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Acute stressor-selective effect on total plasma homocysteine concentration in rats

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Abstract

Stress produces several physiological and behavioral alterations that increase cardiovascular morbidity and mortality. Nonetheless, there is a dearth of studies that have evaluated the effects of stress on total plasma homocysteine, an important amino acid associated with cardiovascular disease. We used four distinct acute stressors in rats, i.e., swimming, restrain, novelty and cold exposure, in order to examine whether any acute effect on total plasma homocysteine concentrations would occur. Plasma corticosterone and adrenocorticotropic hormone concentrations were also measured to demonstrate the ability of the chosen manipulations to activate the hypothalamic–pituitary–adrenal (HPA) axis. Three of the four stressors activated the HPA axis and only restrain affected total plasma homocysteine concentrations (+37%, P=.006) compared with the control group. The complexity of the physiological responses to stress, the peculiarities of stress responses and the intricate regulatory systems involved in homocysteine metabolism must be taken into account in order to clarify the increasing effect of restrain (mainly a psychological stressor) on total plasma homocysteine concentrations in rats and to evaluate its meaning in human pathology.

Keywords: Homocysteine metabolism; Acute stress; Psychological stress; Rats

1. Introduction

Epidemiological studies show that stress has an important impact on human health. Of particular interest are the stress-induced physiological and behavioral alterations that could influence, either directly or indirectly, the function of the cardiovascular system, and thus increase cardiovascular morbidity and mortality (Møller et al., 1996; Fauvel, 2002; Ramey, 2003). Both human and animal studies (Blumenthal et al., 1995; Giorgiades et al., 2000, Yudkin et al., 2000, Muller et al., 2001; Black and Garbutt, 2002) have provided findings on the mechanisms by which stress interferes with immune, neuroendocrine and metabolic changes that may increase cardiovascular risk, even in the absence of other major risk factors (Manuck et al., 1995).

Homocysteine is a sulfur-containing amino acid intermediate in methionine–cysteine metabolism (Finkelstein and Martin, 2000), as it represents a branching point at which it can be remethylated to methionine or converted to cysteine. Plasma homocysteine concentrations are dependent on complex metabolic regulation, including hormone, redox and possibly immune mediators, as well as on availability of nutrients (mainly folate, B6, and B12 vitamins) that serve as cofactors for metabolic reactions (Tobeña et al., 1996; House et al., 1999; Stead et al., 2000; Graham and O'Callaghan, 2002; Vitvitsky et al., 2003). Some of these regulatory systems are activated by stress and could mediate the changes in homocysteine purported to occur in such situations.

Increase of total homocysteine plasma concentrations (the sum of protein-bound, free and disulfide fractions) is now recognized as an independent risk factor for cardiovascular disease (Nygård et al., 1999). Although the strongest associations were demonstrated in case–control and cross-sectional studies, a recent meta-analysis of prospective studies (Hackam and Anand, 2003) also showed that in specific conditions, such as diabetes, chronic renal disease and systemic lupus erythematosus, hyperhomocysteinemia appears to be a predictive factor of mortality (Guilland et al., 2003).

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Very few studies have addressed the effects of stressful manipulations on total plasma homocysteine concentrations. To the best of our knowledge, there is only one study that reports that one session of psychological stress increases plasma homocysteine concentrations (Stoney, 1999), while another did not find any such changes in women (Farag et al., 2003).

In the present study four distinct stressful manipulations were employed. Rats were restrained (considered mainly as a psychological stressor), exposed to swimming (a stressor that interferes with cardiovascular and metabolic homeostasis), cold (physical/metabolic stressor) or to novelty (psychosocial stressor) in order to verify whether stress, in general or in particular, would have any acute effect on total plasma homocysteine concentrations. We also measured corticosterone (CORT) and adrenocorticotropic hormone (ACTH) concentrations as control measures to index the extent to which the animals were stressed.

2. Methods

2.1. Animals

Fifty male, 3-month-old Wistar rats from the animal facility of the Department of Psychobiology were used. They were distributed into five groups corresponding to the different stress manipulations (restrain, swimming, cold, novelty and control). Each stress session, except for swimming, lasted 1 h, beginning approximately at 2:00 p.m. Swimming sessions lasted 40 min because at this point, animals began to drown. Therefore, we established this as the maximum swimming time in order not to make the animals hopeless (an animal model of depression). Animals were sacrificed immediately after the end of each session. All procedures used in the present study complied with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

2.2. Experimental procedures

2.2.1. Control group

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

2.2.2. Restrain

Ten animals were individually placed inside plastic cylinders (21 cm in length × 6 cm in diameter). Both ends of the cylinders were closed with ventilation sliding doors.

2.2.3. Swimming

Animals were placed in a container filled with water at a height of 50 cm. Tap water was not heated, being approximately 20 °C.

2.2.4. Cold

Ten animals were individually placed in a cold chamber at 4 °C inside a wire-mesh cage.

2.2.5. Novelty

Animals were individually placed in a cage filled with used sawdust taken from another cage.

Immediately after the end of each stress session, animals were brought into an adjacent room and decapitated. Trunk blood was collected in precooled plastic vials, containing 0.2 ml of an EDTA solution (60 mg/ml) and centrifuged at 900 × *g* for 20 min at 4 °C. Plasma was extracted, transferred to plastic tubes and stored at − 80 °C until biochemical assays were performed.

2.3. Assay procedures

2.3.1. Homocysteine determination

Total plasma homocysteine values were determined by high-performance liquid chromatography (HPLC) with fluorimetric detection and isocratic elution (Pfeiffer et al., 1999). This methodology involves three steps, namely, reduction of thiol groups using tris(carboxyethyl)phosphine, protein precipitation and derivatization with 7-fluorobenzene-2-oxy-1,3-diazolic-4-ammonium sulfate–SBD-F. The HPLC system used was a Shimadzu apparatus with a SIL-10Dvp automatic sample injector and an RF-10AXL fluorescence detector. Chromatographic separation was performed using a C18 model Shim-pack CLC-ODS column (4.6 × 150 mm, with 5.0-mm microparticles). The fluorescence of the separated compounds was detected with a detector adjusted for excitation at 385 nm and emission at 515 nm. Total homocysteine content was calculated with a calibration curve using known homocysteine concentrations and cystamine as the internal standard. The intra-assay coefficients of variation (CVs) for homocysteine ranged from 1.1% to 1.8% and the interassay CV was 5.6%.

2.3.2. ACTH and corticosterone determination

The ACTH assay was performed by a sequential chemiluminescence immunometric method (DPC Immulite ACTH, Los Angeles, CA), using a monoclonal murine antibody specific for ACTH. The sensitivity of the assay is 9 pg/ml, and intra- and interassay variations are 9.6% and 9.4%, respectively. Corticosterone concentrations were assayed by a double antibody RIA method, specific for rats and mice, using a commercial kit (ICN Biomedical, Costa Mesa, CA). The sensitivity of the assay is 0.25 μg/dl, and intra- and interassay variations are 10.3% and 7.1%, respectively.

2.4. Statistical analysis

The results of each variable were compared using a one-way ANOVA followed, when ANOVA detected a significant difference among groups, by the Newman–Keuls test

for pairwise comparisons. The level of significance was established at $P \leq .05$.

3. Results

Plasma ACTH concentrations (Fig. 1, upper panel): ANOVA revealed a statistically significant difference among groups [$F(4,39) = 27.2$; $P < .00001$], the highest ACTH concentrations having been observed in animals subjected to swimming ($P < .0002$).

Plasma corticosterone concentrations (Fig. 1, middle panel): A significant difference among groups was revealed [$F(4,41) = 32.86$; $P < .00001$]. The highest concentrations were found in animals exposed to swimming ($P < .0002$), followed by those submitted to restrain and cold stress, which were equally higher than novelty-exposed and control animals ($P < .001$).

Plasma homocysteine concentrations (Fig. 1, lower panel): Total homocysteine plasma concentrations were significantly altered by stress [$F(4,43) = 3.70$, $P = .01$], with the highest concentrations found in restrained rats (7.31 μM), as compared to cold-exposed (5.73 μM, $P = .03$) and control (5.30 μM, $P = .006$) groups. Swimming and novelty-exposed groups presented intermediate values, which were not statistically different from the remaining groups.

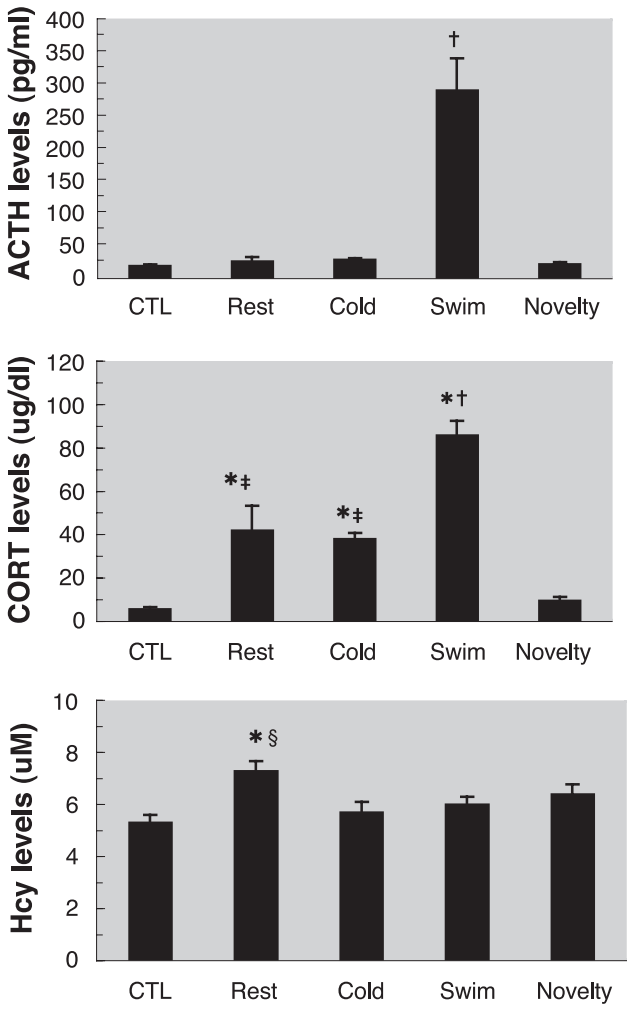

Fig. 1. ACTH (upper panel), corticosterone (middle panel) and homocysteine (Hcy; lower panel) plasma concentrations of animals submitted to different types of acute stress. The stressors used were restrain (rest), cold, swimming (swim) or novelty. Values are presented as mean ± S.E.M. of 8–10 animals/group. * Different from CTL group; †Different from all groups. ‡Different from novelty group. §Different from control (CTL) group.

4. Discussion

As supported by increased activity of the hypothalamic–pituitary–thyroid (HPA) axis, all stress manipulations were effective except novelty. Homocysteine changes occurred in a stressor-specific manner, in that restrain, a model of psychological stress in rats, significantly increased the amino acid concentration (+37%, $P = .006$). There were modest increases in the swimming (+13%) and novelty (+20%) groups that did not reach statistical significance.

The homocysteine increase in the restrain group, a stress mainly considered of psychological nature represents an intriguing finding. Similar results were reported by Stoney (1999), who demonstrated a significant elevation (+7%) of plasma homocysteine in women in response to mental arithmetic and verbal tasks (10-min baseline period and 10-min stress period). In contrast, another recent study (3-min preparation and 3-min stress period), found no effect of a verbal task on plasma homocysteine concentrations (Farag et al., 2003), possibly due to the shorter duration of the stress session in comparison to Stoney's work (1999) (3 vs. 10 min baseline and stress periods). Intrinsic psychological aspects of cardiovascular diseases, e.g., hostility and anger, have also been associated with increased plasma homocysteine concentrations in humans (Stoney and Engebretson, 2000). In the two studies that showed increased homocysteine concentrations, the results were attributed to elevated sympathetic activity of the individuals. Unfortunately our study was not designed to measure such parameters.

In a previous work from our laboratory, we reported that rats sleep deprived for 96 h had lower total plasma homocysteine concentration compared to home cage controls and that this concentration did not increase even when they were allowed to sleep for 24 and 48 h. Differently, rats sleep deprived for 24 h did not present altered homocysteine concentrations compared to controls (De Oliveira et al., 2002).

At first we attributed the differences between sleep deprivation and restrain stress to the duration of the stressor and hypothesized that acute and chronic stressors could have opposite effects on homocysteine concentrations. Although we originally supposed that decreased plasma homocysteine concentrations in sleep-deprived rats could be associated with the hypothyroid state induced by sleep

deprivation, we now believe that these differences may represent the degree to which each of the possible regulatory systems involved in homocysteine metabolism are elicited by a specific stressor, and that duration is related to the latency of the physiological response or sequential activation of these regulatory factors.

Our study indicates that the nature of the stress manipulation is also important, as it may selectively activate the main regulatory systems involved in response to stress (Pacák and Palkovits, 2001). On one hand, stress activates the sympathetic nervous system (SNS) and the HPA axis. Thus, during stressful situations, norepinephrine (from the SNS) and glucocorticoids (from the HPA axis) are released. Stress is also generally associated with increased growth hormone, prolactin, ACTH, norepinephrine and epinephrine concentrations (Lenox et al., 1980; Meyerhoff et al., 1990; Pacak et al., 1998; Pacak and Palkovits, 2001). On the other hand, the effects of stress on the thyroid are variable. Although a review by Pacak and Palkovits (2001) states that restrain inhibits the hypothalamic-pituitary-thyroid axis, Armario et al. (1986) demonstrated increased thyroid stimulating hormone concentrations in rats after 15-min sessions of four different psychological stressors in rats. However, restrain stress was not evaluated.

It is noteworthy that the abovementioned stress-induced hormone changes may interfere with homocysteine metabolism, a fact that may explain the different stress-induced responses. There are some studies in the literature that showed endocrine regulation of homocysteine metabolism. For instance, glucagon has been shown to reduce plasma homocysteine by decreasing homocysteine export from cells and by increasing the activity of cystathionine beta-synthase (CBS) and cystathionine gamma-lyase (CGL) enzymes in the transulfuration pathway (House et al., 1999; Jacobs et al., 2001). Insulin also seems to increase plasma homocysteine concentrations by decreasing CBS and CGL and the remethylation methylenetetrahydrofolate reductase enzyme activities (House et al., 1999; Dicker-Brown et al., 2001). In addition, hypothyroid rats were also shown to present reduced plasma homocysteine concentrations (Jacobs et al., 2000) and it has been proposed that glucocorticoids alter plasma homocysteine concentrations by stimulating CBS expression (Ratnam et al., 2002). Gender-related plasma homocysteine differences also point to an influence of sexual hormones on homocysteine metabolism regulation (Dierkes et al., 2001), as men have increased plasma homocysteine concentrations compared to women, although the mechanisms are still unknown.

Much attention has been paid to the endocrine regulation of homocysteine metabolism, but other physiologic mediators are potentially involved in the changes of plasma homocysteine concentrations induced by specific stressors, such as the case of inflammatory mediators (Tobeña et al., 1996; Black and Garbutt, 2002), and the vasoactive peptide endothelin-1 (Palma et al., 2002; Er et al., 2002). In fact, the extent to which each one of these regulatory systems are activated by a specific stressor will determine the final observed change in homocysteine concentrations, which can be increased, decreased or unaltered.

Particular stressors are associated with hyperhomocysteinemia, which may contribute to the deleterious effect of modern life stress. The complexity of the physiological responses to stress, the peculiarities of stress-selective responses, and the intricate regulatory systems involved in homocysteine metabolism should be better studied so that conclusions concerning the restrain-selective increase in total plasma homocysteine concentrations in rats can be drawn and enable such findings to be extrapolated to human pathology.

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