) contents were assayed in the supernatants using a spectrophotometer SF-46 (LOMO, USSR).

Carbonyl derivatives of proteins were detected by reaction with 2,4-dinitrophenylhydrazine (DNPH) as described previously [6] with resulting 2,4-dinitrophenylhydrazones quantified spectrophotometrically at 370 nm. CP content was expressed as nanomoles of CP per milligram protein in the final guanidine hydrochloride solution.

Low-molecular mass products of lipid hydroperoxide decomposition, including malondialdehyde, were measured by the TBARS assay as described earlier [6]. The values are expressed as nanomoles of TBARS per gram wet mass of tissue (gwm).

Lipid peroxide (LOOH) content was assayed with xylenol orange [24]. For that, tissue samples were homogenized with 5 volumes of 96% cold (∼5 °C) ethanol, centrifuged 5 min at 13,000 × *g*, and supernatants were used for assay as described earlier [6]. The content of LOOH is expressed as nanomoles of cumene hydroperoxide (CHP) equivalents per gram wet mass of tissue.

Free thiols are widely measured spectrophotometrically by the Ellman procedure with DTNB at 412 nm [25]. Tissue homogenates prepared as for the TBARS/CP assays were centrifuged at 4 °C for 15 min at 15,000 × *g* in a K-24 centrifuge (Germany). Total thiol content (the sum of low- and high-molecular mass thiols) was measured in the resulting supernatants as described previously [9]. For measuring low-molecular mass thiol (L-SH) content, supernatants were treated with 10% (final concentration) TCA, centrifuged for 5 min at 13,000 × *g* and the final supernatants were used for the assay. The thiol concentrations are expressed as micromoles of SH-groups per gram wet mass of tissue. The high-molecular mass thiol (H-SH) content was calculated by subtracting the L-SH concentration from the total thiol level.

2.4. Assay of antioxidant enzyme activities

Tissue homogenates prepared as for the TBARS/CP assays were centrifuged at 4 °C for 15 min at 15,000 × *g* in a K-24 centrifuge (Germany). Supernatants were removed and used for enzyme activity assays using a Specord M40 spectrophotometer (Germany). SOD activity was assayed as a function of its inhibitory action on quercetin oxidation [6]. One unit of SOD activity is defined as the amount of enzyme that inhibits the quercetin oxidation reaction by 50% of maximal inhibition. In our case, the maximal inhibition was about 90%. Activities of catalase, glutathione peroxidase (GPx), glutathione-*S*-transferase (GST), glutathione reductase (GR), and glucose-6-phosphate dehydrogenase (G6PDH) were measured as described previously [6]. One unit of enzyme activity is defined as the amount of the enzyme that consumes 1 μmole of substrate or generates 1 μmole of product per minute. All enzyme assays were conducted at 25 ± 1 °C and activities of all enzymes are expressed per milligram soluble protein. Protein concentration was measured by the Bradford method with Coomassie Brilliant Blue G-250 [26] using bovine serum albumin as the standard.

2.5. Statistics

Data are presented as means ± S.E.M. Statistical analysis was performed using a Student's *t*-test. Inhibition values for SOD activity were calculated using an enzyme kinetics computer program [27].

3. Results

3.1. Oxidative stress markers

Fig. 1A shows that DDC and saline injection significantly increased lipid peroxide (LOOH) concentrations in the brain at all time points, DDC treatments resulting in a >2-fold increase in LOOH in brain of fish exposed for 48 and 168 h. In the kidney only DDC treatment caused an elevation of LOOH content with the maximal effect of 4.8-fold after 24 h. In the liver, elevated LOOH levels were seen in the 48 h DDC-treated group and also in 24 h sham-treated fish.

Time-dependent changes in thiobarbituric-acid reactive substances (TBARS) concentrations were similar in all tissues (Fig. 1B). TBARS levels were significantly

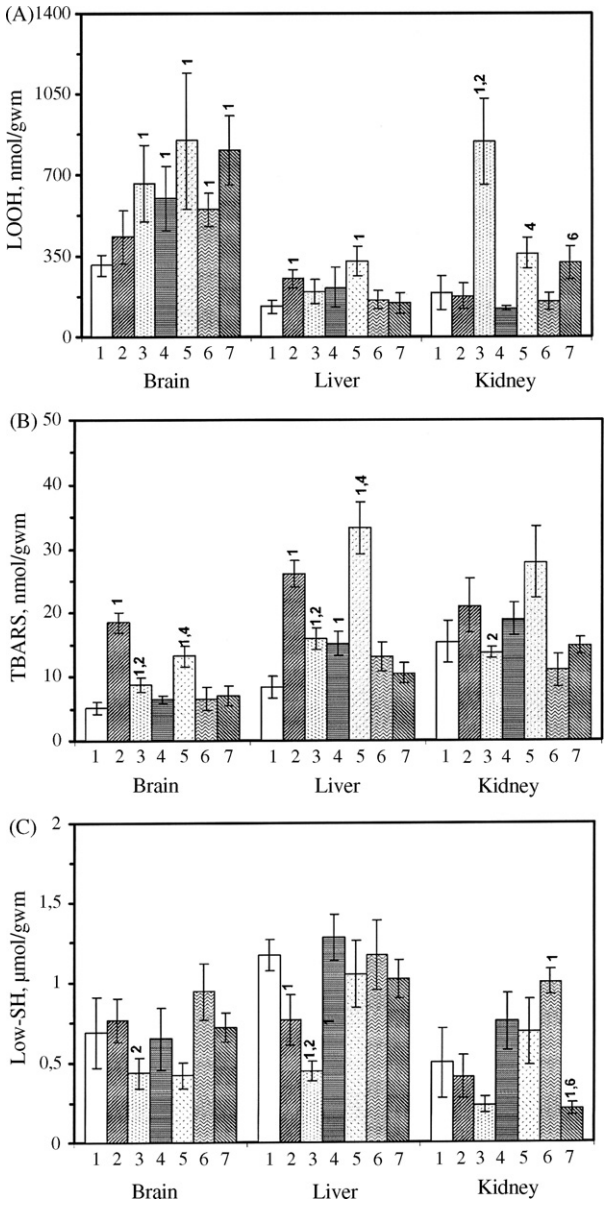

Fig. 1. The effects of DDC on oxidative damage markers in goldfish tissues: (A) lipid peroxides, (B) thiobarbituric-acid reactive substances and (C) low-molecular mass thiols. Experimental conditions are: (1) control uninjected fish; (2), (4) and (6), respectively 24, 48 and 168 h post-injection with 0.9% NaCl; (3), (5) and (7) – 24, 48 and 168 h post-injection with 0.01 mg/gwm DDC. Data are expressed per gram wet mass and are means ± S.E.M., $n = 5–7$. Labels over the histogram bars indicate significant differences from the indicated group, $P < 0.05$.

elevated in brain and liver of 24 h sham-treated fish (and showed an increasing trend in kidney), whereas this parameter was significantly lower in their respective DDC-treated counterparts. After longer post-injection times, however, TBARS content in saline-injected fish was near control values. After 48 h of DDC treatment, TBARS levels were strongly increased in all tissues compared to the respective sham-treated fish, but by 168 h levels returned to the initial values.

A marker of free radical-mediated protein oxidation – protein carbonyls (CP) – showed very little response to DDC treatment in goldfish organs. In the brain of 24 h saline-treated fish CP content was only 50% of the untreated control, whereas DDC counterparts showed increased CP levels at this time point – by 1.6-fold compared to untreated control and ~3.2-fold relative to sham-treated fish. In the liver no changes in CP levels were seen over the entire experiment, whereas in kidney only the 24 h DDC-exposed animals showed higher CP levels (2.7-fold) than the controls (data not shown).

The effects of DDC treatment on the content of low-molecular mass thiols (L-SH) in goldfish tissues are shown in Fig. 1C. After 24 h post-injection with DDC, L-SH levels had decreased by ~40% in the brain and liver, and the same tendency was also seen in the kidney. L-SH levels recovered to initial levels by 48 h in brain and 168 h in liver but remained low in kidney after 168 h (only 20% of the value in the corresponding 168 h sham-injected animals). L-SH levels in the kidney of sham-treated individuals were also elevated after 168 h as compared with untreated controls. The content of sulfhydryl groups in proteins (H-SH) changed little after either saline or DDC injection. The only changes were a slight decrease in the liver of 24 h DDC-treated fish and ~1.7-fold increase in the kidney of 168 h sham-treated animals (data not shown).

3.2. Superoxide dismutase

Fig. 2 shows SOD activities in the brain, liver and kidney of sham- and DDC-treated goldfish. After 24 h

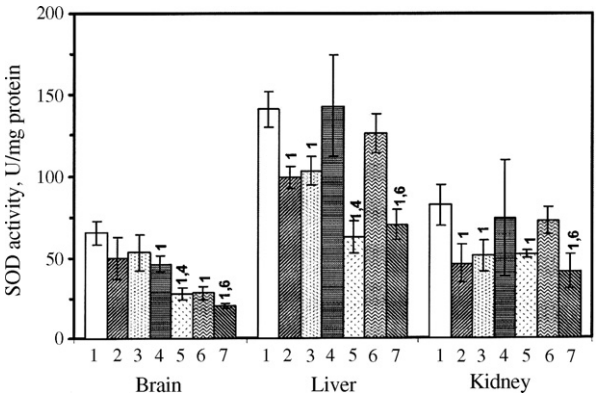

Fig. 2. The effects of DDC on superoxide dismutase activities in goldfish tissues. Enzyme activities are expressed per mg soluble protein. Other information as in Fig. 1.

post-injection, DDC treatment had no effect on SOD activity in brain, but in other organs SOD activities were reduced in both sham- and DDC-treated groups; the activity was reduced by ~30% in liver and ~40% in kidney, compared with uninjected controls. After longer times post-injection (48 or 168 h), SOD activities in sham-treated groups were restored to control levels in both liver and kidney, whereas SOD activities in DDC-treated animals remained low. SOD activity in the kidney remained at ~40% below control levels whereas activity in the liver continued to fall to ~50% of the control value after 48 h and remained low after 168 h. The brain showed a different pattern. SOD activities decreased gradually in both sham- and DDC-injected groups over the experimental course but were significantly lower in DDC-treated fish than in the corresponding sham-treated controls. Thus, brain SOD activities in DDC-treated fish were just 60 and 70% of the corresponding shame-injected control values after 48 and 168 h, respectively.

3.3. Antioxidant and associated enzymes

Effects of sham and DDC injections on catalase activity in brain are shown in Table 1. Neither treatment affected catalase activity in the brain. In the liver and kidney, 24 h DDC treatment resulted in a slight increase in catalase activities relative to untreated control levels (in liver) or to corresponding sham-injected animals (in kidney). However, in both tissues by 48 h post-injection catalase activity was lowered again in DDC groups and showed significantly lower activity (by 35–40%) in DDC-injected fish compared to saline-injected fish.

Glutathione peroxidase (GPx) activity was significantly reduced in all three organs of 24 h DDC-treated fish compared with untreated controls (reduced by ~60, 45 and 65%, respectively) and GPx was also significantly

Table 1
Catalase activities (U/mg soluble protein) in three organs of goldfish treated with physiological saline or diethyldithiocarbamate

Treatment group	Brain	Liver	Kidney
Control	4.57 ± 0.90	25.1 ± 3.2	13.2 ± 2.3
24 h NaCl	4.48 ± 0.31	28.0 ± 4.2	9.86 ± 0.92
24 h DDC	4.78 ± 0.44	35.4 ± 4.8[a]	15.2 ± 1.4[b]
48 h NaCl	4.55 ± 0.58	41.7 ± 3.1[a]	15.8 ± 1.4
48 h DDC	5.18 ± 0.90	25.2 ± 1.9[c]	10.5 ± 1.2[c]
168 h NaCl	3.94 ± 0.07	25.4 ± 3.2	13.5 ± 1.4
168 h DDC	5.20 ± 0.76	37.0 ± 6.9	12.4 ± 2.7

Data are means ± S.E.M., $n = 5–7$.
[a] Significantly different from the control.
[b] 24 h saline-injected groups ($P < 0.05$).
[c] 48 h saline-injected groups ($P < 0.05$).

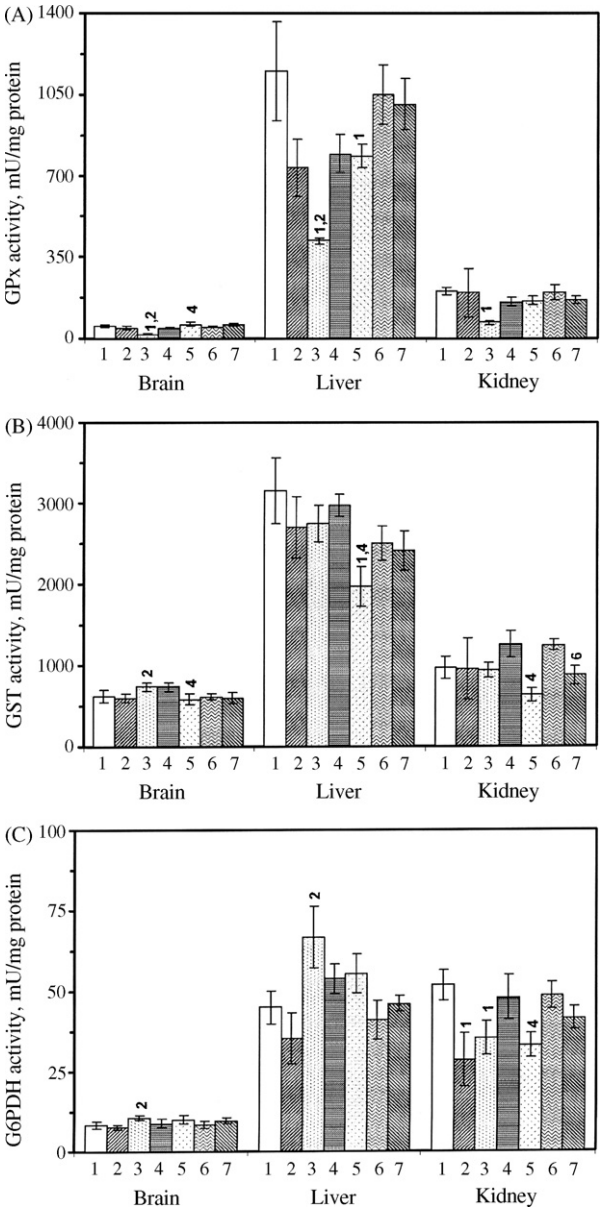

Fig. 3. The effect of DDC on the activities of antioxidant enzymes in goldfish tissues: (A) glutathione peroxidase, (B) glutathione-*S*-transferase and (C) glucose-6-phosphate dehydrogenase. Enzyme activities are expressed per mg soluble protein. Other information as in Fig. 1.

reduced as compared with sham-injected fish in brain and liver (Fig. 3A). However, GPx activities in all organs were restored after longer times post-injection.

Glutathione-*S*-transferase (GST) activities were also reduced by DDC treatment. Activities in 48 h post-injection DDC-treated fish were decreased by ~35% in the liver and ~50% in the kidney compared to the corresponding sham-treated fish (Fig. 3B). However, activities

Table 2
Glutathione reductase activities (mU/mg protein) in three organs of goldfish treated with physiological saline or diethyldithiocarbamate

Treatment group	Brain	Liver	Kidney
Control	10.7 ± 2.2	18.7 ± 1.5	21.4 ± 1.7
24 h NaCl	10.2 ± 1.2	18.1 ± 1.8	16.2 ± 5.2
24 h DDC	12.4 ± 0.8	22.7 ± 1.3[a,b]	23.2 ± 1.9
48 h NaCl	11.0 ± 1.6	22.9 ± 1.8	23.6 ± 2.0
48 h DDC	10.5 ± 2.2	19.8 ± 1.4	17.0 ± 1.6[a,c]
168 h NaCl	15.3 ± 1.9	16.2 ± 1.2	14.9 ± 3.0[a]
168 h DDC	11.8 ± 0.9	20.4 ± 1.3[d]	18.9 ± 1.2

Data are means ± S.E.M., $n = 5–7$.
[a] Significantly different from the control.
[b] 24 h saline-injected groups ($P < 0.05$).
[c] 48 h saline-injected groups ($P < 0.05$).
[d] 168 h saline-injected groups ($P < 0.05$).

returned to control levels thereafter. Brain GST activities increased slightly in DDC-injected fish after 24 h as compared with sham-injected animals, whereas 48 h DDC-treated fish showed a small decrease in GST activities compared with sham-treated animals.

Brain glutathione reductase (GR) activities did not change under the experimental conditions (Table 2). In the liver, GR activities were elevated by 20% in 24 h and by 26% in 168 h DDC-treated fish compared with corresponding sham-injected controls, whereas kidney GR activities were reduced by 39% after 48 h DDC treatment relative to untreated and sham-injected controls.

Similar to GR, glucose-6-phosphate dehydrogenase (G6PDH) brain and liver showed transiently increased activities by 1.4- and 1.9-fold within 24 h after injection with DDC (Fig. 3C). Oppositely, kidney G6PDH activities were lowered in both sham- and DDC-treated 24 h groups and in the 48 h DDC-treated group. Activities in all organs were restored to untreated control values by 168 h.

4. Discussion

All organs in the study experience oxidative stress and all have antioxidant defenses to deal with ROS formed under both normal and stressed conditions. Although a number of similarities were seen between organs in the various parameters measured, a number of significant organ-specific differences cannot be ignored. Levels of measured oxidative stress indices depend on tissue circumstances including the rate of free radical generation, power of enzymatic and/or low-molecular mass antioxidants, sets of specific metabolic pathways in different organs. The net result is control values characteristic to particular tissue. Enzymatic defenses were generally highest in liver, intermediate in kidney and lowest in brain.

The pesticide diethyldithiocarbamate (DDC) is a sulfhydryl-containing metal chelator and has multiple toxicological effects. Depending on the concentration, DDC can act as an antioxidant or prooxidant [20,21,28,29]. DDC action is, in part, related to its ability to chelate copper ions. Therefore, DDC is widely used to deplete Cu,Zn-SOD activity and thereby to investigate the physiological functions of this enzyme [19–22]. DDC and its derivatives inhibit Cu,Zn-SOD both *in vitro* [19] and *in vivo* in animals [20,21], bacteria *Escherichia coli* [33], and budding yeast *Saccharomyces cerevisiae* [22]. DDC probably also inhibits other copper-containing enzymes (e.g. cytochrome c oxidase, xanthine oxidase) and therefore could affect cellular metabolism in other ways. For example, Kojda and coworkers found that DDC inhibited vascular superoxide production in rat aortic rings and the proposed mechanism involved blocking of oxidoreductase enzymes such as xanthine oxidase and those reducing redox active xenobiotics [23]. Another action of DDC is its interaction with thiol systems due to two thiol groups in its molecular structure and depletion of intracellular glutathione [30]. DDC may also affect the transcription factor NF-κB that is known to up-regulate many antioxidant defenses [21]. Low concentrations of DDC also inhibit the activity of the proteasome [32]. Carbamate derivatives are used as pesticides; they may have a broad environmental impact affecting, for example, fish behavior [31].

In goldfish tissues DDC and, in some cases, saline treatment affected lipid peroxidation in a tissue-dependent manner The different patterns of change in LOOH and TBARS may reflect differences in the time dependencies of the production, metabolism and detoxification of different products of lipid peroxidation. Short-term and generally lower increases in LOOH and TBARS in some sham-treated groups are possibly a response to stress during experimental manipulation. It is worthwhile to note that in the 24 h DDC-treated group, TBARS levels in all tissues were substantially lower compared to their sham-injected counterparts. A similar decrease in TBARS content was found by Hungarian scientists who treated carp (*Cyprinus carpio*) by adding DDC into environmental water [20]; the authors explained this effect as an antioxidant action of DDC. Generally, goldfish show a quick response of lipid peroxidation to various kinds of stressful conditions – hyperoxia, heat shock, catalase inhibition [6,9,10,17,18] – and in most cases this parameter rather quickly returned to control values. Unlike lipid peroxidation, DDC injection had very little effect on protein oxidation, assessed

as protein carbonyl content. This indicates powerful protection of goldfish proteins from oxidative modification and/or fast degradation of oxidized proteins.

DDC injection also reduced L-SH concentrations in all tissues after 24 h. The L-SH content of cells is mainly attributed to glutathione, the most important thiol antioxidant. Depletion of glutathione often occurs during the early stages of oxidative stress, including that induced by DDC [20,30]. Reduced glutathione might be consumed by direct interaction with DDC molecules or due to increased consumption of glutathione as a ROS scavenger. Increased glutathione consumption could result as a consequence of reduced SOD activity and a need for glutathione-linked mechanisms to assume a greater role in total cellular antioxidant defense. In most tissues, the decrease in L-SH was short-lived, and thereafter L-SH content was restored, probably due to *de novo* glutathione synthesis. Hence, DDC injection led to oxidative stress development which was assessed as transient by most indices except LOOH levels.

In the present study, a single injection of DDC at a concentration of 0.01 mg/gwm resulted in a reliable decrease in goldfish SOD activity seen by 48 h post-injection in all three tissues tested. Decreased SOD activities as an early (24 h) response in the liver and kidney of sham-treated fish may be connected with a general stress reaction to experimental treatment (injection, handling, etc.) and the comparable suppression of SOD in 24 h DDC-treated groups may have the same cause. Hence, we cannot link the early (24 h) suppression of SOD activities in liver or kidney with DDC action although the continued suppression of SOD activity that was maintained through 168 h post-injection can be attributed to DDC effects in these tissues. Furthermore, although brain of sham-treated fish showed reduced SOD activities at 48 and 168 h post-injection, the SOD suppression was much greater in the corresponding DDC-treated fish, again clearly showing a marked effect of DDC on the suppression of SOD activities in goldfish tissues *in vivo*. Three organs examined showed different extent of decreasing SOD activity with the most marked effect in the liver and the smallest in the brain. Given that we measured total SOD activity, this might be explained by variable ratio between Mn-SOD and Cu,Zn-SOD activities in the organs as well as possible different availability of DDC, for example, to brain.

We expected that SOD inhibition would cause compensatory responses by other antioxidant enzymatic defenses. However, the results seen for different enzymes were quite variable and tissue-specific. Activities of some enzymes like GPx were reduced by 24 h after DDC injection in all three tissues. This may be considered as ROS-mediated inactivation of the enzyme because at this time signs of oxidative stress were clearly seen. Transient reduction of some other enzyme activities was registered later, after 48 h, and was also restored by 168 h.

In this work we aimed to use DDC injection to inhibit SOD and, thereby, to use this as a tool to clarify the role of SOD in goldfish. Certainly, in model organisms like *Escherichia coli* or *Saccharomyces cerevisiae*, the role of SOD has been studied extensively either with knockouts or inhibition models [22,33]. Recently, knockout mice became more available and popular as a model. For example, Sentman et al. demonstrated that mice lacking extracellular SOD and Cu,Zn-SOD showed relatively modest phenotypes [34]. Inactivation of the Cu,Zn-SOD gene reduced mouse lifespan and increased urinary excretion of isoprostanes and plasma TBARS levels, two markers of free radical lipid oxidation. It also halved GPx activities, but increased glutathione and iron concentrations in liver. The authors explained these effects as resulting from superoxide-induced iron release from ferritin and disruption of protein Fe,S-clusters, which was evidenced by a greatly reduced activity of cytosolic aconitase in liver of Cu,Zn-SOD knockouts [34]. The situation with fish is different from that seen in mice because the possibility of generating knockouts in fish lines is still in its infancy and, therefore, the use of effectors to inhibit or activate enzymes of interest is the primary way to study their role. Therefore, we used DDC as an inhibitor of goldfish Cu,Zn-SOD activity, but clearly understood that it also affects other processes in the organism.

Generally, DDC injection resulted in transient oxidative stress and disturbance of antioxidant enzymes, but by 7-day post-injection most parameters returned to the initial levels except for SOD activity and lipid peroxide content. Hence, we can suggest that Cu,Zn-SOD may be particularly important in the protection of goldfish membranes from lipid peroxidation. Furthermore, depletion of SOD by DDC does not result in major compensatory enhancements of other enzymatic defenses. Thus, it appears that the constitutive activities of the enzymes that were examined were largely sufficient for protection against stress associated with SOD suppression. On the other hand DDC may also inhibit superoxide production by operating either directly as an antioxidant, or blocking oxidoreductase enzymes such as xanthine oxidase [23]; this should be investigated further.

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