The effect of vitamin E, L-arginine, N-nitro L-arginine methyle ester and Forskolin on endocrine and metabolic changes of rats exposed to acute cold stress

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ABSTRACT

Objective: It is a well documented fact that under stress conditions the hypothalamic-pituitary-adrenal axis (HPA) and the sympathetic nervous system (SNS) are stimulated. This results in a series of neural and endocrine adaptations known as "the stress response". The current study assessed the effects of acute cold stress on adrenomedullin (ADM) levels in plasma and peripheral tissues (kidneys and heart) of rats, as well as on blood glucose, cholesterol, triglycerides (TG), total proteins both before and after intraperitoneal administration of each of the following: vitamin-E, L-arginine, forskolin and L-NAME.

Methods: The current study was conducted in the Department of Physiology, Faculty of Medicine, King Saud University, Saudi Arabia, between September 2003 and March 2004. We observed 6 groups of Wistar rats for their plasma ADM, tissue plasminogen activator (t-PA), total protein, glucose and cholesterol levels. Following exposure to cold stress (-10°C for 3 hours).

Results: Acute cold stress produced a significant increase in ADM levels in plasma, heart and kidney tissues of rats. Furthermore, acute cold stress produced a reduction in cholesterol and plasma protein levels. On the other hand, acute cold stress caused an increase in TG, glucose plasma levels and tissue plasminogen activator (t-PA). We found hormonal and metabolic changes caused by cold exposure to be decreased or even prevented after vitamin E treatment or after changing nitric oxide (NO) level by L-arginine or L-NAME treatment.

Conclusion: The results suggest a regulatory or protective role for ADM in counteracting HPA activation following a variety of physiological and psychological stressors. Oxidative stress or changes in intracellular signals as NO, cyclic-AMP may play a role in explaining some of the metabolic and hormonal changes occurring during acute cold stress.

Saudi Med J 2006; Vol. 27 (1): 17-22

The up regulation of adrenomedullin (ADM) gene expression and increases in systemic circulatory as well as localized tissue of ADM concentration is well coordinated with the onset and progression of trauma, infection, and sepsis.[1] Adrenomedullin has differential effects on cellular metabolism, immune function, and cardiovascular function.[2] This peptide appears to play a pivotal role during the different phases of the inflammatory response as well as a role in restoring homeostatic equilibrium to the body.[3] Stress (disruption of homeostasis) place demands on the body that is met by activation of 2 systems, the hypothalamic-pituitary-adrenal axis (HPA) and the sympathetic nervous system (SNS).[4] Stressor

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Received 31st May 2005. Accepted for publication in final form 30th October 2005.

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induced activation of the HPA axis, and the SNS results in a series of neural and endocrine adaptations known as "the stress response" or "stress cascade". The stress cascade is responsible for allowing the body to make the necessary physiological and metabolic changes required to cope with the demands of a homeostatic challenge.[5] Adrenomedullin appears to be secreted with catecholamine in response to nicotinic stimulation.[6] Acute restraint stress is known to stimulate sympathetic activity as well as HPA axis, produced a significant increase in ADM levels in the pituitary gland, plasma, and adrenal glands, all of which are key components of the HPA axis.[7] Nitric oxide (NO), which mediates ADM vasodilator action[8] is suggested to modulate stress-induced activation of the HPA axis and the sympathoadrenal medullary system[9] suggesting a regulatory or a protective role for ADM in counteracting HPA activation following a variety of physiological or psychological stressors.[10] Tissue plasminogen activator (t-PA) is an endothelium derived key enzyme in the initiation of endogenous fibrinolysis.[11] Tissue plasminogen activator is reported to be critically important for the development of anxiety-like behavior after stress.[12] Some stressors induce an increase or decrease in the local balance of fibrinolytic activities, resulting in bleeding or thrombosis in the local vessels. Such changes may not be detected in the general circulation due to the neutralization of locally induced fibrinolytic changes or the involvement of other hepatically originated hemostatic factors induced by stressors.[13] From the above mentioned facts, the aim of this study was to investigate the anti-stress effect of intraperitoneal administration of vitamin E, L-arginine, L-NAME (NO inhibitor) and forskolin on some endocrine and metabolic changes caused by acute cold stress in adult male rats.

Methods. The current study was conducted in the Department of Physiology, Faculty of Medicine, King Saud University, Riyadh, Kingdom of Saudi Arabia during the period between September 2003 through March 2004. Adult normal male Wister rats (mean weight 200-300 gm) supplied by the Animal Care Centre, College of Pharmacy, King Saud University, were housed 5 animals in a cage in a room temperature of 22 ± 1ºC and had a free access to water and food (ad libitum). The animals were randomly divided into 10 rats in each group. The study was conducted in accordance with the standards established guidelines of Laboratory Animals of College of Medicine Research Council (CMRC), King Saud University. Rats were divided into 6 experimental groups (10 rates in each), namely control group (not exposed to cold stress or under any medication, received intraperitoneal injection of normal saline). Group 2 exposed to cold stress only (by keeping them at -10°C for 3 hours). Group 3 received intraperitoneal vitamin E injection (600 ng/kg/day)[14] and were exposed to cold stress. Group 4 received L-arginine (20 mg/kg/day)[15] and cold stress. Group 5 received L-NAME (10 mg/kg/day)[15] and cold stress. Finally, group 6 received forskolin (20 mg/kg/day) and cold stress.[16] All drugs used were purchased from sigma-Aldrich chemicals. At the end of the duration of the experiment venous blood samples from the heart were collected in ethylenediaminetetraacetic acid (EDTA) tubes for measurement of plasma proteins, cholesterol, triglycerides (TG), and glucose levels. For measurement of plasma ADM and t-PA levels, blood was collected in shield EDTA tubes, centrifuged immediately in a cooling centrifuge at -4°C and plasma was kept at -80°C until the time of the assay. After collection of the blood samples, animals were sacrificed, the heart and kidney tissues were taken to measure the ADM level. Tissues were homogenized, and the tissue extract was kept at -80°C until the assay time. Tissue extraction procedure as follows: tissues were removed (heart and kidney), chilled in ice-cold isotonic saline, then blotted on filter paper and weighed. Chopped tissues were boiled for 10 min in 1 mol/L acetic acid and homogenized with a Polytron at 4°C. The extract solution was centrifuged at 24,000μg for 30 min. The plasma and tissue extract solution were loaded onto a Sep-Pak C18 cartridge and pre-equilibrated with 0.5 mmol/L acetic acid, and the adsorbed material was eluted with 4 ml of 50% CH_3CN containing 0.1% trifluoroacetyl acid (TFA). After the samples were lyophilized, the residue was dissolved in EIA buffer and assayed according to the manufacturer's instructions.[17] Adrenomedullin and t-PA were measured by enzyme immunoassay kits (Phoenix pharmaceuticals, USA). Total proteins were measured by a colorimetric method by a commercial kit (Spinreact, S.A. Ctra. Santa Coloma, Spain). Glucose and cholesterol were measured by enzymatic colorimetric method (Spinreact, S.A. Ctra. Santa Coloma, Spain).

Results were statistically analyzed using a statistical software program (SPSS for Windows). Data were compared with normal controls and groups together using one-way analysis of variance (ANOVA). Comparison of differences between groups was carried out by the least-significant difference (LSD) tests; the independent sample t-test was also carried out to compare the 2 groups together. Results were shown as mean ± standard deviation and significance were set at 95% confidence limit (p<0.05).

Table 1 - Plasma (pm/ml), heart (pg/g) and kidney (pg/g) adrenomedullin (ADM) concentrations under cold stress, cold stress and vitamin E, cold stress and L-arginine, cold stress and L-NAME as compared to control.

ADM	Control	Cold stress	Cold stress + Vitamin E	Cold stress + Forskolin	Cold stress + L-arginine	Cold stress + L-NAME
Plasma (pm/ml)	0.04 ± 0.007	0.46 ± 0.14*	0.39 ± 0.18*	0.31 ± 0.05*†	0.07 ± 0.007†	0.20 ± 0.04*†
Heart (pg/g)	0.90 ± 0.05	1.14 ± 0.25	0.55 ± 0.23*†	0.54 ± 0.28*†	0.69 ± 0.1†	0.68 ± 0.10†
Kidney (pg/g)	0.61 ± 0.10	1.00 ± 0.15*	0.54 ± 0.23†	0.18 ± 0.05*†	0.59 ± 0.09	0.59 ± 0.12†

*significant difference from the control, †significant difference from the cold stress group at $p<0.05$.

Table 2 - Plasma tissue plasminogen activator (tPA) concentration (pm/ml) under cold stress, cold stress and vitamin E, cold stress and L-arginine, cold stress and L-NAME as compared to control.

Tissue plasminogen activator	Control	Cold stress	Cold stress + Vitamin E	Cold stress + Forskolin	Cold stress + L-arginine	Cold stress + L-NAME
Plasma (pm/ml)	11.88 ± 1.97	27.93 ± 5.3*	18.72 ± 2.4*†	28.15 ± 5.9*	26.0 ± 4.1*	51.57 ± 7.0*†

*significant difference from the control, †significant difference from the cold stress group at $p<0.05$.

Table 3 - Plasma levels (mmol/L) of cholesterol, triglycerides, glucose and total protein in control, cold stress, cold stress and vitamin E, Forskolin, L-arginine or L-NAME treated rats. Cs=cold stress, Vit E=vitamin E, *significant difference from the control, significant difference from the cold stress group, $p<0.05$.

Plasma level (mmol/L)	Control	Cold stress	Cold stress + Vitamin E	Cold stress + Forskolin	Cold stress + L-arginine	Cold stress + L-NAME
Cholesterol	1.96 ± 0.69	0.52 ± 0.39*	1.07 ± 0.35*	1.05 ± 0.95*	1.30 ± 0.36	1.65 ± 0.5†
Triglyceride	1 ± 0.26	1.9 ± 0.14*	2.0 ± 0.7*	1.2 ± 0.4†	1.90 ± 0.48*	1.20 ± 0.65†
Glucose	6.2 ± 0.64	7.4 ± 1.0*	6.4 ± 1.7	6.9 ± 0.9	6.30 ± 0.35	7.70 ± 1.4
Plasma protein	46.7 ± 8	38.8 ± 7*	47.6 ± 7†	50.8 ± 4†	48 ± 3†	45.0 ± 6

*significant difference from the control, †significant difference from the cold stress group at $p<0.05$.

Results. Acute cold stress caused a significant elevation in plasma ADM levels in comparison with control. This significant elevation above the control was still present in the presence of Vitamin E ($p<0.005$), forskolin ($p<0.005$) and L- NAME ($F=18.3$, $p<0.05$) (**Table 1**). Meanwhile, the administration of L-arginine returned the plasma levels of ADM to controls ($p>0.05$). The administration of forskolin, L-arginine, and L-NAME reduced plasma ADM compared to those in cold stress group ($p<0.05$), ($p<0.005$) and ($p<0.05$). Acute cold stress elevated the heart tissue levels of ADM, which did not reach significant level ($p>0.05$). The administration of vitamin E and forskolin reduced heart levels of ADM compared to controls ($p<0.05$ for both). And acute cold stress group ($p<0.05$ for both), meanwhile, the administration of L-arginine and L-NAME reduced heart ADM levels compared to cold stress group ($p<0.05$ for both). Acute cold stress caused significant elevation in kidney tissue levels of ADM ($p<0.05$). Administration of vitamin E, L-arginine and L-NAME reduced kidney levels of ADM compared to acute cold stress group ($p<0.05$ for all). Furthermore, administration of forskolin reduced kidney ADM levels compared to controls and cold stress group ($p<0.05$ for both) (**Table 1**).

Table 2 shows, a significant elevation in t-PA level in rats exposed to cold stress in comparison with control group ($p<0.05$). Plasma levels of t-PA were still significantly high compared to controls after administration of vitamin E, forskolin, L-arginine and L-NMAE to acute cold stress rats ($p<0.05$ for all). On the other hand, in acute cold stress rats, vitamin E administration significantly decreased, while L-NAME administration significantly increased plasma t-PA levels in comparison with cold stress rats ($p<0.05$ for both).

Table 3 shows that, plasma cholesterol level was significantly reduced in rats exposed to cold stress compared to the controls ($p<0.05$). The reduced levels of plasma cholesterol were still present after administration of vitamin E and forskolin, compared to controls ($p<0.05$). Furthermore, administration of L-NAME to cold stress animals leads to elevation of cholesterol levels compared to cold stress group ($p<0.05$). Acute cold stress caused significant elevation of plasma TG levels compared to control group ($p<0.05$). The administration of vitamin E or L-arginine to cold stressed rats caused significant elevation in plasma TG concentration compared to controls ($p<0.05$ for both). On the other hand, forskolin and L- NAME injection in association with cold stress caused significant reduction in plasma TG concentration, compared to its level in cold stressed group ($p<0.05$ for both) (**Table 3**).

In addition, cold stress produced significant elevation of plasma glucose levels compared to controls ($p<0.05$). Vitamin E, L-arginine, L-NAME and forskolin administration returned blood glucose levels to controls ($p<0.05$) (**Table 3**). Acute cold stress led to significant reduction in plasma total protein levels compared to controls ($p<0.05$). On the other hand, administration of vitamin E, forskolin, L-arginine and L-NAME elevated total protein levels compared to acute cold stress group ($p<0.05$ for all).

Discussion. It has previously been suggested that cold is a form of physiological stress which induces an ADM release.[18,19] However, the literature inferences rather than a clear cut evidence to this concept.[10] We found that acute cold stress significantly increased plasma and tissue (heart and kidney) ADM levels in adult male rats. This increase in ADM level may have a protective effect against cold stress associated with an increase in sympathetic activity and vasoconstriction of blood vessels, which may interfere with sufficient blood supply to tissues.[20] Adrenomedullin and pro-adrenomedullin (PADM) were reported to have an inhibitory effect on the in vitro release of ACTH from the pituitary corticotrophs, and this suggests that this effect may become relevant when an exceedingly high ACTH secretion must be counteracted.[21] The concentration of ADM and PADM in the blood rule out the possibility that they act as true circulating hormones. Conversely, their content in the HPA complex and adrenal gland is consistent with a paracrine mechanism of action, which may play an important role in pathological conditions where the function of the HPA axis has to be reset.[21]

Our results also showed elevated tissue plasminogen activator (t-PA) in cold stressed rats, this may suggest that cold stress may exert a protective response by enhancing acute endogenous fibrinolytic capacity that protect the body during stress against thrombosis. Similar results were reported in case of acute systemic inflammation in humans[22] and in surgical stress.[11] Deficiency of inorganic signals, such as NO, which in addition to its vasodilator effect, has anti-thrombotic properties, such as inhibition of platelets aggregation, my be a contributing factor in increased t-PA.[23] In addition, sympathoadrenal system stimulation[24] and oxidative stress[25] may be another contributing cause of increased t-PA in stress conditions. In the current study, acute cold exposure produced reduction in protein, cholesterol levels, and increase TG. This is in accordance with the results of Yuksel et al[10] and Kolosova[26] who reported increase in the common cold fraction of plasma lipids (very low-density lipoprotein, low-density lipoprotein [LDL]) in rats exposed to as long as 15 days of cold acclimatization.[26] This might be a physiological response to cold stress or may result from the elevated ADM level resulting in this condition. In partial consistence with ours, Yuksel et al[10] reported that 48 hours of cold stress at 8°C decreased protein, and cholesterol levels, but they reported reduced TG levels in Sprague-Dawley rats. These results are inconsistency with our results. On the contrary, Jain et al[27] found to increase in protein levels in response to swim stress induced in Sprague-Dawley rats, compared to controls. The water used in this experiment was 15°C and the experiment was carried out for 5 consecutive days. The difference in the results obtains from this study and other studies may be contributed to different experimental conditions and different animals species used. It may be considered that both ADM in addition to cold stress acts via a different kinetic mechanism and causes altered metabolic regulation taking partial or total occupation of ADM and structurally and functionally similar receptor groups such as CGRP receptors.[10] The changes in the physicochemical features of lipoproteins do not always correspond to their quantitative changes.[26] Cold stressed rats in the present study showed an increase in their blood glucose level, this hyperglycemic effect of cold stress is supported by previous reports by Yuksel et al[10] and Larkin et al,[28] who demonstrated elevated serum glucose and lactate concentration in cold stressed rats. The elevated blood glucose level could be due to a rapid rise in plasma glucagon level in cold stressed rats which in turn leads to increase in the concentration of hepatic gluconeogenesis and ketogenesis,[29] or it may be due to increased thyroid stimulating hormone (TSH) secretion and

consequently increased thyroxine level[30-33] which may therefore be responsible for the hyperglycemia. In addition, thyroxin is known to decrease plasma cholesterol concentration due to increased formation of LDL receptors in the liver, resulting in increased hepatic removal of cholesterol from the circulation, which may explain reduced cholesterol level after cold exposure in our experiments. Hyperglycemic effect of cold stress could also be due to activation of the HPA axis, which in turn increases corticotrophin releasing hormone (CRH) leading to cortisone release and hence to hyperglycemia.[34] However, Kioukia et al[35] showed no change in blood glucose level in 14-day cold swim stress, in presence of stimulation of HPA axis and increased ACTH. Like most of the other stressors to which the body may be exposed, cold stress leads to profound alterations in the blood level of several humoral, blood borne signal effectors (such as adrenocortical and locally produced tissue hormones, immune cytokines) and inorganic signals such as NO.[3] In addition, cold stress in rats is reported to be associated with oxidative stress and free radical generation.[36,37] Increased brain nitrite levels was reported in the brain of stressed rats,[36] lipid peroxidation was also found to be increased in brain and retina of stressed rats as evidenced by increased thiobarbituric acid reactive substances (TBARS).[37] In the current study, treatment of the cold stressed rats with vitamin E prevented some of the effects of cold stress, as changes in plasma t-PA, TG and glucose. In addition, it prevented increased kidney ADM level compared to cold stress group. On the other hand, vitamin E lowered heart ADM levels below controls and acute stress group. These effects of vitamin E in cold stressed rats could be due to its anti-oxidative effect. It had been reported previously, that low vitamin E levels in conditions associated with oxidative stress and plasma vitamin E levels correlated significantly with TBARS.[36] Previous reports,[37] find that vitamin E decreased corticosterone level, glutathione peroxidase (GSH-Px) activity and TBARS levels in brain and retina of cold stressed rats. All these findings support a protective role of vitamin E in cold stress conditions. The lowering effect of vitamin E on t-PA is in accordance with similar results of Skrha et al,[38] who suggested that vitamin E may further worsen the hypofibrinolysis caused by decreased t-PA. In the current study, we did not measure the level of plasminogen activator inhibitor (PAI) which if elevated could support this hypothesis. As NO is one of the inorganic signals that may be changed by cold stress,[3] we found that administration of the amino acid precursor of NO (L-arginine) in combination with the cold stress exposure prevented changes in plasma and tissue ADM levels, cholesterol, TG, protein, blood glucose level and elevated t-PA in cold stressed rats. So our results may suggest that NO deficiency may underlie some of the changes occurring in cold stress and thus, correction of this deficiency by giving the NO precursor (L-arginine) abolishes some of these changes. Nitric oxide synthase blockade by L-nitro-arginine methyl ester (L-NAME) augments the increase in t-PA and blood glucose levels, but it shares some of the changes caused by L-arginine on TG, cholesterol, tissue ADM which may suggest that other mechanisms than NO causes these changes. Our findings regarding effect of L-arginine and L-NAME on t-PA are in accordance with the results of Newby[39] and Smith et al[23] who suggest that L-arginine/NO pathways contribute to t-PA release in vivo. Forskolin a cell permeable activator of adenyl cyclase[40] also prevents some of the changes occurring with cold stress as those of plasma TG, glucose, plasma total proteins levels. On the other hand, it reverses the effects of cold stress on tissue ADM. This may be through increasing the level of cAMP which mediates intracellular effects of NO. It may also be suggested that alteration in adenyl cyclase activity or cAMP levels may underlie some of the metabolic and humoral effects of cold stress.

In conclusion, cold stress induced changes appear to be multifactorial and more than one physiologic and homeostatic mechanism might be taking place. A series of neural and endocrine adaptations occur as a result of cold induced stimulation of the HPA axis and SNS known as "stress response", the stress response is responsible for helping the body to overcome and cope with the homeostatic challenge (stressor) by making the necessary physiological and metabolic changes required. Some metabolic and endocrine changes caused by acute cold stress are found to be corrected following supplementation with vitamin E, forskolin or L-arginine. This may indicate that the underlying cause of these changes is multifactorial and that more than one mechanism takes place in these changes. The level of NO, cAMP, free radical generation may be partially responsible. Stress response study is a broad topic that needs a lot of further investigation to elucidate mechanisms of its regulation.

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