

E. Şahin · S. Gümüşlü

Cold-stress-induced modulation of antioxidant defence: role of stressed conditions in tissue injury followed by protein oxidation and lipid peroxidation

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Abstract The aim of this study was to determine the effects of cold stress on antioxidant enzyme activities and examine protein oxidation and lipid peroxidation in various tissues (brain, liver, kidney, heart and stomach). Twenty male Wistar rats (3 months old) weighing 220 ± 20 g were used. The rats were randomly divided into two groups of ten: the control group and the cold stress group. Cold stress was applied to the animals by maintaining them in a cold room (5°C) for 15 min/day for 15 days. Blood samples were taken for measuring plasma corticosterone levels. Tissues were obtained from each rat for measuring the antioxidant enzyme activities, protein oxidation and lipid peroxidation. Corticosterone levels were increased in the cold stress group. Copper, zinc superoxide dismutase activities were increased in the brains, livers and kidneys, whereas they decreased in the hearts and stomachs of rats in the cold stress group. Catalase activities were increased in the brains, livers, kidneys and hearts, whereas they decreased in the stomachs of rats in the cold stress group. Selenium-dependent glutathione peroxidase activities were increased in the brain, liver, heart and stomach. Reduced glutathione levels were decreased, while levels of protein carbonyl, conjugated diene and thiobarbituric-acid-reactive substances were increased in all tissues of the cold stress group. These results lead us to conclude that cold stress can disrupt the balance in an oxidant/antioxidant system and cause oxidative damage to several tissues by altering the enzymatic and non-enzymatic antioxidant status, protein oxidation and lipid peroxidation.

Keywords Cold stress · Antioxidant enzymes · Reduced glutathione · Protein oxidation · Lipid peroxidation

Introduction

Stress is known as the disruption of homeostasis, which is managed by the complex formations of physiological and behavioural adaptive responses of organisms (Johnson et al. 1992). It is well known that cold exposure may be reflected in an elevated metabolic rate and possibly also increased production of reactive oxygen species, e.g. hydrogen peroxide (H_2O_2), hydroxyl radicals (HO^\cdot) and superoxide anion radicals ($\text{O}_2^{\cdot-}$), which cause lipid peroxidation (Selman et al. 2000; Heise et al. 2003). Membrane injury following lipid peroxidation causes disruption of the tissue integrity (Bagchi et al. 1999). Therefore, to neutralize reactive oxygen species the body uses mainly enzymatic copper, zinc superoxide dismutase (Cu,Zn-SOD), catalase and selenium-dependent glutathione peroxidase (Se-GSH-Px) and non-enzymatic antioxidants, e.g. reduced glutathione (GSH).

Cold stress, which is characterized by the increase in metabolic rate, leads to alterations in some antioxidant enzyme activities. These alterations are explained as a mechanism resisting the negative effects of reactive oxygen species (Selman et al. 2000). It is reported that acclimation at temperatures below 10°C for a period of more than 3 weeks should result in observable effects (Kennedy et al. 1977).

Several studies have investigated whether exposure to low temperatures results in compensatory changes taking place in the antioxidant defence system (Barja de Quiroga et al. 1991; Ohno et al. 1991; Bondarenko et al. 1999), although there has been no detailed and comparable study investigating the effects of chronic cold stress on protein modification and lipid peroxidation in different tissues. Additionally we could not find any study clarifying the tissue most affected by chronic cold stress according to the oxidative parameters. In our previous study we investigated the influences of different stress models on the antioxidant status and lipid peroxidation in rat erythrocytes (Gumuslu et al. 2002). In the present study, we have investigated the effects of chronic cold stress (at 5°C for 15 min/day for 15 days) on tissue antioxidant

E. Şahin · S. Gümüşlü (✉)
Department of Biochemistry, Faculty of Medicine,
Akdeniz University, 07070 Antalya, Turkey
e-mail: sgumuslu@akdeniz.edu.tr
Tel.: +(90)-242-227-4343
Fax: +(90)-242-227-4495

status (Cu,Zn-SOD, catalase and Se-GSH-Px activities and GSH levels), protein modification [protein carbonyl (PC) levels] and lipid peroxidation [conjugated diene (CD) and thiobarbituric-acid-reactive substances (TBARS) levels] by using brain, liver, kidney, heart and stomach tissues.

Materials and methods

Animals

In this study, 20 male Wistar rats (3 months old) weighing 220 ± 20 g were used. Four animals were housed per cage. The rats were randomly divided into two groups of ten rats each to form the control group and the cold stress group. Control animals were acclimatized to standard animal laboratory conditions (12 h/12 h light/dark cycle, temperature 22 ± 2 °C and humidity $50 \pm 5\%$). The cold stress model was designed according to the methods described by Finlay et al. (1995). The animals in the cold stress group were placed in a cold room (ambient temperature of 5 °C) for 15 min/day for 15 days.

Preparations of tissue homogenates

At the end of the experimental periods, the rats were deprived of food for 24 h and then prepared for experimental procedure under ether anaesthesia. The whole brain, liver, kidney, heart and stomach were immediately removed and washed with cold saline. The whole tissues were homogenized in cold potassium phosphate buffer (50 mM, pH 7.4). Some of the homogenate was separated for measuring GSH levels on the day the animals were sacrificed and the remaining part was divided into two portions. The first portion of homogenate was centrifuged at $700 \times g$ for 20 min in order to determine catalase activity (Liotti et al. 1987; Gumuslu et al. 2001), PC, CD and TBARS levels. The second portion of the homogenate was centrifuged at $20,000 \times g$ for 30 min in order to determine Cu,Zn-SOD and Se-GSH-Px activities (Carrillo et al. 1992; Gumuslu et al. 2001). Tissue protein levels were measured after appropriate dilutions had been made in the supernatants.

Plasma corticosterone level

The whole blood was centrifuged at $1,500 \times g$ for 10 min at 4 °C to separate plasma from erythrocytes. Plasma was used for the measurement of corticosterone levels. The ImmChem™ ^{125}I -corticosterone radioimmunoassay kit (ICN Biomedicals Inc., Costa Mesa, CA 92626) that is designed for use in laboratory mice and rats was used for measuring plasma corticosterone levels in all experimental groups. Plasma and standard samples that were diluted with steroid diluent were incubated with ^{125}I -corticosterone and corticosterone antiserum for 2 h. After the precipitation step, the samples were centrifuged at $1,000 \times g$ and 4 °C for 15 min. The supernatant was removed. The radioactivity of the remaining pellet was counted by gamma counter GC-20. The resulting concentration of plasma corticosterone was expressed as ng/ml, using corticosterone standards prepared in different concentrations.

Cu,Zn-SOD activity

Cu,Zn-SOD activity was measured according to the method of Misra and Fridovich (1972). This method is based on the ability of Cu,Zn-SOD to inhibit the auto-oxidation of adrenalin to adrenochrome at alkaline pH. Enzymatic activity was expressed as U/g protein at 30 °C.

Catalase activity

Catalase activity was measured by the method of Aebi (1987) using hydrogen peroxide as substrate. The disappearance of H_2O_2 was followed spectrophotometrically at 240 nm. Enzymatic activity was expressed as k/g protein (k : rate constant of a first-order reaction) at 25 °C.

Se-GSH-Px activity

Se-GSH-Px activity was measured by the method of Fecondo and Augusteyn (1983) using *t*-butylhydroperoxide as substrate. Enzymatic activity was expressed as U/g protein at 37 °C.

GSH level

GSH levels were assayed using the methods of Sedlak and Lindsay (1968) and Fairbanks and Klee (1986). 5,5'-Dithiobis-(2-nitrobenzoic acid) is a disulfide chromogen that turns to dark yellow on reduction with sulfhydryl compounds. The absorbance of the reduced chromogen was followed spectrophotometrically at 412 nm. The GSH level was determined from a standard curve and expressed as mg/g protein.

PC level

The protein carbonyl content was measured by the method of Levine et al. (1990). Protein was precipitated with 20% trichloroacetic acid (TCA). After centrifuging at $11,000 \times g$ and 4 °C for 15 min, the supernatant was removed. The pellet was resuspended in 0.5 ml 10 mM 2,4-dinitrophenylhydrazine/2 M HCl (50 °C). Samples were held in the dark for 1 h where they were vortexed every 10 min. The samples were precipitated with 0.5 ml 20% TCA and centrifuged at $11,000 \times g$ at 4 °C for 3 min. The same procedure was repeated three times with 10% TCA. The precipitate was dissolved in 2 ml NaOH at 37 °C. The PC level was calculated from the maximum absorbance (360 nm) using a molar absorption coefficient (ϵ) of $22,000 \text{ M}^{-1} \text{ cm}^{-1}$. The results were expressed as nmol carbonyl/mg protein.

CD level

The CD level was measured by the method described by Recknagel and Glende (1984). Lipids were extracted with 2/1 (v/v) chloroform/methanol; the extract was evaporated to dryness under a stream of nitrogen and then redissolved in cyclohexane. The cyclohexane solution was assayed at 234 nm. The results were expressed as nmol hydroperoxide/mg protein using $\epsilon_{\text{max}} = 2.52 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

TBARS level

The TBARS level was measured by a fluorometric method described by Wasowicz et al. (1993) and Gumuslu et al. (2001) using 1,1,3,3-tetraethoxypropane as standard, and the results were given as nmol malondialdehyde/mg protein.

Protein content

The protein content of the samples was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Statistical analysis

Data were expressed as the mean \pm standard error of the mean (SEM). The parameters of the control and cold stress groups were compared using the independent-samples *t*-test in an SPSS package program (Version 10.01; SPSS, Chicago, Ill.). *P* values less than 0.05 are considered statistically significant.

Results

The plasma corticosterone level of the cold stress group (751.00 ± 7.92 ng/ml) was significantly higher than that of the control group (274.50 ± 10.39 ng/ml) ($P < 0.001$).

Cu,Zn-SOD activities were higher in the brain, liver and kidney, and were lower in the heart and stomach than in the control ($P < 0.001$) (Fig. 1). The highest percentage of increased Cu,Zn-SOD activity (118.45%) was found in the liver. We saw that catalase activities were increased significantly in the brain, liver, kidney and heart while being decreased in the stomach ($P < 0.001$)

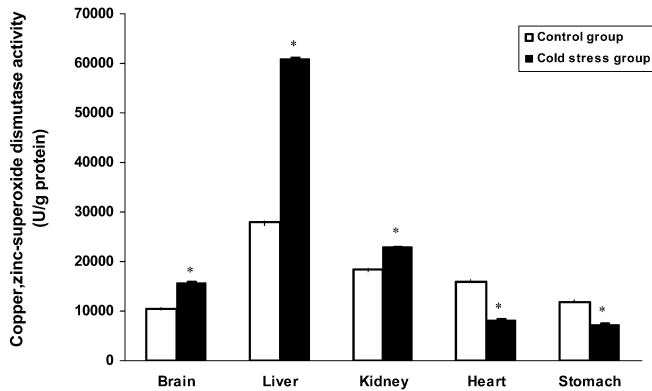


Fig. 1 Copper,zinc-superoxide dismutase (Cu,Zn-SOD) activities in the brain, liver, kidney, heart and stomach of control and cold stress groups. Each value represents the mean \pm SEM of ten animals per group. * $P < 0.001$ different from control

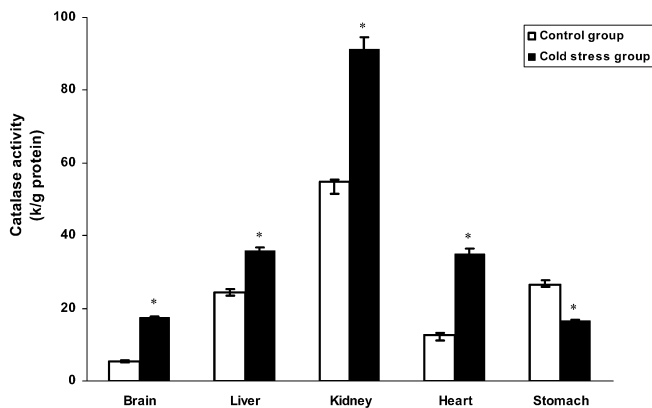


Fig. 2 Catalase activities in the brain, liver, kidney, heart and stomach of control and cold stress groups. Each value represents the mean \pm SEM of ten animals per group. * $P < 0.001$ different from control

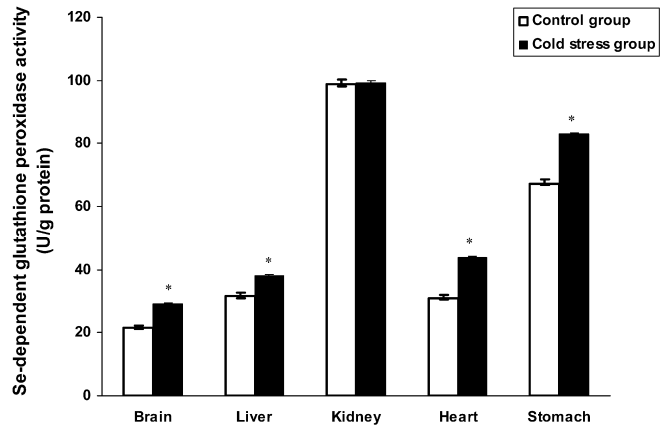


Fig. 3 Selenium-dependent glutathione peroxidase (Se-GSH-Px) activities in the brain, liver, kidney, heart and stomach of control and cold stress groups. Each value represents the mean \pm SEM of ten animals per group. * $P < 0.001$ different from control

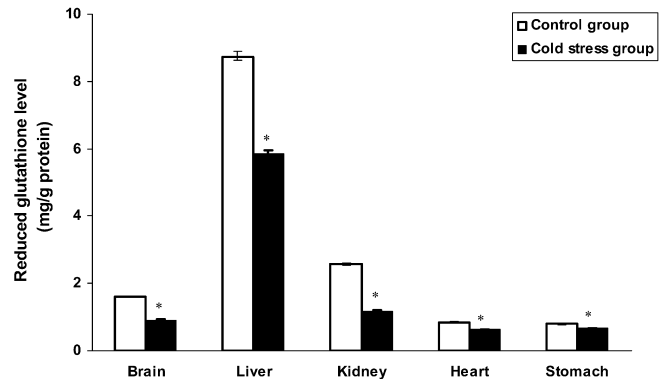


Fig. 4 Reduced glutathione (GSH) levels in the brain, liver, kidney, heart and stomach of control and cold stress groups. Each value represents the mean \pm SEM of ten animals per group. * $P < 0.001$ different from control

(Fig. 2). The highest percentage of increased catalase activity (217.18%) was found in the brain. Se-GSH-Px activities were higher in the brain, liver, heart and stomach than in the control ($P < 0.001$), while it did not change significantly in kidney (Fig. 3). The highest change in Se-GSH-Px activity (41.21%) was observed in the heart. GSH levels were lower in all tissues than in the control ($P < 0.001$) (Fig. 4). The highest percentage decrease of GSH (53.91%) was found in kidney. PC, CD and TBARS levels were increased in all tissues ($P < 0.001$) (Figs. 5, 6 and 7 respectively). The highest percentage (210.00%, 75.00% and 186.21%) increases were found in the liver, brain and stomach respectively.

There was a negative correlation between brain PC and GSH levels ($r = -0.637$, $P < 0.05$), between plasma corticosterone and liver CD levels ($r = -0.721$, $P < 0.05$) and between kidney GSH and PC levels ($r = -0.683$, $P < 0.05$), and a positive correlation between plasma corticosterone and liver TBARS levels ($r = 0.644$,

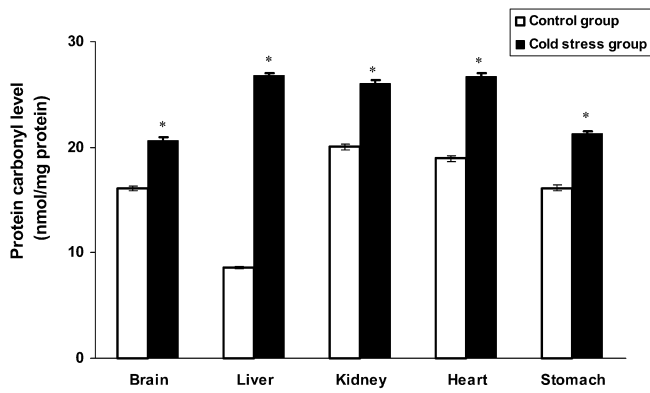


Fig. 5 Protein carbonyl (PC) levels in the brain, liver, kidney, heart and stomach of control and cold stress groups. Each value represents the mean \pm SEM of ten animals per group. * $P < 0.001$ different from control

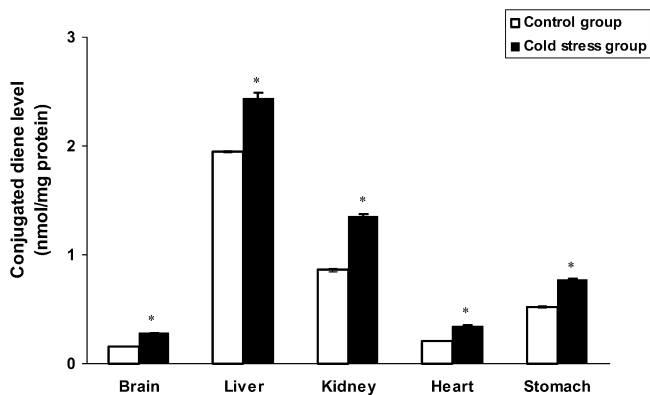


Fig. 6 Conjugated diene (CD) levels in the brain, liver, kidney, heart and stomach of control and cold stress groups. Each value represents the mean \pm SEM of ten animals per group. * $P < 0.001$ different from control

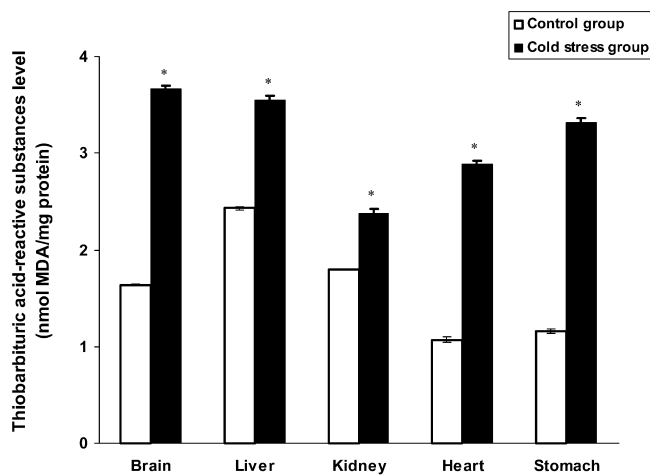


Fig. 7 Thiobarbituric acid-reactive substance (TBARS) levels in the brain, liver, kidney, heart and stomach of control and cold stress groups. Each value represents the mean \pm SEM of ten animals per group. * $P < 0.001$ different from control

$P < 0.05$) and between heart GSH and TBARS levels ($r = 0.807$, $P < 0.05$).

Discussion

This is the first study to examine the tissue most affected by cold stress for the changes in antioxidant enzyme activities, GSH, protein oxidation and lipid peroxidation levels.

The plasma corticosterone level of the cold stress group was found to be significantly higher than that of the control group. Our result is in accordance with the previous studies (Kim et al. 1999; Malendowicz et al. 1996; Djordjevic et al. 2003) demonstrating that the plasma corticosterone level is one of the most important indicators of stress.

We found a significant increase in brain Cu,Zn-SOD, catalase and Se-GSH-Px activities of rats exposed to cold stress. Kaushik and Kaur (2003) and Yaras et al. (2003) also found that Se-GSH-Px activity was increased in the brain after chronic cold exposure. The elevations of all antioxidant enzyme activities suggest that cold stress may increase the production of reactive oxygen species on a large scale in brain tissue. When we compared catalase activities in different tissues, the highest percentage of increased activity was observed in the brain. This result may suggest that the H_2O_2 concentration is higher in the brain of rats in the cold stress group than in the control animals, because catalase functions in a state of high H_2O_2 concentration. H_2O_2 has been reported to be especially toxic to brain neurons (Abe and Saito 1998).

In the liver, as in the brain, cold stress elevated the activities of all antioxidant enzymes. The increment in Cu,Zn-SOD activity suggests that cold stress may increase the rate of O_2^- formation and subsequently H_2O_2 formation. The mechanism of accelerated formation of reactive oxygen species in stress is not known exactly. However, the hormones produced as part of the natural adaptation to stress have been demonstrated to increase the activity of the hepatic cytochrome- P_{450} -dependent mixed-function oxidase system (Kato 1977), which is probably the principal source of the activated oxygen species, the precursors from which the lipoperoxides are generated. A major source of cellular lipoperoxide generation is the microsomal NADPH-cytochrome- P_{450} enzyme system, and liver cells have an extensive endoplasmic reticulum (Mead 1976). When we compared the Cu,Zn-SOD activities in different tissues, the highest increase was found in the liver.

In the kidney, Cu,Zn-SOD and catalase activities were increased in the cold stress group. Kaushik and Kaur (2003) found that chronic exposure to cold stress increased catalase activity, as in our study, although it decreased total superoxide dismutase and GSH-Px in the kidney. The contradictory results may derive from the different laboratory conditions used in carrying out the cold stress procedure. The increases in Cu,Zn-SOD and

catalase activities may indicate that the concentrations of O_2^- and H_2O_2 may be higher than in the controls.

Cu,Zn-SOD activities were observed to be decreased in the hearts of the cold stress group. A previous study of chronic cold stress, which was performed by Kaushik and Kaur (2003) supports our results. The reason for this decrease may be product inhibition due to considerable H_2O_2 formation catalyzed by Cu,Zn-SOD. Higher catalase and Se-GSH-Px activities support the suggestion that H_2O_2 occurs in large amounts in the heart. The heart is an aerobic organ and has one of the highest mass-specific oxygen consumption rates in the body; therefore the heart copes with high rates of oxidant formation and stress (Liu et al. 2000). Somani and Arroyo (1995) found that the heart has four times less SOD activity than the liver. It is possible that stress may upset the balance between reactive oxygen species and antioxidant defences and lead to myocardial injury. When we compared Se-GSH-Px activities in different tissues the highest increase was observed in the heart. This enzyme may not only detoxify H_2O_2 , but also reduce organic hydroperoxides to less reactive compounds mainly in this tissue.

We found that Cu,Zn-SOD and catalase activities were decreased in the stomachs of rats in the cold stress group while Se-GSH-Px activities were higher in this group than in the control. Uchida and Kawakishi (1994) explained that histidine residues at the active site of Cu,Zn-SOD oxidized when this enzyme was exposed to H_2O_2 . SOD is inactivated by oxidative damage of the histidine residue caused by site-specific generation of OH^\cdot at its Cu^{2+} binding site (Yim et al. 1990; Uchida and Kawakishi 1994). This study supports our observations of the activity of Cu,Zn-SOD. Das et al. (1997) concluded that OH^\cdot is the major causative factor in the oxidative damage of the gastric mucosa by stress. Therefore, OH^\cdot formed by the Haber-Weiss reaction may inactivate catalase in this tissue. The increased activity of Se-GSH-Px may show that cold stress not only produces H_2O_2 but also causes the formation of organic hydroperoxides which are detoxified by Se-GSH-Px in large amounts. According to our results, Se-GSH-Px serves as a major antioxidant enzyme in this tissue.

GSH is very important for the cellular defence against reactive oxygen species. GSH levels were found to be decreased in all tissues of the cold stress group in our study. Our result is in agreement with the results of Kaushik and Kaur (2003) who reported decreased GSH levels in all tissues after chronic cold stress, Ohno et al. (1991) who reported decreased levels of GSH in erythrocytes and Barja de Quiroga et al. (1991) who found a decrease in the GSH level in brown adipose tissue after stress. GSH can react chemically with singlet oxygen (1O_2), O_2^- and HO^\cdot and therefore function directly as a free-radical scavenger. It has been reported that stress reduces GSH levels and leads to increased levels of reactive oxygen species (Liu et al. 1996). When we compared the GSH levels in the tissues, the kidney was found to have the lowest GSH concentration.

The differences in antioxidant enzyme activities and GSH levels among different organs may be dependent on several factors, such as oxygen consumption, susceptibility to oxidants, environmental conditions, sex and age.

Oxidative damage to a specific protein, especially at the active site, can induce a progressive loss of a particular biochemical function. The accumulation of oxidized proteins is often measured by the content of reactive carbonyls (Stadtman et al. 1992). We found that the PC content was increased in all tissues. When we compared the PC levels in the different tissues, the liver was found to have the highest PC content. The increase in PC level of the stomach is in agreement with a previous study by Das et al. (1997) who found an increase in carbonyl levels in gastric mucosa after restraint/cold stress for 2 h. Selman et al. (2002) also found a significant increase in PC level in the liver after acute cold ($7 \pm 3^\circ C$) exposure (1, 10, or 100 h duration).

An important indicator of lipid peroxidation is CD, which is formed by molecular rearrangement of carbon-centred radicals (Gutteridge 1995). Another particular product, malondialdehyde, is the important biomarker of lipid peroxidation. There have been many reports of stress-induced lipid peroxidation in plasma (Liu et al. 1994), erythrocytes (Gumuslu et al. 2002) and other tissues (Giralt et al. 1993; Bian et al. 1997; Seckin et al. 1997) of animals. CD and TBARS levels were found to be increased in all tissues after cold stress. The results we obtained for TBARS agree with previous reports, as shown above. The highest increases in CD and TBARS levels were found in the brain and stomach respectively. The differences in lipid peroxidation and PC levels among tissues may be due to the differences in mitochondrial biogenesis and the degree of oxidant-induced degeneration during stress.

The negative correlation between PC and GSH levels in the brains and kidneys of rats in the CS group is evidence of the susceptibility of these organs to protein oxidation during stress. The increased protein modification may be due to the decrease of GSH, which protects proteins against oxidation of the sulfhydryl groups of the globin chains, denaturation and formation of precipitates (Robbins and Kumar 1994). The positive correlation between plasma corticosterone and liver TBARS levels is supported by a study by Hidalgo et al. (1991) who suggested that glucocorticoids appeared to increase liver TBARS in stressed rats. In contrast, we found a negative correlation between plasma corticosterone and liver CD levels. This result supports the idea that glucocorticoids keep lipid peroxidation at a certain level (Hidalgo et al. 1991). When we compared the CD levels in different tissues, the lowest increase in CD levels was found in the liver.

As a result, the most affected tissue was the liver for Cu,Zn-SOD activity, the brain for catalase activity and the heart for Se-GSH-Px activity. The most decreased GSH level was seen in the kidney. The most increased PC, CD and TBARS levels were found in the liver, brain and stomach respectively. The observed changes in

antioxidant enzyme activities are tissue-specific, but it is evident that exposure to cold stress (at 5 °C for 15 min/day for 15 days) leads to oxidative stress by decreasing GSH levels and increasing protein oxidation and lipid peroxidation in all tissues.

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