

Met-Enkephalin Modulation of Age-Related Changes in Red Cell Antioxidant Status

S. SOBOČANEC, T. BALOG, V. ŠVERKO, T. MAROTTI

Division of Molecular Medicine, Rudjer Bošković Institute, Zagreb, Croatia

Received January 15, 2004

Accepted March 23, 2004

Summary

Opioid peptides have been recognized as modulators of reactive oxygen species (ROS) in mouse macrophages and human neutrophils. Since the effect cannot be ascribed to its direct scavenger properties, in this study, we tested the hypothesis that methionine-enkephalin (MENK) modulates ROS by alteration of antioxidant enzyme activity (AOE). For this purpose superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) are measured in red blood cells of 1, 4, 10, and 18-month-old CBA mice of both sexes injected with 10 mg/kg MENK. The results indicate that MENK-affected antioxidant enzyme activity of red blood cells is age- but not sex-related. The most abundant effects were observed at the reproductive stage. Increased sensitivity to oxidative stress by opioid peptides was in both sexes mainly due to increased SOD activity followed by GPX decrease. Thus, the damage ascribed to opioid peptides might be, at least partly, ascribed to deleterious effects of accumulated hydrogen peroxide (H₂O₂).

Key words

Antioxidant enzymes • Mice • Red blood cells • Gender • Age

Introduction

Reactive oxygen species (ROS) are physiologically generated in biological systems but they are also involved in many pathological processes. The generation of ROS is a constant process; their number can fluctuate even under normal conditions. Consequently, ROS defense systems must also change in order to adapt to a particular oxidative status.

One of the aspects of the expression of immunity, which is altered by opioids is the capacity of macrophages and PMN to convert oxygen to microbicidal metabolites. Opioid peptides have been recognized as modulators of ROS in mouse peritoneal macrophages (Efanov *et al.* 1994) and human neutrophils (Marotti *et*

al. 1990). In humans, methionine-enkephalin (MENK) can reverse suppressed superoxide anion release from human neutrophils treated with dexamethasone *in vitro* (Haberstock and Marotti 1993). The effect of MENK on lipid peroxidation (LPO) level in mouse liver is age- and sex-dependent; it stimulates LPO production in younger male and female mice, but suppresses this production only in older male mice (Šverko *et al.* 2002). The effect of MENK on ROS production does not seem to be related to its scavenger property as demonstrated in cell-free xanthine: xanthine oxidase system (Marotti *et al.* 1996). Another possibility is that MENK alters ROS production by affecting the quantity and/or activity of AOE, which scavenges oxygen free radicals. These free-radical scavenging enzymes include SOD which can convert

superoxide anion ($O_2^{\cdot-}$) to H_2O_2 , CAT, and/or GPX, which then further reduce H_2O_2 to H_2O .

In this study, as an initial approach to test the above hypothesis, we assessed the effect of MENK on SOD, CAT and GPX activity in the blood of 1-, 4-, 10-, and 18-month-old male and female CBA mice.

Methods

Animals

Male and female CBA/HZgr mice aged 1, 4, 10, or 18 months raised in the breeding colony of Rudjer Bošković Institute, Zagreb, Croatia, were used. Animals were kept in conventional circumstances (light/dark rhythm 12/12 h, temperature 22 °C, humidity 55 %), fed a standard diet (Domžale, Slovenia) and given water *ad libitum*.

The experiments were performed in accordance with the current laws of the Republic of Croatia and with guidelines of European Community Council Directive of November 24, 1986 (86/609/EEC).

Drugs

The MENK (Sigma, St. Louis, MO, USA) was prepared freshly on the testing days, dissolved in physiological saline, and injected intraperitoneally (ip) in a dose of 10 mg/kg body weight. Controls were given saline following the same schedule.

Acquisition of sample

Two hours after a single injection of MENK, mice of both sexes were anesthetized with diethyl ether and bled from the jugular vein into heparinized tubes. Whole blood was centrifuged (10 min at 3000 rpm), plasma discarded and in the remaining pellet antioxidant enzyme activities were determined in a lysate of erythrocytes. Hemoglobin concentration was measured by Cell-Dyn 1600 (Abbot, USA).

Assay for antioxidant enzyme activities

GPX activity was assayed spectrophotometrically (340 nm) using Camspec M330 equipped with M330 Camspec software package (Camspec LTD, Cambridge, UK) based on the method of Paglia and Valentine (1967). GPX was determined by RANSEL kit (RANDOX, San Diego, CA, USA) which measures GPX activity indirectly by determining the rate of NADPH oxidation to $NADP^+$, which is accompanied by a decrease in absorbance at 340 nm. One GPX unit is proportional to

the amount of NADPH consumed in nmol per minute at 37 °C and pH 7.2. GPX activity was expressed as U/mg Hb.

SOD activity was measured spectrophotometrically (550 nm) by RANSOD kit (RANDOX, San Diego, CA, USA) which is based on xanthine oxidase-mediated reduction of cytochrome C as described by Flohé and Ötting (1984). One unit of SOD activity is defined as the amount of enzyme required to give 50 % inhibition in the typical calibration curve obtained with standard SOD. SOD activity was expressed as U/mg Hb.

CAT activity was determined spectrophotometrically (240 nm) using 30 mM H_2O_2 as a substrate according to the method of Aebi (1984). Changes in absorbance in the reaction mixture were measured during 30 s after addition of the sample. One unit of activity corresponds to the loss of 1 μ mol of H_2O_2 per min. CAT activity was expressed as U/mg Hb.

Statistical analysis

Results were processed with SPSS v. 10.0 statistical package. Data were expressed as means \pm S.E.M. For the age-related studies, the data were analyzed statistically by means of analysis of variance (ANOVA) followed by *post hoc* analysis using Scheffé's test. Statistical significance was considered when $p < 0.05$. Student's t-test was applied to define the difference between males and females of the same age or the difference between control and MENK-treated animals.

Results

The effect of MENK on SOD activity

As presented in Figure 1A, in males (ANOVA; $F_{3,24}=7.6$, $p=0.001$), erythrocyte SOD activity from 18-month-old mice was significantly higher than in mice aged one month ($p=0.033$), 4 months ($p=0.001$) or 10 months ($p=0.036$). In females (ANOVA; $F_{3,27}=5.5$, $p=0.004$) a difference in SOD activity was observed only between erythrocytes of 4- and 18-month-old mice ($p=0.004$). No sex-related difference in SOD activity was observed.

MENK (Figs 1B and 1C) significantly and to the same degree up-regulated SOD activity in erythrocytes of adult (4-month-old) male and female mice ($p < 0.001$). In older mice, SOD activity was up-regulated with MENK in the blood of 10-month-old males and 18-month-old females ($p < 0.01$).

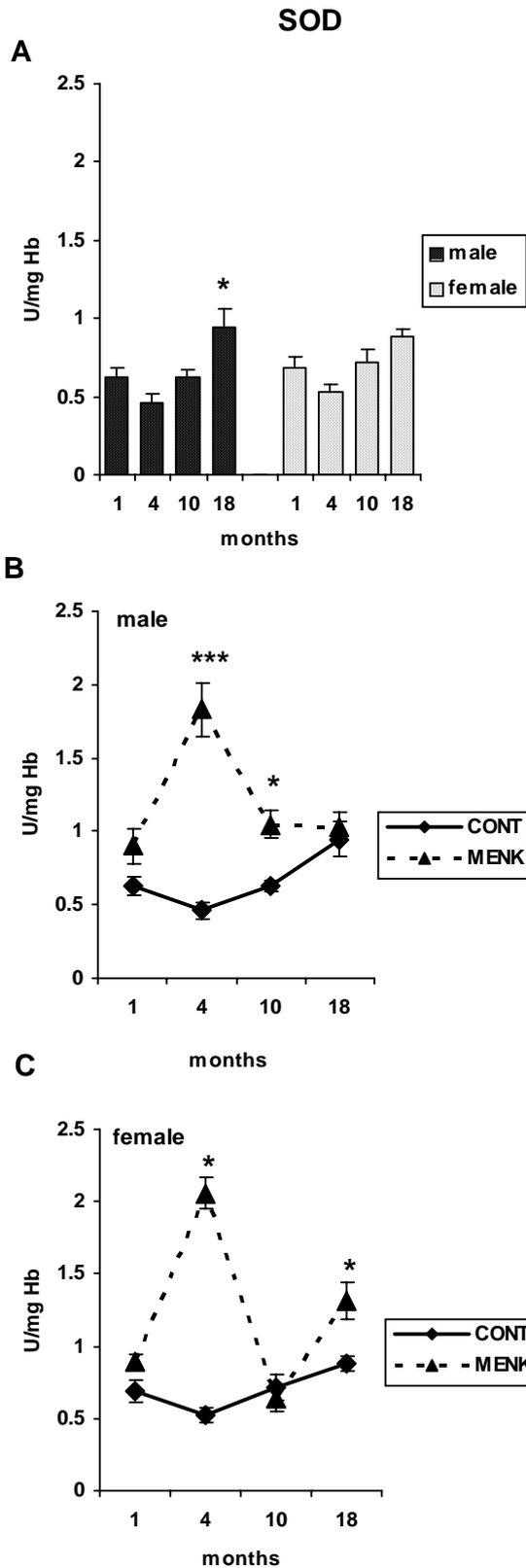


Fig. 1. Levels of erythrocyte SOD activity (U/mg Hb) in 1-, 4-, 10- and 18-month-old control mice (A) or male (B) and female (C) mice treated ip with MENK (10 mg/kg). Each point represents mean \pm S.E.M. of 6-9 animals. A * p <0.05 vs. 1-month-old mice; B, C ** p <0.01, *** p <0.001 MENK vs. control

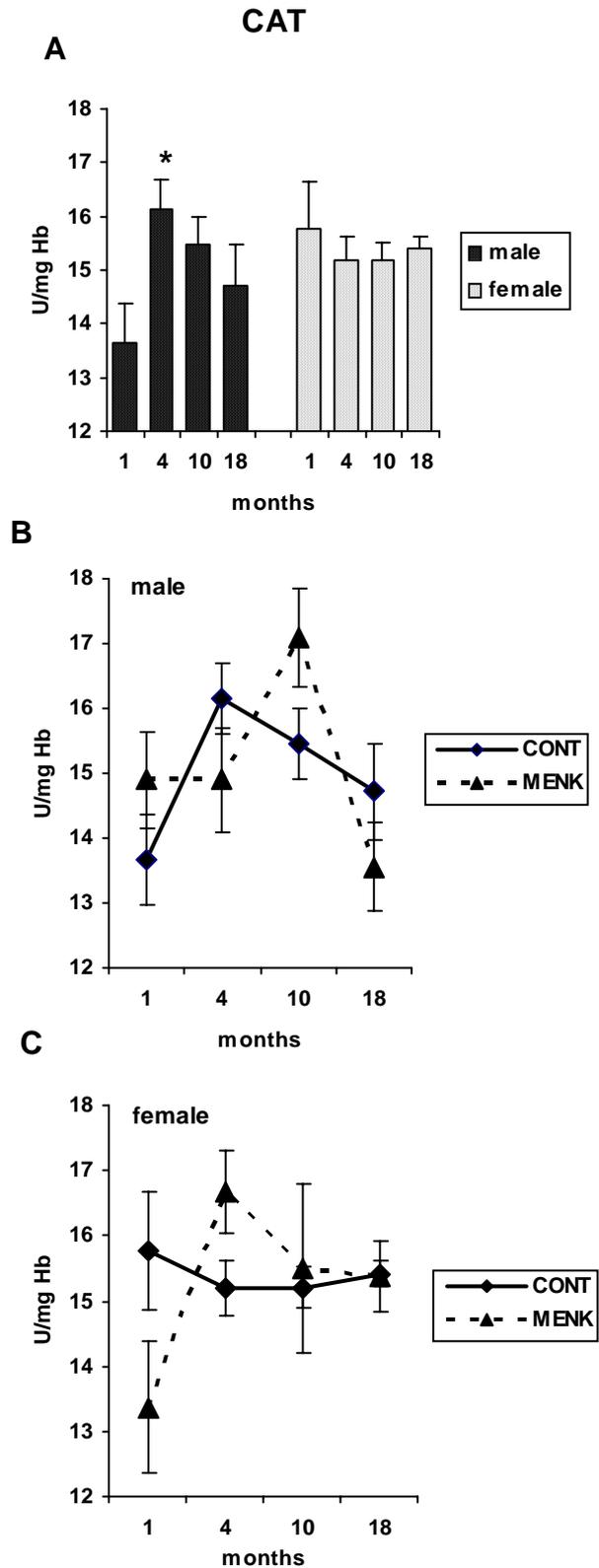


Fig. 2. Levels of erythrocyte CAT activity (U/mg Hb) in 1-, 4-, 10- and 18-month-old control mice (A) or male (B) and female (C) mice treated ip with MENK (10 mg/kg). Each point represents mean \pm S.E.M. of 6-9 animals. A * p <0.05 vs. 1-month-old mice

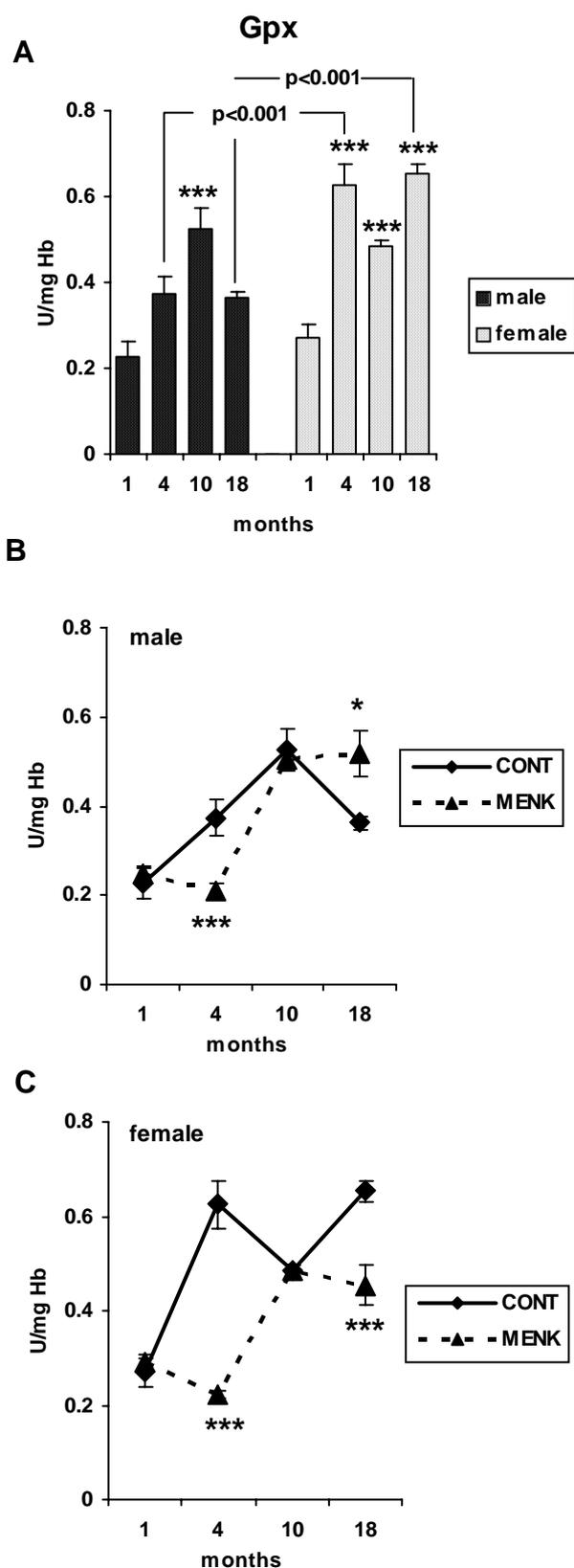


Fig. 3. Levels of erythrocyte GPX activity (U/mg Hb) in 1-, 4-, 10- and 18-month-old control mice (**A**) or male (**B**) and female (**C**) mice treated ip with MENK (10 mg/kg). Each point represents mean \pm S.E.M. of 6-9 animals. **A** *** p <0.001 vs. 1 month old mice; **B, C** * p <0.05, *** p <0.001 MENK vs. control

The effect of MENK on CAT activity

As demonstrated in Figure 2A, no age-related difference in CAT activity of female mice erythrocytes was detected. In males (ANOVA; $F_{3,32}=3.1$, $p=0.04$), only blood of 1-month-old mice had marginally ($p=0.05$) lower CAT activity than blood of adult (4-month-old) mice. No difference in CAT activity between males and females was observed. MENK did not affect CAT activity in the blood of either female or male mice (Figs 2B and 2C).

The effect of MENK on GPX activity

GPX activity in the blood of male and female CBA mice was differently regulated with age as demonstrated in Figure 3A. In males, (ANOVA; $F_{3,27}=10.1$ p <0.001) GPX activity increased gradually with age, reaching significant difference at 10 months of age ($p=0.001$) compared to 1-month-old mice. In females, (ANOVA; $F_{3,33}=32.9$ p <0.001) GPX activity was significantly higher ($p=0.001$) at 4-, 10-, or 18-month-old mice than at 1-month-old mice. Blood of adult (4 months) and old males (18 months) had less (p <0.001) GPX activity than the blood of corresponding females.

MENK down-regulated GPX activity in adult mice (4 months, p <0.001) of both sexes (Figs 3B and 3C). At old age (18 months) MENK up-regulated GPX activity (p <0.05) in males, while in females GPX activity was down-regulated (p <0.001).

The effect of MENK on SOD/CAT+GPX ratio

As demonstrated in Figure 4A, SOD/CAT+GPX ratio increased in male and female mice with age. The lowest ratio (compared to senescent mice) was observed in males (44 %) and females (60 %) at 4 months of age. MENK treatment (Fig. 4B) markedly increased SOD/CAT+GPX ratio only in 4-month-old mice of both sexes (males 4.5 and females 3.5 times greater than 4-month-old control mice).

Discussion

The aim of this study was to determine if enzymatic antioxidant levels in erythrocytes of CBA mice are age- and/or sex-related and to examine the effect of opioid peptide MENK on these parameters. The analysis revealed that a) there was an age- but no sex-related increase in SOD activity, b) no evidence of any age- or sex-related changes in CAT was observed, and c) an age-related increase in GPX activity was also sex-related.

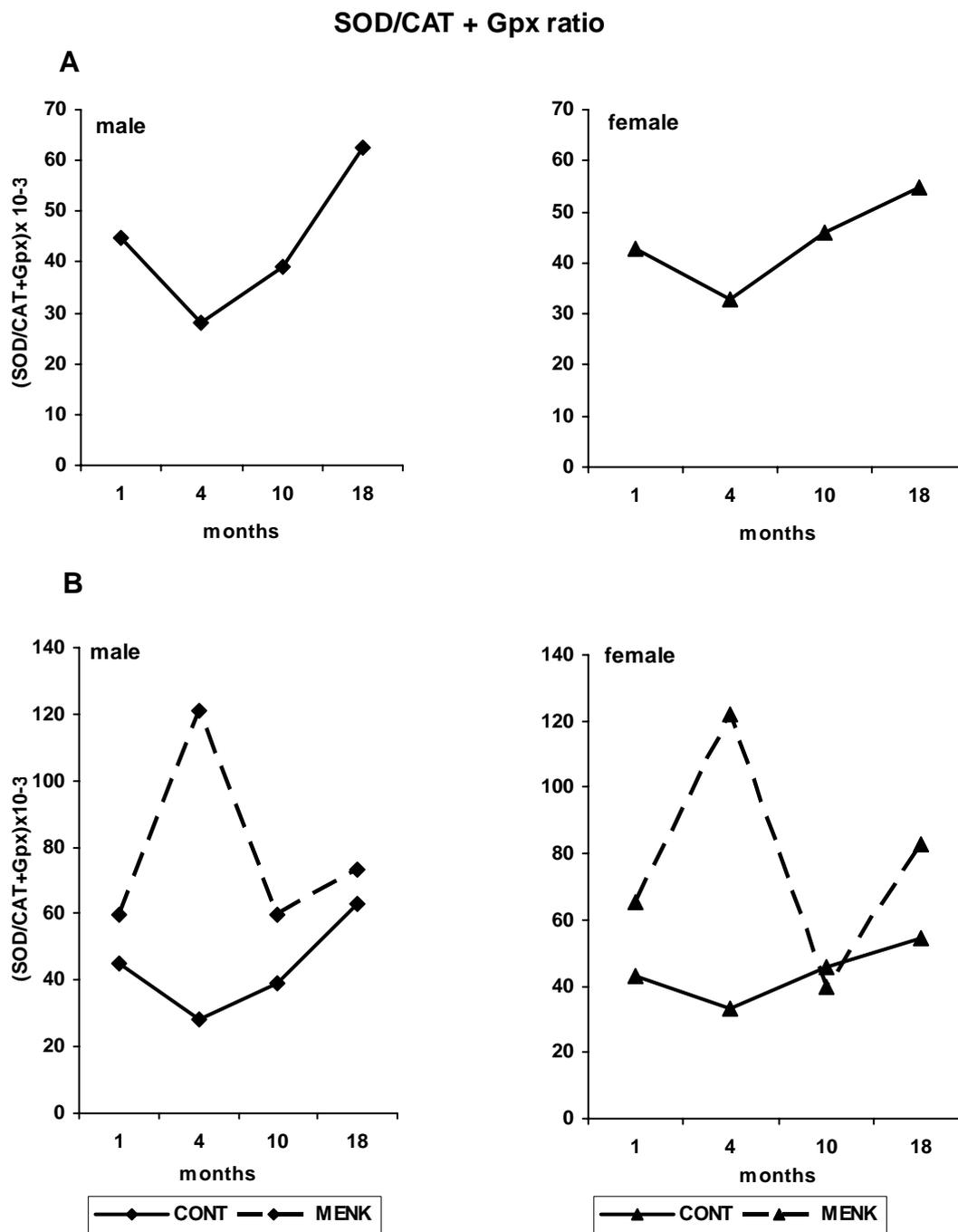


Fig. 4. Ratio of antioxidant enzymes ($R = \text{SOD}/\text{CAT} + \text{GPX}$) of 1-, 4-, 10- and 18-month-old male or female control mice (**A**) or mice injected ip with 10 mg/kg MENK (**B**).

These results indicate that an acute dose of MENK affects antioxidative enzyme activity of male and female red blood cells from CBA mice 2 h after treatment. MENK significantly stimulated SOD activity and suppressed GPX activity. The effect was not sex-related (demonstrated in both males and females), but it was age-related. The most abundant effects were observed at the reproductive stage in 4-month-old mice. In 4-month-old mice of both sexes, increased SOD

activity by MENK could be a direct consequence of MENK-induced IL-1 activity as a primary effect and IL-1-induced increase in SOD expression and activity as a secondary effect, since there are data showing both the induction of IL-1 upon MENK stimulation (Marotti *et al.* 1998) as well as the up-regulation of SOD activity by IL-1 (O'Donnell and Lynch 1998). Since both IL-1 and SOD are proteins derived from genes that fall into the functional category of genes expressed within 2 h upon

stimuli (Utsuyama *et al.* 2002, Weigel *et al.* 2003), SOD increase upon MENK stimulation within 2 h might be related to this category of genes. The absence of MENK stimulation of SOD activity in older animals could be explained by a shift in cytokine profile from Th1 to Th2 that has been observed both in aging humans and in mice (O'Mahony *et al.* 1998). Stronger suppression of GPX activity upon MENK stimulation in 4-month-old females could be explained by different response to stress in males and females associated with diverse activation of HPA axis (Kudielka *et al.* 2000). There are also evidences showing sex-related differences in blood GPX activity due to hormone specific serum levels (Massafra *et al.* 2002).

Cell sensitivity to a free radical attack apparently depends on the relationship between SOD and CAT+GPX ratio rather than on absolute amounts of individual antioxidant enzymes (Michiels *et al.* 1994). As indicated by Husain and Somani (1997), the ratio of SOD/CAT+GPX is a significant indicator of tissue ability to cope with oxidative stress. Four-month-old animals of both sexes in the reproductive stage had the lowest ratio of SOD/CAT+GPX, demonstrating high resistance to oxidative damage, whereas senescent mice had the highest sensitivity to oxidative stress.

Thus, the age-associated increases in antioxidant enzyme activities may be an attempt by an organism to counterbalance the reduced ability of coping with stress, as demonstrated by the increase of SOD/CAT+GPX ratio in old mice of both sexes.

The ratio SOD/CAT+GPX, which is increased in MENK-treated 4-month-old animals of both sexes (430 % in males and 370 % in females), indicates that opioids increase sensitivity to oxidative stress. Given ratio is a consequence of SOD increase followed by GPX decrease. Since MENK does not affect CAT activity simultaneously, the associated H₂O₂ accumulation might induce the damage ascribed to opioid peptides.

The lack of sex differences in the effect of MENK in our study is not in accordance with the study of Bartok and Craft (1997) who found no sex difference in the antinociceptive effects of *mu* opioid agonist, whereas *kappa* and *delta* opioid agonists showed sex differences.

The reasons for these discrepancies might depend on the assay, the dose of the drug and whether it was administered acutely or chronically. One of the reasons for the absence of sex differences observed in our study might be the difference in opioid pharmacokinetics. Namely, previous studies have shown sex differences in

the level of opioids in the brain (Craft *et al.* 1996), but no sex difference in blood levels of opioids has been reported.

In our study, we demonstrated no effect of MENK in very young 1-month-old mice. We have also previously observed an age-related effect of MENK on NO release in mice which was observed only in mature, 4-months-old mice and not in young 2-month-old mice (Balog *et al.* 2001).

There is an increasing evidence in the literature of animal studies that opioids have qualitatively different effects in immature compared to mature nervous system (Rahman *et al.* 1998). Several reasons could explain this. One of them was observed by Barr and Zadina (1999) who noticed that stress-induced responses, mediated by endogenous opioids, which occur late in development, may be related to the late appearance of endomorphin-2. Another reason could be associated with receptors of opioid peptides. Changes in opioid receptor level on human erythrocytes during chronic opiate use were reported by Zeiger *et al.* (2000). Their finding indicated the presence of opioid receptors on human erythrocytes, although with considerable variation in receptor levels. In the study of Negri *et al.* (1997), the relative percentage of low and high affinity sites for *delta* opioid receptor agonist (DPDPE) was shifted to high affinity sites from 15 days of age onwards (up to 2-month-old mice). The rank of potency of *delta* agonists increased with age due to maturation of the *delta* receptor subtypes. Abundance of mRNA for *delta* opioid receptor is not altered during development of the rat brain but is increased in adult caudate putamen and nucleus accumbens at the time when these receptors are believed to be functional (La Moine *et al.* 2002). Finally, an additional reason might be associated with the hydrolytic enzyme that cleaves opioid peptides. Neutral endopeptidase (NEP), a proteolytic enzyme that hydrolyzes opioids, demonstrated age-related changes in the expression of its parental gene. It was differently expressed in 2-month-old and 12-month-old rats (Raizada *et al.* 2002).

So far, the only effect of opioids on antioxidant status was described by Tsao *et al.* (1998) who found that *delta* opioid agonist DADLE *in vitro* blocked methamphetamine "speed"-induced neurotoxicity ascribed to ROS. To the best of our knowledge, this study is the first report of *in vivo* effect of opioids on antioxidative enzyme activity. The present results suggest that increased sensitivity of blood erythrocytes to stress induced by MENK *in vivo* is not sex- but rather age-related.

Acknowledgment

We thank V. Matešić for her most helpful technical support and preparation of the manuscript. This work was

supported by Grant 14MP096 Croatian Ministry of Science and Technology.

References

- AEBI H: Catalase in vitro. *Method Enzymol* **105**: 121-126, 1984.
- BALOG T, MAROTTI T, MUSANI V, SOBOČANEC S, ŠVERKO V: The effect of methionine-enkephalin on nitric oxide release in mice is age and gender related. *Pharmacol Res* **44**: 287-292, 2001.
- BARR GA, ZADINA JE: Maturation of endomorphin-2 in the dorsal horn of the medulla and spinal cord of the rat. *Neuroreport* **10**: 3857-3860, 1999.
- BARTOK RE, CRAFT RM: Sex differences in opioid antinociception. *J Pharmacol Exp Ther* **282**: 769-773, 1997.
- CRAFT R, KALIVAS P, STRATMANN J: Sex differences in discriminative stimulus effects of morphine in the rat. *Behav Pharmacol* **7**: 764-778, 1996.
- EFANOV AM, KOSHKIN AA, SAZANOV LA, BORODULINA OI, VARFOLOMEEV SD, ZAITSEV SV: Inhibition of the respiratory burst in mouse macrophages by ultra-low doses of an opioid peptide is consistent with a possible adaptation mechanism. *FEBS Lett* **335**: 114-116, 1994.
- FLOHÉ L, ÖTTING F: Superoxide dismutase assays. *Method Enzymol* **105**: 93-104, 1984.
- HABERSTOCK H, MAROTTI T: Met-enkephalin induced escape from dexamethasone immunosuppression. *Neuropeptides* **25**: 199-206, 1993.
- HUSAIN K, SOMANI SM: Interaction of exercise training on chronic ethanol ingestion on hepatic and plasma antioxidant system in rat. *J Appl Toxicol* **17**: 189-194, 1997.
- KUDIELKA BM, HELLHAMMER DH, KIRSCHBAUM C: Sex differences in human stress response. *Encycl Stress* **3**: 424-428, 2000.
- LA MOINE C, FAUCHEY V, JABER M: Opioid receptor gene expression in dopamine transporter knock-out mice in adult and during development. *Neuroscience* **112**: 131-139, 2002.
- MAROTTI T, ŠVERKO V, HRŠAK I: Modulation of superoxide anion release from human polymorphonuclear cells by met- and leu-enkephalin. *Brain Behav Immun* **4**: 13-22, 1990.
- MAROTTI T, HABERSTOCK H, GABRILOVAC J, BORANIĆ M, ŠVERKO V, RABATIĆ S: Opioid peptides in immunomodulation: a view. *Period Biol* **98**: 169-174, 1996.
- MAROTTI T, BALOG T, MAŽURAN R, ROČIĆ B: The role of cytokines in met-enkephalin modulated nitric oxide release. *Neuropeptides* **32**: 57-62, 1998.
- MASSAFRA C, GIOIA D, DE FELICE C, MUSCETTOLA M, LONGINI M, BUONOCORE G: Gender-related differences in erythrocyte glutathione peroxidase activity in healthy subjects. *Clin Endocrinol* **57**: 663-667, 2002.
- MICHIELS C, RAES M, TOUSSAINT O, REMACLE J: Importance of Se-glutathione peroxidase, catalase and Cu/Zn-SOD for cell survival against oxidative stress. *Free Radic Biol Med* **17**: 235-248, 1994.
- NEGRI L, SEVERINI C, LATTANZI R, POTENZA RL, MELCHIORRI P: Postnatal development of delta-opioid receptor subtypes in mice. *Br J Pharmacol* **120**: 989-994, 1997.
- O'DONNELL E, LYNCH MA: Dietary antioxidant supplementation reverses age-related neuronal changes. *Neurobiol Aging* **19**: 461-467, 1998.
- O'MAHONY L, HOLLAND J, JACKSON J, FEIGHENY C, HENNESSY TPJ, MEALY K: Quantitative intracellular cytokine measurement: age-related changes in preinflammatory cytokine production. *Clin Exp Immunol* **113**: 213-219, 1998.
- PAGLIA DE, VALENTINE WN: Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J Lab Clin Med* **70**: 158-169, 1967.
- RAHMAN W, DASHWOOD MR, FITZGERALD M, AYSLEY-GREEN A, DICKENSON AH: Postnatal development of multiple opioid receptors in the spinal cord and development of spinal morphine analgesia. *Dev Brain Res* **108**: 239-254, 1998.

-
- RAIZADA V, LUO W, SKIPPER BJ, MCGUIRE PG: Intracardiac expression of neutral endopeptidase. *Mol Cell Biochem* **232**: 129-131, 2002.
- ŠVERKO V, SOBOČANEC S, BALOG T, MAROTTI T: Met-enkephalin modulates lipid peroxidation and total sialic acid level in CBA mice in age- and sex-dependent manners. *Exp Gerontol* **37**: 1413-1421, 2002.
- TSAO LI, LADENHEIM B, ANDREWS AM, CHIUEN C, CADET JL, SU TP: Delta opioid peptide [D-Ala²,D-Leu⁵] enkephalin blocks the long-term loss of dopamine transporters induced by multiple administration of metamphetamine: involvement of opioid receptors and reactive oxygen species. *J Pharmacol Exp Ther* **287**: 322-331, 1998.
- UTSUYAMA M, HIROKAWA K: Differential expression of various cytokine receptors in the brain after stimulation with LPS in young and old mice. *Exp Gerontol* **37**: 411-420, 2002.
- WEIGEL AL, IDA H, BOYLAN SA, HJELMELAND LM: Acute hyperoxia-induced transcriptional response in the mouse RPE/choroid. *Free Radic Biol Med* **35**: 465-474, 2003.
- ZEIGER AR, PATKAR AA, FITZGERALD R, LUNDY A, BALLAS SK, WENSTEIN SP: Changes in mu opioid receptors and rheological properties of erythrocytes among opioid abusers. *Psychol Arena* **7**: 207-217, 2000.
-

Reprint requests

Tatjana Marotti, Division of Molecular Medicine, Rudjer Bošković Institute, Bijenička 54, 10000 Zagreb, Croatia.
E-mail: marotti@irb.hr