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Induction of oxidative stress in *Rana ridibunda* during recovery from winter hibernation

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Abstract

The effect of transfer from winter hibernation at 5°C to a warm temperature (20°C for 1 or 24 h) on oxidative stress and antioxidant defenses was assessed in the frog, *Rana ridibunda*. The temperature increase had little effect on the levels of thiobarbituric acid reactive substances but carbonylprotein levels (581, 740, 1270 and 614 pmol/mg protein in brain, liver, kidney and muscle, respectively, at 5°C) rose 3.2-fold in brain and 2.6-fold in liver after transfer to 20°C. The switch to 20°C also affected the activities of antioxidant enzymes: catalase in liver, kidney and muscle rose 33%, 55% and 126%, respectively, whereas superoxide dismutase increased 2.4-fold in liver and 2.5-fold in muscle. Glutathione reductase and glucose-6-phosphate dehydrogenase activities were also affected in some organs. The data show that recovery from winter hibernation in frogs causes increased oxidative stress and stimulates an elevation of organ antioxidant defenses.

Keywords: Oxidative stress; Carbonylproteins; Antioxidant enzymes; Winter hibernation; *R. ridibunda*

1. Introduction

Oxidative stress in animals arises when the generation of reactive oxygen species (ROS) exceeds the capacity of endogenous antioxidant defense systems. The situation is often related to disease, aging, irradiation, pollutants, or many other factors (Halliwell and Gutteridge, 1989; Stadtman and Levine, 2000). However, oxidative stress can also result during normal physiological activities including exercise, arousal from hibernation, starvation, etc. (Barja de Quiroga, 1992; Storey, 1996; Drew et al., 2001). The mitochondrial respiratory chain is considered to be a main source of ROS production in vivo (Halliwell and Gutteridge, 1989). In fact, any physiological processes that stimulate respiration may cause oxidative stress.

The antioxidant defense mechanisms of frogs have received some attention. Responses to physiological stresses of anoxia, freezing and thermal acclimation have been analyzed by several groups (Perez-Campo et al., 1990; Hermes-Lima and Storey, 1996; Joanisse and Storey, 1996). Arousal from hibernation may be among other putative conditions that provoke oxidative stress in frogs. Winter dormancy of ectotherms is accompanied by a significant depression in aerobic metabolic rate (Hazel, 1989; Storey and Storey, 1996). When animals return to normal activity, therefore, the intensity of metabolism and oxygen consumption increases. Since the rate of ROS generation is related to oxygen consumption, it could be expected that the intensification of respiration at higher temperatures would result in enhanced ROS production and require enhanced antioxidant defenses for protection. Thus, we investigated the recovery of frogs, *Rana ridibunda*, when transferred from winter hibernation at 5°C to a higher temperature (20°C), monitoring two indices of oxidative

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stress in tissues as well as the activities of selected antioxidant enzymes.

Oxidative stress was assessed by measuring the levels of thiobarbituric acid reactive substances (TBARS) and carbonylproteins (CP). The former method has been widely used to assess oxidative stress in many organisms from bacteria to mammals, whereas the latter is not yet in common use. It is known that the oxidation of some amino acid residues such as lysine, arginine, and proline by ROS leads to the formation of carbonyl derivatives, aldehydes and ketones (Stadtman, 1993). The appearance of such carbonyl groups is taken as a presumptive evidence of oxidative stress (Stadtman and Levine, 2000), and their assay provides a convenient technique for detecting and quantifying the oxidative modification of proteins. The antioxidant enzymes analyzed included superoxide dismutase (SOD) and catalase, two primary antioxidant enzymes that are directly involved in the processing of ROS, as well as glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PDH) that are secondary enzymes involved in the synthesis and supply of reduced glutathione to antioxidant enzymes. This investigation evaluates the intensity of oxidative stress and the response of some antioxidant enzymes to the resumption of physiological activities at warm temperatures after the end of winter hibernation in *R. ridibunda*.

2. Methods

2.1. Animals

Adult *R. ridibunda* Pallas of both sexes, weighing 14–88 g, were collected at the end of October from local lakes in Ivano-Frankivsk (Western Ukraine). Animals were kept in the dark at 5°C without feeding for 2 months. In this state, frogs have a low metabolic rate and exhibit very little physical activity. For experimental exposure, the frogs were placed into a controlled temperature incubator at 20 ± 2°C and were held at this temperature for 1 or 24 h. This treatment did not result in enhanced physical activity of the animals, but their respiration intensity was increased. After experimental exposure animals were killed by transspinal dissection and brain, liver, kidney, and leg skeletal muscle were dissected out within 5 min. Control frogs were sampled from the 5°C holding containers.

2.2. Chemicals

Phenylmethylsulfonyl fluoride (PMSF), Sephadex G25-150, butylated hydroxytoluene, and glucose-6-phosphate (G6P) were purchased from Sigma Chemical Co whereas *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), NADPH, NADP and oxidized glutathione (GSSG) were from Reanal (Hungary), and guanidine-HCl was from Fluca. All other reagents were of analytical grade.

2.3. Indices of oxidative stress

Fresh tissue samples were quickly weighed, washed in ice-cold 0.9% NaCl and homogenized using a Potter-Elvjeham glass homogenizer in 50 mM potassium phosphate (KP_i) buffer, pH 7,0, containing 0.5 mM EDTA and a few crystals of PMSF. Homogenates were 1:10 (w:v) extracts for all organs and in some cases 1:15 for brain. The homogenates were used for TBARS and CP tests.

2.3.1. Thiobarbituric acid reactive substances assay

Lipid hydroperoxides decompose to low molecular weight products, including malondialdehyde, which can be measured by the TBARS assay (Rice-Evans et al., 1991). A 100–200 μl aliquot of homogenate was mixed with 1 ml of 30% (w:v) trichloroacetic acid (TCA) in 0.2 M HCl and then centrifuged for 10 min at $5000 \times g$. The pellet was discarded and the supernatant was combined with the same volume of TBA-reagent, containing a saturated solution of TBA in 0.1 M HCl and 10 mM butylated hydroxytoluene (BHT) dissolved in ethanol; pH was adjusted to 2.5. BHT was added to avoid tissue peroxidation during/following heating. The mixture was then boiled for 60 min. After quick cooling, the same butanol volume as the mixture total volume was added and mixed vigorously. Samples were centrifuged for 10 min at $5000 \times g$ and the butanol phase was removed and used to evaluate the level of TBARS. Absorption was measured using a spectrophotometer SF-46 (USSR) at 535 nm and a molar extinction coefficient of $156 \times 10^3\,M^{-1}cm^{-1}$ was used for the calculation of TBARS concentration (Rice-Evans et al., 1991). The values are expressed as moles of TBARS per gram tissue.

2.3.2. Carbonylproteins assay

Carbonyl derivatives of proteins may be detected by reaction with 2,4-dinitrophenylhydrazine (DNPH). This compound reacts with protein carbonyl groups that have been formed by ROS attack on side chains of amino acids. Resulting 2,4-dinitrophenylhydrazones are quantified spectrophotometrically (Lenz et al., 1989). A 100 μl aliquot of homogenate was mixed with 1 ml of 10 mM DNPH in 2 M HCl. Control samples contained 1 ml of 2 M HCl instead of the DNPH solution. Samples were incubated for 1 h at room temperature, then precipitated with 1 ml of 20% w:v TCA and centrifuged for 10 min at $5000 \times g$. The supernatants were discarded and the pellets were washed three times with 1 ml of ethanol-butylacetate (1:1 v:v)

mixture. The pellets were then dissolved in 1.5 ml of 6 M guanidine–HCl and centrifuged to pellet insoluble particles. The amount of CP was evaluated spectrophotometrically at 370 nm using a molar extinction coefficient of $22 \times 10^3\ M^{-1}cm^{-1}$ (Lenz et al., 1989). The values are expressed as moles of CP per milligram protein in guanidine solutions.

2.4. Assay of antioxidant enzyme activities

Homogenates were prepared as above, centrifuged for 15 min at $15{,}000 \times g$ and the supernatant was removed for use for enzyme activity assays. In preliminary studies, enzyme extracts were desalted by centrifugation through small columns of Sephadex G25-150 equilibrated in homogenization buffer. However, activity loss was high and desalting gave no appreciable reduction in blank activity, so the procedure was not used for the data reported here.

The activity of SOD was assayed using its inhibitory action on quercetin oxidation (Kostyuk et al., 1990, with modifications). The reaction mixture contained (final concentrations): 30 mM Tris-HCl buffer (pH 9.0), 0.5 mM EDTA, 0.8 mM TEMED, 0.05 mM quercetin, and 1–80 μl of supernatant. The reaction was monitored at 406 nm for 6–8 different volumes of supernatant. One unit of SOD activity is defined as the amount of enzyme (per protein milligram) that inhibits quercetin oxidation reaction by 50%. The maximal inhibition was about 90%. For the other enzymes, blanks were run in all cases that omitted the most specific substrate (indicated by an asterisk in the assays below). Catalase activity was measured at 240 nm in a medium containing 50 mM KP_i (pH 7.0), 0.5 mM EDTA, 10 mM H_2O_2*, and 0.2–5.0 μl of supernatant. GR activity was assayed at 340 nm in medium containing 50 mM KP_i (pH 7.0), 0.5 mM EDTA, 0.25 mM NADPH, 1 mM GSSG*, and 50 μl of supernatant. G6PDH activity was measured at 340 nm in 50 mM KP_i (pH 7.0), 0.5 mM EDTA, 5 mM $MgCl_2$, 0.2 mM NADP, 2 mM G6P* with 50 μl of supernatant. One unit of catalase, GR, and G6PDH activity is defined as the amount of the enzyme that consumes 1 μmol of substrate per minute; activities were expressed per milligram of soluble protein.

2.5. Protein measurements and statistics

Protein concentration was measured by the Bradford method with Coomassie Brilliant Blue G-250 (Bradford, 1976) using bovine serum albumin as a standard. Data are presented as mean ± SEM. Statistical analysis was performed using the Student's *t*-test. Inhibition constants were calculated using an enzyme kinetics computer program (Brooks, 1992).

3. Results

The levels of TBARS were tissue-specific (Table 1). The maximum levels were found in liver (27.2 nmol/g wet weight) with intermediate concentrations in brain and kidney and the lowest concentration in muscle (8.87 nmol/gww). The temperature increase from 5°C to 20°C had only a small effect on this parameter. In brain the TBARS concentration had decreased significantly by 50% after the 1 h exposure at 20°C. Other organs showed a similar tendency for reduced TBARS content at the higher environmental temperature.

Levels of CP were evaluated per milligram of protein. In this case, soluble protein content was determined in the same samples in which CP levels were measured (in guanidine solutions). CP concentrations were 581, 740, 1270 and 614 pmol/mg protein in brain, liver, kidney and muscle of control animals, respectively. CP levels did not change significantly in either kidney or skeletal muscle, but in brain CP rose 3.2-fold after 24 h at warmer temperatures (Fig. 1). In liver CP levels rose quickly by 2.6-fold after 1 h, but were reduced again after 24 h.

In assays of enzyme activity in homogenates, gel filtration is widely used to remove low molecular weight metabolites and ions that can affect the activity or cause high blank values (Hermes-Lima and Storey, 1996; Lushchak et al., 2001). Initial tests with catalase, GR and G6PDH showed negative effects of gel filtration on the activity of some enzymes (data not shown). For example, catalase activity was reduced 2–3-fold in liver and kidney of filtered extracts, skeletal muscle GR activity was lost and G6PDH activity disappeared from extracts of all tissues after gel filtration. These results led us to abandon gel filtration of the extracts for the data reported below.

Fig. 2 shows the effect of recovery from hibernation on SOD activity in three organs. The activity of SOD was high in liver (56.4 U/mg protein) and kidney (47.4 U/mg) of control frogs and low in muscle (6.42 U/mg). It was unchanged after 1 h at 20°C, but had risen significantly in liver (2.4-fold) and skeletal muscle (2.5-fold) after 24 h.

Table 1
Effect of temperature increase from 5°C to 20°C on the levels of TBARS (nmol/gww) in *Rana ridibunda* tissues

	Control	1 h at 20°C	24 h at 20°C
Brain	24.6 ± 3.2 (7)	12.2 ± 1.9 (3)[a]	19.7 ± 3.9 (4)
Liver	27.2 ± 2.8 (6)	26.2 ± 2.9 (4)	22.8 ± 0.8 (3)
Kidney	19.0 ± 2.4 (6)	15.9 ± 3.2 (4)	15.1 ± 1.9 (4)
Muscle	8.87 ± 0.89 (4)	5.51 ± 1.72 (4)	1.69 (2)

Data are mean ± SEM; number of animals is shown in parentheses.

[a] Significantly different from the control value, $P < 0.025$.

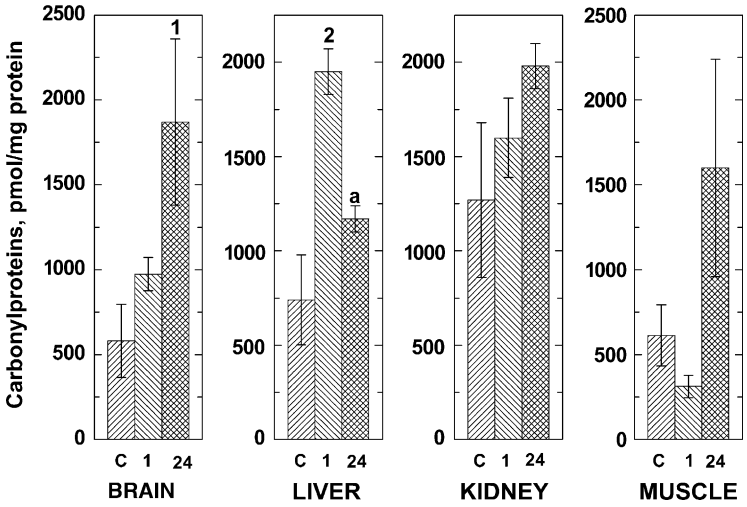

Fig. 1. The effect of recovery from hibernation on the level of carbonylproteins in tissues of *R. ridibunda*. Data are mean ± SEM, $n = 3-4$. Columns show C (control, hibernated), and 1 and 24 h of exposure to 20°C. 1—significantly different from the control group $P<0.05$, 2—$P<0.005$ and from the 1 h group, a—$P<0.001$.

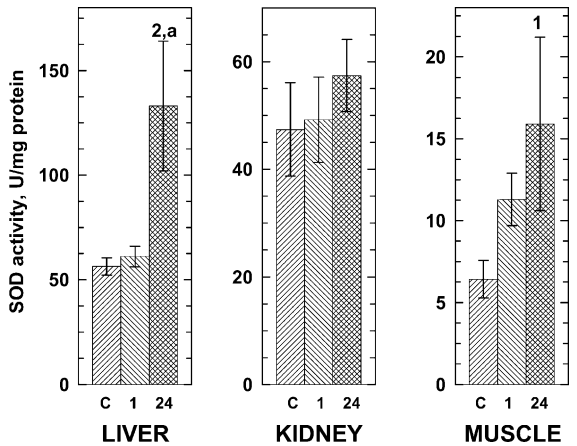

Fig. 2. Activity of superoxide dismutase in three tissues of hibernated and recovered *R. ridibunda*. Data are mean ± SEM, $n = 3-5$. 1—significantly different from the control group, $P<0.05$, 2—$P<0.025$ and from the 1 h group, a—$P<0.025$.

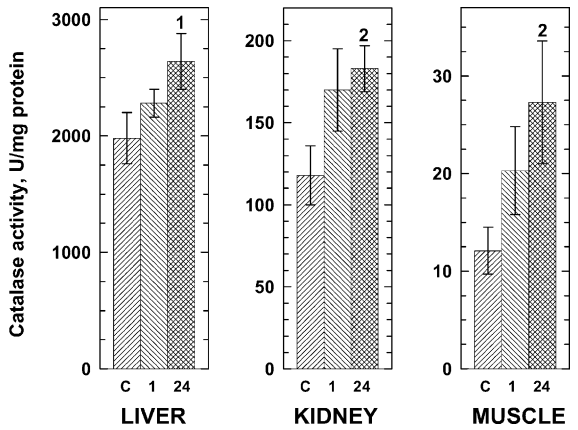

Fig. 3. Activity of catalase in different tissues of hibernated and recovered *R. ridibunda*. Data are mean ± SEM, $n = 4-7$. 1—significantly different from the control group, $P<0.05$, 2—$P<0.025$.

The activity of catalase was very high in liver (1980 U/mg) of control frogs and much lower in kidney (118 U/mg) and in muscle (12.1 U/mg). The pattern of catalase response to 20°C exposure was similar in all three tissues (Fig. 3). After 1 h all organs showed a trend of increasing activity that developed into a significant increase after 24 h when catalase activity was 33%, 55% and 126% higher than the control values in liver, kidney and muscle, respectively.

GR activity was the highest in the kidney (27.1 mU/mg) of control frogs and substantially lower in liver (9.66 mU/mg) and skeletal muscle (5.05 mU/mg). Responses to the temperature increase were tissue-specific (Fig. 4). GR activity had dropped 2.6-fold in liver after 1 h of 20°C exposure whereas muscle showed a 2.7-fold decrease after 24 h. By contrast, GR activity in kidney had risen 1.7-fold after 24 h.

G6PDH activity in liver and kidney of control frogs was 13.6 and 14.2 mU/mg protein, respectively. G6PDH activity could not be detected in frog muscle, being below the sensitivity of the assay used (< 1 mU/mg). Exposure of frogs to 20°C increased G6PDH activity in both tissues with a 2.1-fold increase seen in liver after 24 h and a 1.4-fold increase in kidney within 1 h that was sustained after 24 h (Fig. 5).

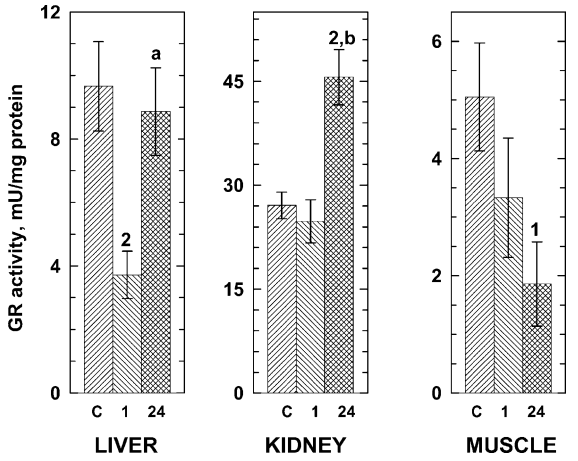

Fig. 4. Activity of glutathione reductase in different tissues of hibernated and recovered *R. ridibunda*. Data are mean ± SEM, $n = 4-7$. 1—significantly different from the control group with $P < 0.025$, 2—$P < 0.005$ and from the 1 h group, a—$P < 0.01$, b—$P < 0.005$.

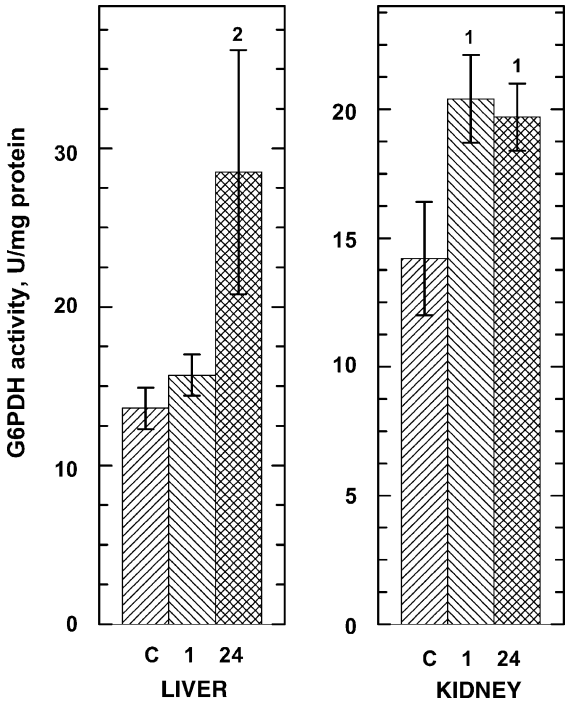

Fig. 5. Activity of glucose-6-phosphate dehydrogenase in different tissues of hibernated and recovered *R. ridibunda*. Data are mean ± SEM, $n = 4-7$. 1—Significantly different from the control group, $P < 0.05$, 2—$P < 0.025$.

4. Discussion

Different kinds of physiological factors can result in oxidative stress. For example, the antioxidant status of animals has been investigated in fish under anoxia and reoxygenation (Lushchak et al., 2001), in frogs under anoxia and recovery (Hermes-Lima and Storey, 1996) and with respect to temperature changes (Perez-Campo et al., 1990; Joanisse and Storey, 1996), in garter snakes during freezing and anoxia (Hermes-Lima and Storey, 1993), and in cold-acclimated rats (Barja de Quiroga et al., 1991). To our knowledge, this is the first examination of how antioxidant systems respond in frogs to the change from a cold, semi-dormant state (that characterizes winter hibernation) to a warmer temperature where animals resume normal activities. Since the metabolic rate of ectotherms is proportional to environmental temperature, we hypothesized that the increase in metabolic rate (oxygen consumption) that would accompany the rise in temperature from 5°C to 20°C would also require adjustments to antioxidant defenses because the rate of ROS generation increases in proportion to the rate of oxygen consumption.

TBARS are widely used as index of lipid peroxidation, although the method has very low specificity (Halliwell and Gutteridge, 1989). In studies with ectotherms, it was found that an increase in body temperature was accompanied by enhanced TBARS levels (Perez-Campo et al., 1990; Parihar and Dubey, 1995). However, changes in the amount of this product of lipid peroxidation did not always correlate well with oxidative stress (Halliwell and Gutteridge, 1989).

In *Rana perezi* acclimated to 10°C for several months the TBARS content in liver was several times higher than that measured in our study of *R. ridibunda* (Perez-Campo et al., 1990). By contrast, TBARS levels in the freeze tolerant wood frog *Rana sylvatica* were low in 5°C-acclimated frogs (8.05 nmol/g tissue in liver and 3.76 nmol/g tissue in muscle) and not changed by freeze/thaw exposure (even decreasing in liver of 4 h thawed frogs) (Joanisse and Storey, 1996). However, in *Rana pipiens*, values for this parameter were similar to those found in *R. ridibunda* and did not change over anoxia and reoxygenation (Hermes-Lima and Storey, 1996). This suggests that animals that are well adapted to survive in anoxic or ischemic conditions have mechanisms to prevent or minimize the production of ROS during tissue reoxygenation (Storey, 1996). The rise in body temperature of *R. ridibunda* from 5°C to 20°C and the increase in oxygen consumption and accompanying ROS generation that this would produce little effect on TBARS levels in this species. Levels of TBARS in brain decreased transiently after 1 h of 20°C exposure but, as assessed by these products of lipid peroxidation, the temperature change did not result in oxidative damage to tissue lipids in any of the other tissues. It would appear that existing mechanisms for the detoxification of ROS and/or the destruction of lipid peroxides via

peroxidases can prevent or minimize net oxidative damage to lipids when the rate of ROS generation increases along with oxygen consumption when body temperature is warmed. However, it must also be appreciated that the present study examined only short-term (1 and 24 h) responses to elevated temperature and that accumulation of oxidative damage to membrane lipids at the higher temperature may take a longer time to develop. For example, after 4 months of temperature acclimation, *R. perezi* showed significantly higher TBARS levels in liver of high (29°C) versus low (10°C) temperature acclimated animals (Perez-Campo et al., 1990).

ROS attack not only membrane lipids but also other cellular macromolecules including proteins. Oxidation of some amino acid residues leads to the formation of carbonyl derivatives (Stadtman, 1993). Quantification of carbonyl groups on proteins is an accepted way to measure damage caused by oxidative stress (Gladstone and Levine, 1994). The expression of CP per mg protein reflects oxidative stress development and the advantage of this method is that both protein concentration and oxidized carbonyl groups were measured in the same guanidine–HCl dissolved protein fractions. The data demonstrated an increase in CP content in both liver and brain in frogs transferred to 20°C and a similar tendency was observed in kidney and muscle. To our knowledge, the present study is the first devoted to measuring carbonyl groups in proteins of oxidatively stressed frogs. It can be concluded from our data that the CP assay may serve as a reliable method to assess the intensity of oxidative stress in animals.

The measurement of oxidative damage by TBARS versus CP levels obviously produced different assessments of damage. The TBARS method suggested little or no oxidative damage to membrane lipids with the temperature change whereas the changes in CP levels suggested that the same temperature stress caused oxidative damage to proteins in some organs. Evaluation of oxidative stress effects on tissues is, indeed, a difficult task that is complicated by many factors. Firstly, there may be inherent differences in the susceptibility of different cellular macromolecules to oxidative stress as well as in the rate with which detectable damage accumulates. Secondly, damage detection can also vary depending on the type of damage product that is assessed; for example, three different methods that are routinely used to asssess lipid peroxidation each measure different parameters and each can give somewhat different assessments of the degree of oxidative damage to lipids (Storey, 1996). Thirdly, there can be inherent differences in the levels of enzymatic defenses available to reverse damage to lipids versus proteins (e.g. lipid peroxides are decomposed by peroxidases, carbonylproteins are hydrolyzed by specific or nonspecific proteases) that can result in a differential net accumulation of damage to different macromolecule types under the same oxidative stress conditions. Fourthly, there can be differences in the long-term stability and maintenance of damage products in cells; for example, proteins damaged by oxidative stress tend to accumulate. During aging, for example, the cellular level of oxidized proteins rises and carbonyl derivatives are used as a marker of this process (Stadtman, 1998). Hence, it is apparent that a comprehensive evaluation of oxidative stress effects on tissues should involve the assessment of at least two markers of oxidative damage, preferably assessing different macromolecular targets.

Changes in environmental temperature have been related to respiratory intensity in ectotherms and, hence, to ROS generation. Increased ROS production is well known to stimulate increased levels of antioxidant defenses including enzymes (Michiels et al., 1994; Storey, 1996). Thus, the effect of the temperature increase in stimulating elevated activities of all four antioxidant enzymes agrees with the prediction that a higher metabolic rate and higher physiological activity requires enhanced antioxidant defenses. Significant increases in enzyme activities were readily apparent after 24 h at the elevated temperature whereas G6PDH showed a significant increase in kidney within 1 h.

The activity of SOD measured in *R. ridibunda* liver in the present study was higher than that found by other authors for the same species (Barja de Quiroga et al., 1984), but the earlier study used a different assay method. Our data on the tissue distribution of SOD agree with comparable analyses of *R. sylvatica* and *R. pipiens* that showed high SOD activities in liver and kidney of control frogs and lower values in brain and muscle (Joanisse and Storey, 1996). However, catalase activity in *R. ridibunda* tissues was substantially higher than that in the other two frog species (Joanisse and Storey, 1996; Hermes-Lima and Storey, 1996). A study on tadpoles of two amphibian species, *Discoglossus pictus* and *R. ridibunda perezi*, showed that catalase activities were higher in *D. pictus* (Gil et al., 1987). Basal SOD level was also higher in *D. pictus*. The authors concluded that the elevated antioxidant enzyme activities confer a resistance to hyperoxia in *D. pictus* over *R. ridibunda perezi*. Therefore, if catalase levels reflect the power of antioxidant defense mechanisms, adult *R. ridibunda* can be considered to have high resistance to oxidative stress when compared with *R. sylvatica* or *R. pipiens*.

Catalase and GR activities correlated inversely in two metabolically active organs, liver and kidney. Catalase activity was much higher in liver than in kidney whereas the opposite was true for GR in kidney. It may be suggested that the main enzyme involved in the decomposition of hydrogen peroxide in liver is catalase, whereas in kidney glutathione peroxidase may dominate with its dual ability to react with both H_2O_2 and organic

hydroperoxides. High GR in kidney would support glutathione peroxidase function.

The activities of the primary antioxidant defense enzymes, SOD and catalase, showed a general and consistent response during recovery from hibernation in *R. ridibunda* tissues. In most cases an increase was implicated within 1 h of the transfer (although the increase was not significant) with a statistically significant increase of 33–126% for catalase and 2.4–2.5-fold for SOD recorded after 24 h except for SOD activity in kidney. Parallel changes in the activities of these two enzymes may be explained by the fact that they act in concert to process ROS. Thus, SOD reduces superoxide anions to hydrogen peroxide and catalase converts hydrogen peroxide to water and molecular oxygen. The increased activities of both enzymes when frogs were transferred to the higher temperature probably represent a compensatory adjustment of activities to deal with a new steady-state level of ROS generation associated with the higher metabolic rate at the warmer temperature.

G6PDH activity also showed the same pattern of response to the temperature increase with a significant 40% increase in kidney within 1 h and a 2.1-fold rise in liver after 24 h at 20°C. G6PDH is the initial enzyme of the pentose phosphate pathway and has an important role to play in many biosynthetic functions that require either NADPH reducing power or sugar phosphate units. The increase in G6PDH activity with the rise in temperature may be linked with a general increase in the biosynthetic capacity of liver and kidney triggered by the warmer temperature or may be more specifically associated with the elevation of antioxidant defenses. The role of G6PDH in antioxidant defense is peripheral but important. This enzyme is one of the main cellular sources of NADPH, which is needed for the reduction of oxidized glutathione by GR. A constant supply of reduced glutathione is required to support the activities of the primary antioxidant enzymes glutathione peroxidase and glutathione-*S*-transferase.

The rise in GR activity in kidney after 24 h at 20°C followed the same trend as was seen for the other enzymes, but GR in liver and muscle responded differently to the temperature stress. In both cases, activity was strongly reduced within 1 h in liver and after 24 h in muscle. At a time when antioxidant defenses are being generally elevated, a reduction in GR activity seems anomalous. One factor that may be involved is the sensitivity of many enzymes, GR among them, to inactivation by different compounds including ROS (Fucci et al., 1983). Oxidative modification can lead to loss of catalytic activity and increased sensitivity to denaturation. It has been suggested that thiol groups at the active site of GR are oxidized causing inactivation of the enzyme (Worthington and Rosemeyer, 1976). Thus, GR may be affected, perhaps only temporarily, by an increased rate of ROS generation brought about by the increased metabolic rate at the higher body temperature.

In summary, the present data show that recovery from hibernation in the frog *R. ridibunda* is accompanied by oxidative stress, as documented by the increase in carbonylprotein content in organs within 1–24 h after the animals were moved to 20°C. Organs responded to this with an increase in the activities of primary antioxidant enzymes, SOD and catalase, and with selective increases in the activities of two other enzymes that support antioxidant defenses, GR in kidney and G6PDH in liver and kidney. Overall, the power of antioxidant defenses in some species of frogs (*R. ridibunda, R. sylvatica, R. pipiens*) seems to be high to withstand to stressful environmental factors. For example, the levels of TBARS (an indicator of lipid peroxidation) were not altered significantly by oxidative stress arising during recovery from anoxia, freezing or hibernation. High activities of catalase in liver and kidney of these species also reflect a powerful potential of these tissues to neutralize ROS. The rapid increase in antioxidant enzyme activities in tissues of *R. ridibunda* within 1–24 h in response to the rise in environmental temperature also indicates that these frogs possess well-developed regulatory mechanisms that allow their metabolism to response quickly to oxidative stress.

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