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Pharmacology, Biochemistry and Behavior 85 (2006) 400–407
PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR
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Acute stressor-selective effects on homocysteine metabolism and oxidative stress parameters in female rats☆

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Received 12 April 2006; received in revised form 4 September 2006; accepted 11 September 2006
Available online 23 October 2006

Abstract

Homocysteine levels are affected by diet factors such as vitamin deficiencies, non-diet factors such as genetic disorders, and stress exposure. Hyperhomocysteinemia has been implicated in several disorders, including cardiovascular disease, depression, schizophrenia, Alzheimer's and Parkinson's disease. Since sex differences play a role both in stress responses and in susceptibility to various diseases, the objective of this study was to evaluate possible alterations in homocysteine metabolism including cysteine, folate, and vitamin B_6, and oxidative stress markers in female rats exposed to different types of acute stress. Female rats were randomly distributed into eight groups according to stress manipulation (restraint, swimming, cold and control) and estrous cycle (diestrus and estrus). In general no significant differences were seen between rats in estrus and diestrus. Restraint stress was the only type of stress that altered homocysteine concentrations (+33% relative to controls). An increase in levels of thiobarbituric acid reactive substances (TBARS) and a decrease in total glutathione (GSHt) concentration were also observed in animals subjected to restraint and swimming stress, suggesting the possibility of oxidative damage. Thus, both the homocysteine results and the oxidative stress data indicated that restraint stress was the most powerful stress manipulation in female rats, as previously observed in male rats.

These findings indicate that hormonal and gonadal differences do not interfere with stress responses related to homocysteine metabolism and suggest that putative gender-related differences in homocysteine responses are probably not involved in the differential prevalence of some diseases in human males and females.

Keywords: Homocysteine metabolism; Acute stress; Psychological stress; Oxidative stress; Female rats

1. Introduction

Stress has been associated with several pathological conditions. In particular, human and animal studies have provided findings on mechanisms by which stress interferes with immune (Yudkin et al., 2000), neuroendocrine (Muller et al., 2001; Black and Garbutt, 2002) and metabolic changes (de Oliveira et al., 2004; Setnik et al., 2004) that may increase cardiovascular risk (Blumenthal et al., 1995; Manuck et al., 1995) and psychiatric illness (Agid et al., 2000).

Homocysteine is a sulphur-containing amino acid that is an intermediary in the metabolism of methionine–cysteine (Finkelstein and Martin, 2000). This metabolic pathway is important due to the production of *S*-adenosylmethionine (SAM), the main donor of methyl groups for methylation reactions in the organism (Chiang et al., 1996). Homocysteine can be converted into methionine via a remethylation pathway that requires folate and vitamin B_{12}, and/or into cysteine via a transsulfuration pathway that requires vitamin B_6. Homocysteine can also be exported to the extracellular environment (Selhub, 1999).

☆ This work was supported by the grants from FAPESP (97/01870-4), Brazil and Associação Fundo de Incentivo à Psicofarmacologia (AFIP). F.G. Souza was recipient of fellowship from Capes; M.D.B. Rodrigues, V. D'Almeida and S. Tufik are recipients of fellowships from CNPq (Brazil).
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doi:10.1016/j.pbb.2006.09.008

Several studies have shown that levels of homocysteine are affected by diet factors such as vitamin deficiencies, by non-diet factors such as stress and genetic background, and by several pathological conditions (Minner et al., 1997; Ueland et al., 2001). Increases in total homocysteine plasma concentrations are recognized as an independent risk factor for cardiovascular disease (Nygård et al., 1999).

High homocysteine levels are also related to cellular damage caused by oxidative stress. Studies have demonstrated that formation of reactive oxygen species (ROS) can be induced by high levels of this amino acid (Blom et al., 1992; Dudman et al., 1993; Blom, 2000; Perna et al., 2003). Recently various stressors have been associated with enhanced free radical generation causing oxidative stress (Sies, 1997). The first event and one of the most important consequences of free radical production is the membrane lipoperoxidation. Moreover, stress has been suggested to decrease the levels of glutathione (GSH) and vitamin C and, both substances that play an important role in tissue protection from oxidative damage (Liu et al., 1994; Levi and Basuaj, 2000). In the present study we evaluated thiobarbituric acid reactive substances (TBARS) levels as a consequence of different stressors which could be indicative of oxidative stress.

Both oxidative stress and increased homocysteine levels have been implicated in neurodegenerative illnesses such as Alzheimer's and Parkinson's diseases, and are also associated with aging, depression and schizophrenia (Ben-Shachar et al., 1991; Richardson, 1993; Ames et al., 1993; Bottiglieri, 2005; Levine et al., 2005).

Since regulation of homocysteine levels is dependent on hormones and immunological factors, homocysteine concentrations can be altered by stress. Yet few studies have attempted to correlate stress and homocysteine levels. Our group has previously reported that when male Wistar rats were subjected to various acute stressors, only restraint stress resulted in increased homocysteine concentrations (de Oliveira et al., 2004). While several important studies have shown that sex differences have a large influence on stress responses using behavioral paradigms (Bowman et al., 2001; Conrad et al., 2004), the role of sex differences in homocysteine concentration using classical stressors has not been examined.

Studies in humans have demonstrated a hormonal effect on homocysteine metabolism in response to stress (Stoney, 1999; Farag et al., 2003). In particular, the neuroprotective effects of estrogen, *in vivo* and *in vitro*, have been well documented (Behl, 2002) and include antioxidant effects (Behl et al., 1995), beneficial effects on coronary heart disease with changes in lipid profile, direct effects on the arterial wall, and a decrease in stress reactivity (Fredrikson and Matthews, 1990; Moerman et al., 1996). Furthermore, the prevalence of some diseases related to homocysteine metabolism is significantly different in men and women (e.g. depression, stroke, coronary artery disease), and stress plays a critical role in most of them (Kessler et al., 1993; Nygård et al., 1999).

All stressors activate the HPA axis and the sympatho-adrenomedullary system, the degree of activation depends on stress duration, type and intensity. In the present study, three distinct stressful manipulations were employed, namely restraint stress (possibly the strongest stress by combining physical and emotional stress), swimming (a stressor that interferes with cardiovascular and metabolic homeostasis), and cold exposure (a moderate physical/metabolic stressor). Our first objective therefore was to verify whether these different types of acute stress would have effects on homocysteine metabolism and oxidative stress parameters in female rats. In addition, two stages of the estrous cycle were studied. The estrus and the diestrus phases were chosen because they are associated with differences in estrogen and progesterone levels, as well as differences in sexual behavior rats. While higher estradiol levels occur in the proestrus phase, proestrus has a very short duration and usually happens at the end of the day. Since previous work from our group demonstrated a circadian variation in homocysteine levels (Martins et al., 2005), we opted to avoid collection of samples at different times of day.

2. Methods

2.1. Animals

Eighty 3-month-old female Wistar rats from the animal facility of the Department of Psychobiology, Universidade Federal de São Paulo were used (initial $n=100$). These animals derived from the Charles River Laboratories (Wilmington, MA, USA) foundation colony. The animals were housed in groups of 5 in a colony maintained at 22 °C with a 12:12 h light–dark cycle (lights on at 0700 h) and were allowed free access to food and water. They were randomly divided into eight groups according to stressor (restraint, swimming, cold and control) and estrous cycle (diestrus and estrus). Animal care and use procedures were carried out by trained personnel (FELASA Category C) and conducted in accordance with the Ethical and Practical Principles of the Use of Laboratory Animals (Andersen et al., 2004a,b). The experimental protocol was approved by the Ethical Committee of UNIFESP (CEP no. 1272/03).

2.2. Estrous phase determination

Estrous phase was determined by microscope examination of daily vaginal smears for at least 15 days (three complete estrous cycles) according to well established cytological criteria (Maeda et al., 2000). Only females showing three consistent estrous cycles were used in the study ($n=80$). All female rats were again smeared 1 h before application of stress procedures to confirm estrous phase status.

2.3. Experimental procedures

Each stress session, except for swimming, lasted 1 h; beginning approximately at 2:00 p.m.

2.4. Control group

Animals were maintained in their original home cages in groups of five rats.

2.5. Restraint

Animals were individually placed in plastic cylinders (21 cm in length × 6 cm in diameter). Both ends of the cylinders were closed with ventilated sliding doors.

2.6. Swimming

Animals were placed in a container (60 cm in height × 30 cm in diameter) filled with water to a height of 50 cm. Water temperature was approximately 20 °C. The duration of swim stress was no longer than 40 min. Animals that showed signs of exhaustion before 40 min were removed from the container. Following the trial, rats were dried with a towel and returned to their home cages.

2.7. Cold

Animals were individually placed in a cold chamber at 4 °C inside wire-mesh cages (30 cm in length × 18 cm in width × 25 cm in height).

2.8. Biochemical measurements

After stress procedures animals were returned to their home cages for appropriate periods of time in order to ensure a constant time interval between the beginning of stress and sacrifice. Rats were sacrificed by decapitation and blood aliquots were collected in tubes containing either heparin, ethylenediaminetetraacetic acid (EDTA) or no anticoagulant (Becton Dickinson, New England, UK). Samples were centrifuged at 4 °C for 10 min at 3000 rpm to obtain plasma and serum, respectively. Homocysteine and cysteine values were determined in plasma (EDTA) by high performance liquid chromatography (HPLC; Shimadzu, Kyoto, Japan) with fluorimetric detection and isocratic elution (Pfeiffer et al., 1999). Total homocysteine and cysteine content were calculated with calibration curves using known concentrations of these amino acids using cystamine as the internal standard. Results were expressed in μmol/L. Folate and vitamin B_6 were quantified in serum using HPLC with ultraviolet detection (UV) and isocratic elution, as described by Sharma and Dakshinamurti (1992). Pyridoxal-5′-phosphate and folic acid were used as internal standards and the wavelength of the UV detector was set at 290 nm. Folate and vitamin B_6 results were expressed in nmol/L. Corticosterone concentrations were assayed by a double antibody RIA method specific for rats and mice, using a commercial kit (ICN-Biomedical, Orangeburg, NY, USA) and results were expressed in ng/mL. Oxidative stress parameters were analyzed in plasma by levels of thiobarbituric acid reactive substances (TBARS), a product of lipid peroxidation, using a colorimetric assay ($\lambda = 535$ nm) (Ohkawa et al., 1979). The results were expressed as nmol of malondyaldehyde (MDA) per milliliter (nmol/mL). Total glutathione (GSHt) assays were carried out using a method described by Tietze (1969). Erythrocyte GSHt levels were obtained spectrophotometrically at 412 nm and the results expressed as μmol/g Hb.

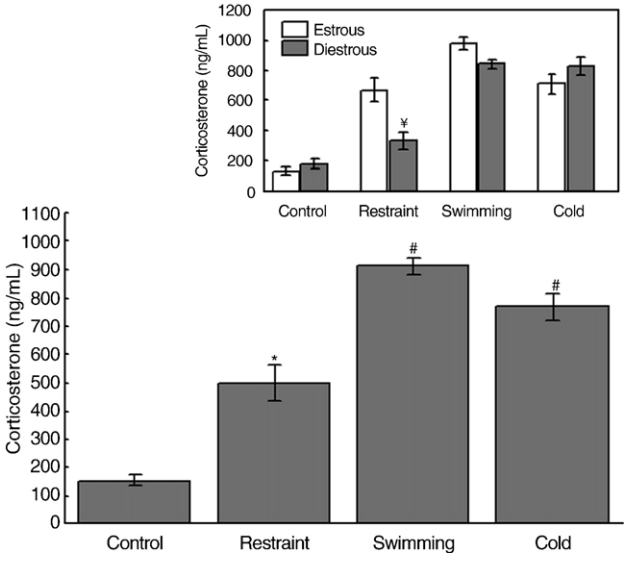

Fig. 1. Corticosterone concentrations in female Wistar rats subjected to various stressors. The inset shows data for estrus and diestrus groups separately ($n = 10$ per group) and the main panel shows combined estrus and diestrus data ($N = 20$ per group). Values are means ± S.E.M. ¥ Different from same stressor group in estrus ($p < 0.05$). * Different from control group; # Different from control and restraint groups ($p < 0.05$).

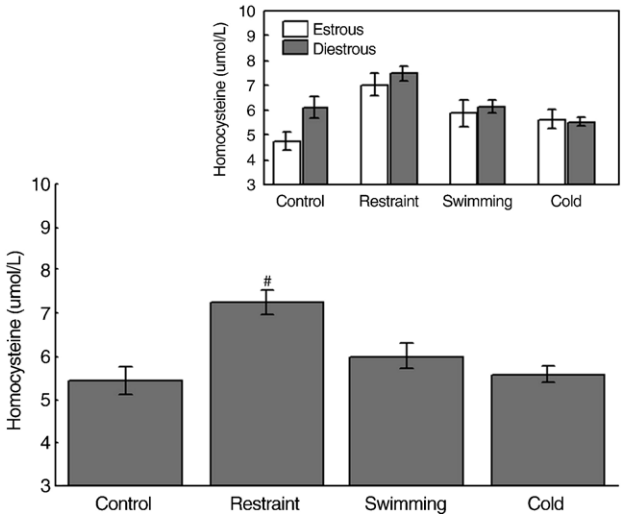

Fig. 2. Homocysteine concentrations in female rats subjected to different stressors. The inset shows data for estrus and diestrus groups separately ($n = 10$ per group) and the main panel shows combined estrus and diestrus data ($N = 20$ per group). Values are means ± S.E.M. # Different from control and other stress groups ($p < 0.05$).

2.9. Statistical analysis

The data are presented as mean ± S.E.M. Each variable was first analyzed using two-way analysis of variance (ANOVA) using Stress Type and Cycle Phase as factors. Where these analyses did not reveal a significant main effect of Cycle or a significant Stress × Cycle interaction, data for estrus and diestrus rats were pooled and subjected to a one-way ANOVA using

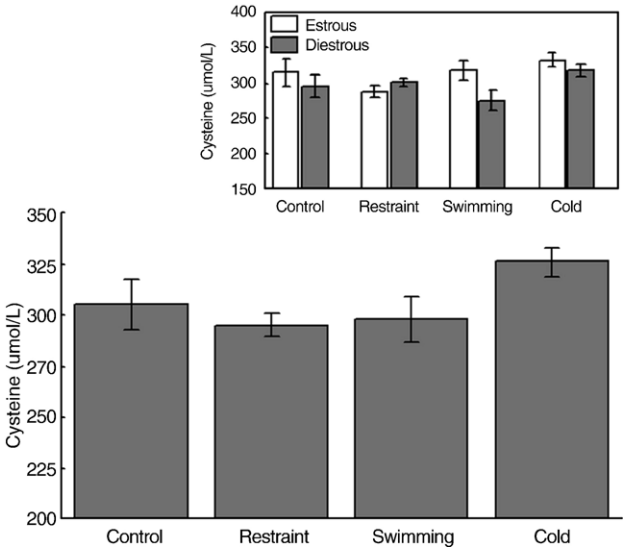

Fig. 3. Cysteine concentrations in female rats subjected to different stressors. The inset shows data for estrus and diestrus groups separately ($n=10$ per group) and the main panel shows combined estrus and diestrus data ($N=20$ per group). Values are means±S.E.M.

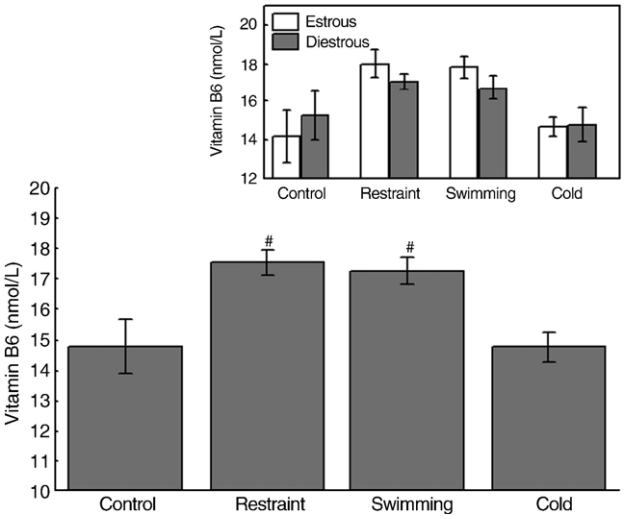

Fig. 5. Vitamin B_6 concentrations in female rats subjected to different stressors. The inset shows data for estrus and diestrus groups separately ($n=10$ per group) and the main panel shows combined estrus and diestrus data ($N=20$ per group). Values are means±S.E.M. # Different from control and cold stress groups ($p<0.05$).

Stress type as a factor, followed, when appropriate, by post-hoc Tukey tests to identify differences among specific stressors. An alpha level of 0.05 was used as the criteria for statistical significance.

3. Results

Plasma corticosterone concentrations are shown in Fig. 1. A two-way ANOVA indicated a significant main effect of Cycle Phase [$F(1,72)=4.35$; $p=0.041$], a significant main effect of Stress manipulation [$F(3,72)=2.32$; $p=0.0001$], and a significant interaction between Cycle Phase and Stress factors [F $(3,72)=7.67$; $p=0.0002$]. Rats subjected to restraint stress showed the lowest increases in corticosterone when compared to other stress groups. Animals in diestrus subjected to restraint stress showed significantly smaller increases in corticosterone levels compared to estrus rats subjected to this stressor ($p<0.01$; Fig. 1 inset). Overall, the highest concentrations were found in animals exposed to swimming and cold stress ($p<0.0001$), followed by those subjected to restraint stress ($p<0.0001$) when compared to the control group.

Fig. 2 shows the effect of stress manipulations on homocysteine concentrations. Since a preliminary two-way

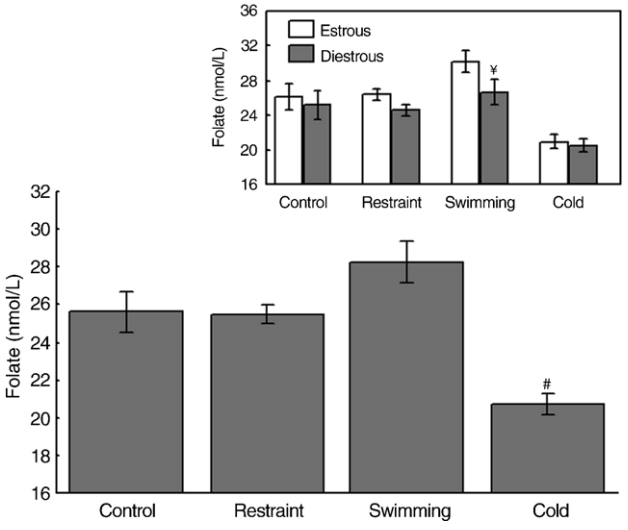

Fig. 4. Folate concentrations in female rats subjected to different stressors. The inset shows data for estrus and diestrus groups separately ($n=10$ per group) and the main panel shows combined estrus and diestrus data ($N=20$ per group). Values are means±S.E.M. ¥ Different from same stressor group in estrus ($p<0.05$). # Different from control and other stressor groups ($p<0.05$).

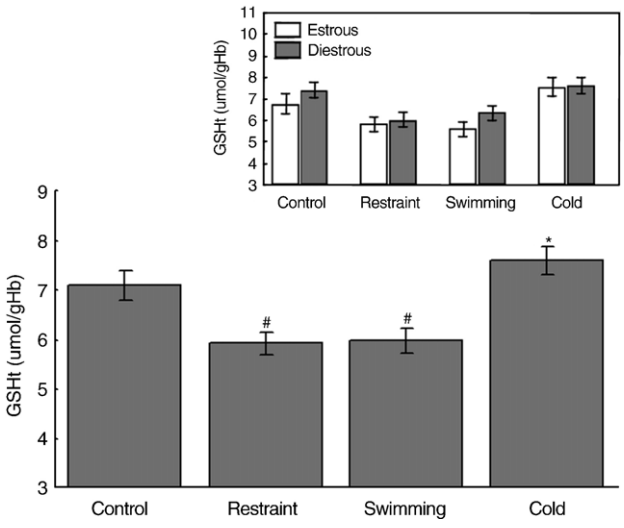

Fig. 6. Total glutathione concentrations in female rats subjected to different stressors. The inset shows data for estrus and diestrus groups separately ($n=10$ per group) and the main panel shows combined estrus and diestrus data ($N=20$ per group). Values are means±S.E.M. * Different from control group; # Different from control and cold stress groups ($p<0.05$).

ANOVA indicated no main effect of Cycle Phase and no Cycle Phase × Stress interaction, estrus and diestrus groups were pooled and subjected to a one-way ANOVA, which confirmed a strong effect of Stress type ([$F(3,76)=9.00$; $p=0.00004$]. Post hoc tests revealed that restraint stress had a significant effect on homocysteine concentrations when compared to control and other stressors groups ($p<0.001$ and $p<0.005$, respectively).

Cysteine concentrations did not show statistical differences among groups (Fig. 3). No significant effects were seen in the initial two-way ANOVA, and when Cycle Phase data were pooled a one-way ANOVA did not reveal a significant effect of Stress type [$F(3,71)=2.18$; $p=0.097$]).

Results for folate are presented in Fig. 4. A two-way ANOVA revealed a significant main effect of Cycle Phase [F (1,69)=15.06; $p=0.045$] but no Cycle Phase × Stress interaction [$F(3,69)=0.65$; $p=0.58$]. Rats subjected to swimming stress in the estrus phase had higher folate levels than rats exposed to this stressor during the diestrus phase (Fig. 4, inset, $p<0.05$). When estrus and diestrus groups were pooled, a one-way ANOVA confirmed a significant Stress effect [$F(3,73)=14.135$; $p=0.0001$]. The group subjected to cold stress had the lowest folate levels when compared to control and other stress groups ($p<0.001$).

For vitamin B_6 the two-way ANOVA revealed no Cycle Phase effect and no Cycle Phase × Stress interaction (Fig. 5 inset). When Cycle Phase data were pooled, a one-way ANOVA confirmed a significant difference among stressors [$F(3,70)=6.2167$; $p=0.008$] and post-hot comparisons indicated that groups subjected to restraint and swimming had significantly higher B_6 levels when compared to control and cold groups ($p<0.05$).

Fig. 6 shows the effects of stress manipulations on the antioxidant glutathione. Since no main effect of Cycle Phase and no Cycle Phase × Stress interaction were seen (Fig. 6 inset), the Cycle Phase data were pooled. A one-way ANOVA confirmed a significant Stress effect [$F(3,71)=9.2025$; $p=0.00003$] and pairwise group comparisons indicated that rats subjected to restraint and swimming stress had lower concentrations of GSHt when compared to control and cold stress groups ($p<0.05$). Rats subjected to cold stress had the highest GSHt concentrations of all groups ($p<0.05$).

For TBARS plasma concentration, an index of lipid peroxidation, the preliminary two-way ANOVA did not reveal an Cycle Phase main effect or a significant Cycle Phase × Stress interaction (Fig. 7 inset). When Cycle Phase data were pooled a one-way ANOVA confirmed a very significant Stress effect [F (3,72)=24.838; $p=0.00001$] and pairwise comparisons revealed that animals subjected to restraint and swimming had significantly higher TBARS concentrations in relation to control and cold stress groups ($p<0.05$). Rats in the cold stress group showed the lowest TBARS concentrations ($p<0.05$).

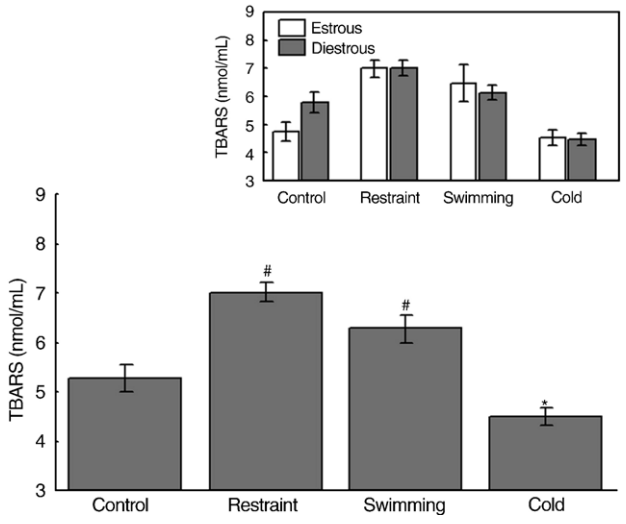

Fig. 7. TBARS concentrations in female rats subjected to different stressors. The inset shows data for estrus and diestrus groups separately ($n=10$ per group) and the main panel shows combined estrus and diestrus data ($N=20$ per group). Values are means±S.E.M. * Different from control group; # Different from control and cold stress groups ($p<0.05$).

4. Discussion

As expected, all of the stress manipulations produced an activation of the hypothalamic–pituitary–thyroid (HPA) axis, as indicated by increased corticosterone levels in all cases. Among stressors, restraint resulted in a significant alteration in homocysteine plasma concentration (+33%, $p=0.0001$). These results are in agreement with previous observations by our group using male Wistar rats, in which restraint resulted in a 37% increase in plasma homocysteine concentrations (de Oliveira et al., 2004). We can conclude, therefore, that alterations observed in plasma homocysteine concentrations induced by specific stress manipulations do not depend on gender or estrous cycle phase since they occurred in the same direction in all situations analyzed.

Restraint is a model that simulates physical and psychological stress in rats, producing an activation effect on various systems including those involved in regulation of body temperature and pain sensitivity (Pacák and Palkovits, 2001). Homocysteine concentration is regulated by a complex metabolic pathway which involves hormones, oxide-reduction reactions and, possibly, immunological mediators, as well as specific nutrients (mainly folate, vitamins B_6 and B_{12}) which act directly on homocysteine metabolism (Tobeña et al., 1996; House et al., 1999; Stead et al., 2000). Part of this regulatory system is activated by stress. Homocysteine metabolism can be altered by specific stress manipulations such as, for example, sleep deprivation. Interestingly, sleep deprivation induces hypohomocysteinemia (de Oliveira et al., 2002; Andersen et al., 2004a,b), whereas in the present study restraint stress increased homocysteine concentrations. Increases or decreases in homocysteine concentrations in response to stress do not seem to be directly related to corticosterone levels, since we did not observe a correlation between homocysteine and corticosterone in the present study. In a previous study we analyzed various biochemical indices of cardiovascular risk in rats after sleep deprivation stress. We found that although some of those indices did increase after sleep deprivation, the increases did not correlate with homocysteine levels (Andersen et al., 2004a,b). These differences suggest that the regulation of homocysteine concentrations can be modulated by different systems activated

during the response to stress. These may also relate to the duration and latency of the physiological response, or to the subsequent activation of other regulatory factors (de Oliveira et al., 2004).

Our results suggest that different stressors vary in their ability to activate the main regulatory systems of the stress response, such as the sympathetic nervous system and the HPA axis. Increases in hormonal concentrations, including increases in prolactin, ACTH, growth hormone, as well as increases in norepinephrine and epinephrine levels are known to be induced by stress (Pacák and Palkovits, 2001). Our interest was to determine possible relationships between these systems and homocysteine levels.

Although there are few studies in the literature showing endocrine effects on homocysteine metabolism, hormonal alterations do seem to participate in homocysteine regulation. It has been demonstrated that glucagon decreases homocysteine concentrations by reducing the export of homocysteine by cells and by increasing the activity of enzymes responsible for the transsulfuration pathway, namely, cystathionine beta-synthase (CBS) and cystathionine lyase (CL) (House et al., 1999; Jacobs et al., 2001). Insulin seems to exert an opposite effect, as it increases homocysteine concentration by decreasing the activity of these same enzymes, as well as by acting on the remethylation pathway modifying the activity of the enzyme methylenetetrahydrofolate reductase (House et al., 1999; Dicker-Brown et al., 2001). In this context it is interesting to note that the same effect observed here with restraint stress had been previously observed in humans. Women subjected to psychological stress (e.g. mentally performing arithmetic calculations) showed a significant increase of 7% in homocysteine plasma concentration (Stoney, 1999).

In general, under physiological conditions alterations in folate and B_6 concentrations, two main cofactors in homocysteine regulation, are explained by differences in the intake of these vitamins, since both folate and vitamin B_6 are obtained mainly through the diet. It is not known, however, if situations of acute stress can result in significant changes in the plasma concentration of these vitamins. Here we have observed a reduction in folate concentrations in animals subjected to cold stress, and this did not result in a homocysteine increase independent of estrous phase. Although some studies have suggested an estrogenic influence on the availability of folate (O'Connor et al., 1997), the differences observed in this study could not be related to estrogen variations associated to estrous phases. In line with this view, homocysteine increases observed after stress manipulations could not be explained by variations in folate and B_6 levels.

Several studies have demonstrated that estrogen has a protective effect that could prevent high homocysteine plasma levels (Dimitrova et al., 2002a,b; Collins et al., 2002). Comparing homocysteine values obtained in various female groups (controls in estrus=5.4 mM; controls in diestrus=6.1 mM; restraint in estrus=7.0 mM; restraint in diestrus=7.5 Mm) to data that we previously reported for males (5.5 mM and 8.0 mM for control and restraint groups, respectively) (de Oliveira et al., 2004), we can suggest a possible action of sex hormones on homocysteine regulation, since females showed a lower increase compared to males, independent of estrous phase. It has been well described in the literature that gender is a determining factor in homocysteine concentrations in humans, the male sex being associated with higher homocysteine levels, and having a higher risk of cardiovascular disease (Selhub, 1999). In Wistar rats, basal homocysteine values in females and males are similar (Martins et al., 2005), but changes in response to restraint stress were more pronounced in males, which showed a 37% increase in the homocysteine levels (de Oliveira et al., 2004). On the other hand, in Sprague–Dawley rats the same pattern observed in humans was found (Setnik et al., 2004).

el-Swefy and co-workers (2002) demonstrated that ovariectomized rats (OVX) have high homocysteine and TBARS concentrations (6.1 μmol/L and 141 nmol/L, respectively) compared to intact controls. When OVX animals were subjected to hormonal replacement therapy with estradiol the values of these two parameters returned to normal control values (2.5 μmol/L and 93 nmol/L for homocysteine and TBARS, respectively), thus demonstrating a protective effect of estrogen against high homocysteine and TBARS concentrations.

Our data demonstrate an increase in plasma TBARS concentration (a product of lipid peroxidation) in groups subjected to restraint and swim stress in the estrus phase, as well as in the group subjected to restraint in the diestrus phase. Interestingly, the restraint group in both estrus and diestrus phases also showed the highest alterations in homocysteine levels. Some studies have suggested a pro-oxidant effect of homocysteine, whereby high homocysteine concentrations could induce oxidative damage (Olszewski and McCully, 1993; Loscalzo, 1996; Hultberg et al., 1997; Welch et al., 1997). Animals subjected to restraint and swimming in the diestrus phase presented a decrease in GSHt concentrations and an increase in TBARS concentrations, which, in turn could suggest a state of oxidative stress. The cell response to stress often involves changes in glutathione content, as glutathione may be initially consumed in reactions that protect the cell by removing deleterious compounds, and may subsequently be restored to levels that exceed those found before exposure to the stressor (Ghizoni et al., 2006). Acute stress induced by sleep deprivation has been shown to increase levels of lipid peroxidation indicators and to produce a decrease in antioxidant enzymatic activities and in glutathione levels (D'Almeida et al., 1998, 2000; Ramanathan et al., 2002; Silva et al., 2004).

Considering the results for the oxidative stress parameters together with the observed increases in homocysteine concentrations, our results indicate that restraint stress, particularly in the diestrus phase, is the most potent stress manipulation. It will be of interest to follow homocysteine variations for longer periods of restraint stress, since data from the literature suggest that, similar to acute restraint stress, prolonged periods of restraint induce increases in TBARS in rat plasma and hippocampus (Liu et al., 1994; Fontella et al., 2005). This suggests that formation of reactive oxidizing species may be one of the major consequences of restraint stress.

Finally, the absence of estrous cycle phase effects supports the conclusion that hormonal and gonadal differences do not interfere with stress responses related to homocysteine metabolism and

that, in turn, putative gender-related differences in homocysteine responses probably do not account for differences in the prevalence of some diseases in human males and females.

References

Agid O, Kohn Y, Lerer B. Environmental stress and psychiatric illness. Biomed Pharmacother 2000;54:135–41.

Ames B, Shigenaga MK, Hagen TM. Oxidants, antioxidants, and degenerative disease of aging. Proc Natl Acad Sci U S A 1993;90:7915–22.

Andersen ML, D'Almeida V, Ko GM, Kawakami R, Martins PJF, Magalhães LE, Mázaro R. Ethical and Practical Principles of the Use of Laboratory Animals. Universidade Federal de São Paulo (Ed): São Paulo, 2004.

Andersen ML, Martins PJ, D'Almeida V, Santos RF, Bignotto M, Tufik S. Effects of paradoxical sleep deprivation on blood parameters associated with cardiovascular risk in aged rats. Exp Gerontol 2004b;39:817–24.

Behl C. Oestrogen as a neuroprotective hormone. Nat Rev Neurosci 2002;3:433–42.

Behl C, Widmann M, Trapp T, Holsboer F. 17-Beta estradiol protects neurons from oxidative stress-induced cell death in vitro. Biochem Biophys Res 1995;216:473–82.

Ben-Shachar D, Riederer P, Youdim MB. Iron–melanin interaction and lipid peroxidation: implication for Parkinson's disease. J Neurochem 1991;57:1609–14.

Black PH, Garbutt LD. Stress, inflammation and cardiovascular disease. J Psychosom Med 2002;52: 1–23.

Blom HJ. Consequences of homocysteine export and oxidation in vascular system. Semin Thromb Hemost 2000;26:227–32.

Blom HJ, Engelen DP, Boers GH, Stadhouders AM, Sengers RC, de Abreu R, et al. Lipid peroxidation in homocysteinemia. J Inherit Metab Dis 1992;15:419–22.

Blumenthal JA, Jiang W, Waugh RA, Frid DJ, Morris JJ, Coleman RE, et al. Mental stress-induced ischemia in the laboratory and ambulatory ischemia during daily life. Association and hemodynamic features. Circulation 1995;92:2102–8.

Bottiglieri T. Homocysteine and folate metabolism in depression. Prog Neuropsychopharmacol Biol Psychiatr 2005;29:1103–12.

Bowman RE, Zrull MC, Luine VN. Chronic restraint stress enhances radial arm maze performance in female rats. Brain Res 2001;904:279–89.

Chiang PK, Gordon RK, Tal J, Zeng GC, Doctos BP, Pardhasaradhi K, et al. *S*-adenosylmethionine and methylation. FASEB J 1996;10:471–80.

Collins P, Stevenson JC, Mosca L. Spotlight on gender. Cardiovasc Res 2002;53:535–7.

Conrad CD, Jackson JL, Wieczorek L, Baran SE, Harman JS, Wright RL, et al. Acute stress impairs spatial memory in male but not female rats: influence of estrous cycle. Pharmacol Biochem Behav 2004;78:569–79.

D'Almeida V, Lobo LL, Hipolide DC, de Oliveira AC, Nobrega JN, Tufik S. Sleep deprivation induces brain region-specific decreases in glutathione levels. Neuroreport 1998;9:2853–6.

D'Almeida V, Hipolide DC, Lobo LL, de Oliveira AC, Nobrega JN, Tufik S. Melatonin treatment does not prevents decreases in brain glutathione levels induced by sleep deprivation. Eur J Pharmacol 2000;390:299–302.

de Oliveira AC, D'Almeida V, Hipolide DC, Nobrega JN, Tufik S. Sleep deprivation reduces total plasma homocysteine levels in rats. Can J Physiol Pharm 2002;80:193–7.

de Oliveira AC, Suchecki D, Cohen S, D'Almeida V. Acute stressor-selective effect on total plasma homocysteine concentration in rats. Pharmacol Biochem Behav 2004;77:269–73.

Dicker-Brown A, Fonseca VA, Fink LM, Kern PA. The effect of glucose and insulin on the activity of methylene tetrahydrofolate reductase and cystathionine-beta-synthase: studies in hepatocytes. Atherosclersosis 2001;158:297–301.

Dimitrova KR, DeGroot KW, Myers AK, Kim YD. Estrogen and homocysteine. Cardiovasc Res 2002a;53:577–88.

Dimitrova KR, DeGroot KW, Pacquing AM, Suyderhoud JP, Pirovic EA, Munro TJ, et al. Estradiol prevents homocysteine-induced endothelial injury in male rats. Cardiovasc Res 2002b;53:589–96.

Dudman NP, Wilcken DE, Stocker R. Circulating lipid hydroperoxide levels in human hyperhomocysteinemia: Relevance to development of arteriosclerosis. Arterioscler Thromb 1993;13:512–6.

el-Swefy SE, Ali SI, Asker ME, Mohamed HE. Hyperhomocysteinemia and cardiovascular risk in female ovariectomized rats: role of folic acid and hormone replacement therapy. J Pharm Pharmacol 2002;54:391–7.

Farag NH, Barshop BA, Milss PJ. Effects of estrogen and psychological stress on plasma homocysteine levels. Fertil Steril 2003;79:256–60.

Finkelstein JD, Martin JJ. Homocysteine. Int J Biochem Cell Biol 2000;32: 385–9.

Fontella FU, Siqueira IR, Vasconcellos AP, Tabajara AS, Netto CA, Dalmaz C. Repeated restraint stress induces oxidative damage in rat hippocampus. Neurochem Res 2005;30:105–11.

Fredrikson M, Matthews KA. Cardiovascular responses to behavioral stress and hypertension: a meta-analytic review. Ann Behav Med 1990;12:30–9.

Ghizoni DM, Pavanati KCA, Arent AM, Machado C, Faria MS, Pinto CMH, et al. Alterations in glutathione levels of brain structures caused by acute restraint stress and by nitric oxide synthase inhibition but not by intraspecific agonistic interaction. Behav Brain Res 2006;166:71–7.

House JD, Jacobs RL, Stead LM, Brosnan ME, Brosnan JT. Regulation of homocysteine metabolism. Adv Enzyme Regul 1999;39:69–91.

Hultberg B, Andersson A, Isaksson A. The effects of homocysteine and copper ions on the concentration and redox status of thiols in cell line cultures. Clin Chim Acta 1997;262:39–51.

Jacobs RL, Stead LM, Brosnan JT. Hyperglucagonemia in rats results in decreased plasma homocysteine and increased flux through the transsulfuration pathway in liver. J Biol Chem 2001;276:43740–7.

Kessler RC, Megonagle KA, Swartz M, Blazer DG, Nelson CB. Sex and depression in the National Comorbidity Survey i: lifetime prevalence, chronicity and recurrence. J Affect Disord 1993;29:85–96.

Levi L, Basuaj E. In an introduction clinical and neuroendocrinology, Karger, Basel, 1 (2000) New York, 78.

Levine J, Sela BA, Yamina O, Belmaker RH. High homocysteine serum levels in young male schizophrenia and bipolar patients and in an animal model. Prog Neuropsychopharmacol Biol Psychiatr 2005;29:11181–91.

Liu J, Xiaoyan W, Mori A. Immobilization stress-induced antioxidant defense changes in rat plasma: effect of treatment with reduced glutathione. Int J Biochem 1994;26:511–7.

Loscalzo J. The oxidant stress of hyperhomocysteinemia. J Clin Invest 1996;98:5–7.

Maeda K, Okhura S, Tsukamura H. Physiology of reproduction: estrous cycle, identification of the estrous cycle. In: Krindle GJ, Bullock G, Bunton TE, editors. The handbook of experimental animals: the laboratory rat. San Diego: Academic Press; 2000. p. 152–5.

Manuck SB, Marsland AL, Kaplan JR, Williams JK. The pathogenicity of behavior and its neuroendocrine mediation: an example from coronary artery disease. Psychosom Med 1995;57:275–83.

Martins PJF, Galdieri LC, Souza FG, Andersen ML, Benedito-Silva AA, Tufik S, et al. Physiological variation in plasma total homocysteine concentrations in rats. Life Sci 2005;76:2621–9.

Minner SE, Evrovski J, Cole DEC. Clinical chemistry and molecular biology of homocysteine metabolism: an update. Clin Biochem 1997;30: 189–201.

Moerman CJ, Witteman JC, Collete HJ, Gevers Leuven JA, Kluft C, Kenemans P, et al. Hormone replacement therapy: a useful tool in the prevention of coronary artery disease in postmenopausal women? Working group on women and cardiovascular disease of the Netherlands Heart Foundation. Eur Heart J 1996;17:658–66.

Muller JR, Le KM, Haines WR, Gan Q, Knuepfer MM. Hemodynamic response pattern predicts susceptibility to stress-induced elevation in arterial pressure in the rat. Am J Physiol 2001;281:R31–7.

Nygård O, Vollset SE, Refsum H, Brattstrom L, Ueland PM. Total homocysteine and cardiovascular disease. J Intern Med 1999;246:425–54.

O'Connor D, Grenn T, Picciano M. Maternal folate status and lactation. J Mammary Gland Biol Neoplasia 1997;2:279–89.

Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979;95:351–8.

Olszewski AJ, McCully KS. Homocysteine metabolism and the oxidative modification of proteins and lipids. Free Radic Biol Med 1993;14:683–93.

Pacák K, Palkovits M. Stressor specificity of central neuroendocrine responses: implications for stress-related disorders. Endocr Rev 2001;22:502–48.

Perna AF, Ingrosso D, De Santo NG. Homocysteine and oxidative stress. Amino Acids 2003;25:409–17.

Pfeiffer CM, Huff DL, Gunter EW. Rapid and accurate HPLC assay for plasma total homocysteine and cysteine in a clinical laboratory setting. Clin Chem 1999;45:290–2.

Ramanathan L, Gulyani S, Nienhuis R, Siegel JM. Sleep deprivation decreases superoxide dismutase activity in rat hippocampus and brainstem. Neuroreport 2002;13:1387–90.

Richardson JS. Free radicals in the genesis of Alzheimer's disease. Ann NY Acad Sci 1993;695:73–6.

Selhub J. Homocysteine metabolism. Annu Rev Nutr 1999;19:217–46.

Setnik B, Souza FG, D'Almeida V, Nobrega JN. Increased homocysteine levels associated with gender and stress in the learned helplessness model of depression. Pharmacol Biochem Behav 2004;77:155–61.

Sharma SK, Dakshinamurti K. Determination of vitamin B6 vitamers and pyridoxic acid in biological samples. J Chromatogr 1992;578:45–51.

Sies H. Oxidative stress. New York: Academic Press; 1997. p. 1–8.

Silva RH, Abilio VC, Takatsu AL, Kameda SR, Grassl C, Chehin AB, et al. Role of hippocampal oxidative stress in memory deficits induced by sleep deprivation in mice. Neuropharmacology 2004;46:895–903.

Stead LM, Brosnan ME, Brosnan JT. Characterization of homocysteine metabolism in the rat liver. Biochem J 2000;350:685–92.

Stoney CM. Plasma homocysteine levels increase in women during psychological stress. Life Sci 1999;64:2359–65.

Tietze F. Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal Biochem 1969;27:502–22.

Tobeña R, Horikawa S, Calvo V, Alemany S. Interleukin-2 induces gamma- *S*-adenosyl-L-methionine synthetase gene expression during T-lymphocyte activation. Biochem J 1996;319:929–33.

Ueland PM, Hustad S, Schneede J, Refsum H, Vollset SE. Biological and clinical implications of the MTHFR C677T polymorphism. Trends Pharmacol Sci 2001;22:195–201.

Welch GN, Upchurch Jr GR, Loscalzo J. Homocysteine, oxidative stress, and vascular disease. Hos Pract (Off Ed) 1997;32:81–2.

Yudkin JS, Kumari M, Humphries SE, Mohamed-Ali V. Inflammation, obesity, stress and coronary heart disease: is interleukin-6 the link? Atherosclerosis 2000;148:209–14.