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DIFFERENTIAL ENDOCRINE RESPONSES OF ANIMALS TO COLD-HYPOXIA-RESTRAIN STRESS

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ABSTRACT
There are large and enduring differences between individuals in the magnitude of their response to stress. The aim of the present study is to differentiate the animals exposed to simulated cold and hypoxia in a restrain condition (C-H-R) and to evaluate the hormonal profile responsible for individual variation in response to the stress. Time taken to attain the rectal temperature (Trec) of 23^{o}C was measured after exposing the animals to Cold (5^{O}C), Hypoxia (428 mmHg) Restrain (C-H-R) stress and screened with reference to their timing to attain Trec 23^{O}C and segregate them as susceptible, normal and resistant. The hormonal profiles of these animals were evaluated after exposure to the test. The study showed a distinct individual difference in response to C-H-R test. There was a significant increase in plasma norepinephrine concentration ($p<0.05$) in resistant group than susceptible rats. Total circulating corticosterone (CORT) level also increased but this was not significant between those two groups. Corticosteroid binding globulin (CBG) concentration differ significantly ($p<0.05$) between resistant and susceptible groups of rats resulting in significant changes in circulating free CORT that in turn may be responsible for individual differences in stress response. Present study showed that multiple components rather than only plasma glucocorticoid are responsible for individual differences in physiological responses to stress. The results also indicate that these endocrine parameters can serve as biomarkers for screening individuals for resistance/susceptibility to high altitude stress.

KEYWORDS: C-H-R stress, catecholamine, corticosterone, ACTH, CBG.

INTRODUCTION
Stress occurs when a challenge overwhelms, or is assessed by the brain as being likely to overwhelm, the body's normal homeostatic response mechanisms.[2,9] One promising new avenue of discovery that has gained prominence in recent years involves the investigation of the factors associated with individual differences in responses to stress and, by extension, susceptibility to stress-related illness.[13,20,21,28] High terrestrial altitude is generally characterized by low barometric pressure (hypobaria), low partial pressure of oxygen (hypoxia), severe cold and low humidity. Hypoxia is a well known stress and the impact of hypoxic stress may cause several illnesses.[12] The occurrence of high altitude illness is dependent upon elevation, the rate of ascent and individual susceptibility. Some people readily develop Acute Mountain Sickness (AMS) on an ascent to high altitude, while others are able to ascend rapidly without difficulty.[19,27] Some people readily develop Acute Mountain Sickness (AMS) on an ascent to high altitude, while others are able to ascend rapidly without difficulty. Several techniques have been used for humans to predict who is likely to develop AMS[4,6,7], but their clinical usefulness needs further study, while there is lack of methods for animals to detect the differential response at simulated hypoxic condition and factors that contribute to this variability.

In the present study a multiple stress animal model which simulates the conditions of high altitude has been selected for screening the animals for individual variation. The animals were exposed to multiple stresses of cold, hypoxia and restrain (C-H-R) conditions following the method of Ramachandran et al., 1990.[24] In the C-H-R animal model, the capacity of the rat to maintain a physiological function, that is thermal balance in a challenging atmosphere has been used as a measure of the capacity to adapt to unfavorable environment. Previously in our laboratory, Cold-Hypoxia-Restrain (C-H-R) stress was followed to evaluate the adaptogenic activity of herbal extracts.[11,25] For the first time we have used the C-H-R stress to differentiate the rats as the susceptible or resistant according to their time taken to attain T_{rec} 23^{0}C. The deep core body temperature was measured during the exposure and the time taken to attain the rectal temperature was taken as a measure of physical endurance. The study evaluates the differential

response of the animals to the C-H-R stress related to endocrine parameters.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (150± 10 g) maintained in the Defence Institute of Physiology and Allied Sciences' animal house were used for experiments. Animals were given food and water *ad libitum* and were maintained at 24°C with a 12-h light–dark cycle. The animals were treated humanely and experiments were carried out according to the guidelines of the ethical committee of this institute.

Experimental design

A Cold (5^{O}C) Hypoxia (428mmHg) Restrain (C-H-R) multiple stressanimalmodel was used to differentiate the animals during exposure to such stressful environment following the method of Ramachandran et al., 1990.[24] The C-H-R exposure was performed in an animal decompressionchamber maintained at 5°C and 428mmHg pressure, equivalent to an altitude of 4572 m. The wind flow was 2 Lmin−1. The rectal probe was inserted 2 cm past the rectum ofthe rat and retained there with the help of adhesive plaster. The rat was kept in a restrainer. The deep core body temperature was measured during the exposure and the time taken to attain the rectal temperature was taken as a measure of physical endurance. The rectal temperature of therat was monitored continuously, every minute by Isothermax Temperature Recorder (Columbus Instruments, USA). Whenthe rat attained a rectal temperature of 23°C, it was taken outof the chamber. The rats were exposed to C-H-R stress and screened with reference to their timing to attain rectal temperature (Trec) 23^{O}C. About 200 animals were screened with C-H-R model and then divided or segregated to the following groups:

Susceptible: Rats showing less than 60 min to attain Trec 23^{O}C.

Normal: Rats showing 60 min - 90 min to attain Trec 23^{O}C

Resistant: Rats showing more than 90 min to attain Trec 23^{O}C.

Rats were sacrificed immediately after exposure to C-H-R stress and blood samples were collected in heparinizedvacutainers (BD). The blood samples were centrifuged immediately at 3500rpm for 20 min at4^{0}C. The plasma samples were then separated and stored at -80^{0}C.

Estimation of plasma catecholamine

The plasma catecholamines were analyzed by HPLC (Waters) after preparation of the samples using kit supplied by M/S CHROMSYSTEMS (USA).[17] The concentrations of the catecholamines were expressed in terms of pg/ml of plasma.

Estimation of Adrenocorticotropic hormone (ACTH) in plasma

Adrenocorticotropic hormone (ACTH) in plasma samples were estimated using commercially available ELISA kits from DRG, USA. The sensitivity of ACTH assay was 0.46pg/ml. The intra and inter-assay coefficients of variations were 3.1 and 5.8% respectively.

Estimation of Prolactin (PRL) in plasma

Prolactin(PRL) in plasma samples were estimated using commercially available ELISA kits from DRG, USA. The sensitivity of PRL assay was 0.026ng/ml. The intra and inter-assay coefficients of variations were 2.9 and 3.5% respectively.

Estimation of Corticosterone (CORT)in plasma

Corticosterone (CORT) in plasma samples were estimated using commercially available ELISA kits from NEOGEN, USA. The sensitivity of CORT assay was less than 0.40ng/ml. The intra and inter-assay coefficients of variations were 2.7 and 5.4% respectively.

Estimation of Testosterone in plasma

Testosterone in plasma samples were estimated using commercially available ELISA kits from ADALTIS, Italy. The sensitivity of Testosterone assay was 0.01ng/ml. The intra and inter-assay coefficients of variations were 6.2 and 3.9 % respectively.

Measurement of plasma corticosteroid binding globulin (CBG)

Plasma CBG was assayed by Radio Immuno Assay (RIA) using commercially available kit from BIO-LINE (USA). Plasma sample was diluted 25 times with supplied dilution buffer. 100µl of diluted samples were used for the assay. The sensitivity of CBG assay was 0.25µg/ml. The intra and inter-assay coefficients of variations were 3.9 and 5.5% respectively.

Measurement of free corticosterone in plasma

CBG-bound and unbound, *i.e.*, free, corticosterone was calculated using mass equation of Barsano and Baumann (1989).[3]

$$H_{free} = 0.5 \times [H_{total} - B_{max} - 1/k_a \pm \sqrt{(B_{max} - H_{total} + 1/k_a)^2 - 4(H_{total}/k_a)}$$

in which H_{free} = free hormone, H_{total} = total hormone, B_{max} = total binding capacity of CBG, and $K_a = 1/K_d$; K_d = equilibrium dissociation constant for corticosterone and CBG.

Free corticosterone is the total concentration of corticosterone minus the concentration of CBG-bound corticosterone.

Statistical analysis

All data were statistically analyzedand presented in the table, as mean ± SEM. Level of significance of all the parameters was determined using Student's t-test using SPSS Vr 14.

RESULTS

Cold-Hypoxia- Restrain stress

Matched animals were divided into three groups according to their time required to attain the $T_{rec}23^0C$.

Susceptible - 29.27%

Normal-48.78%

Resistant-21.95%

In our study we have one control group of animals unexposed to CHR stress. We have seen a significant difference in the level of catecholamine, corticosterone, ACTH, and prolactin hormones and in all the three groups listed above as compared to unexposed. However, since our study is designed to evaluate the differential response between resistant and susceptible groups of animals, data of unexposed control has not been shown in the results.

Concentrations of Catecholamine in plasma

There was significant difference in plasma norepinephrine and epinephrine concentration between susceptible and resistant rats ($p<0.001$). (Fig.1).

Concentrations of ACTH, Prolactin, Corticosterone and Testosterone in plasma

ACTH concentration in plasma was significantly higher in resistant rats than susceptible rats ($p<0.001$). Plasma prolactin level was significantly lower in resistant rats when compared with susceptible rats ($p<0.05$). Plasma testosterone level was significantly ($p<0.001$) higher in resistant rats when compared to susceptible rats(Table 1). However, there was significant difference in plasma concentration of Total corticosterone between resistant and susceptible rats (Fig 2).

Concentrations of corticosteroid binding globulin (CBG) and free corticosterone in plasma

Plasma CBG concentration was significantly ($p<0.05$) lower in resistant rats when compared with susceptible rats (Fig 3). Once CBG capacity and total CORT are known, free CORT titers can be estimated using the mass action-based equation of Barsano and Baumann (1989). Total CORT levels and CBG capacity were used to estimate a mean for free CORT (Fig 4). Error bars for free CORT were estimated by putting total CORT mean ± SE and CBG mean ± SE into the Barsano and Baumann (1989)[3] equation. Free corticosterone level was significantly higher in resistant group when compare to susceptible rats.

Table 1: Plasma ACTH, Prolactin and Testosterone concentration of susceptible and resistant rats exposed to C-H-R stress.

Groups	ACTH (pg/ml)	Prolactin (ng/dl)	Testosterone (ng/ml)
Susceptible (n=18)	49 ± 6.19	1.06 ± 0.090	3.12 ± 0.29
Normal (n=22)	58.9 ± 3.01	0.69 ± 0.054	3.83± 0.4
Resistant (n=15)	64 ± 2.60*	0.71 ± 0.060*	4.64 ± 0.46*

*$p<0.05$ when compared with susceptible rats.

Figure Legends

Fig 1. Norepinephrine (NE) and epinephrine (E) level in plasma of susceptible and resistant groups of rats.

Fig 2. Plasma level of total corticosterone (CORT) in susceptible and resistant groups of rats.

Fig 3. Plasma level of corticosteroid binding globulin (CBG) in susceptible and resistant groups of rats.

Fig 4. Plasma level of free corticosterone (CORT) in susceptible and resistant groups of rats.

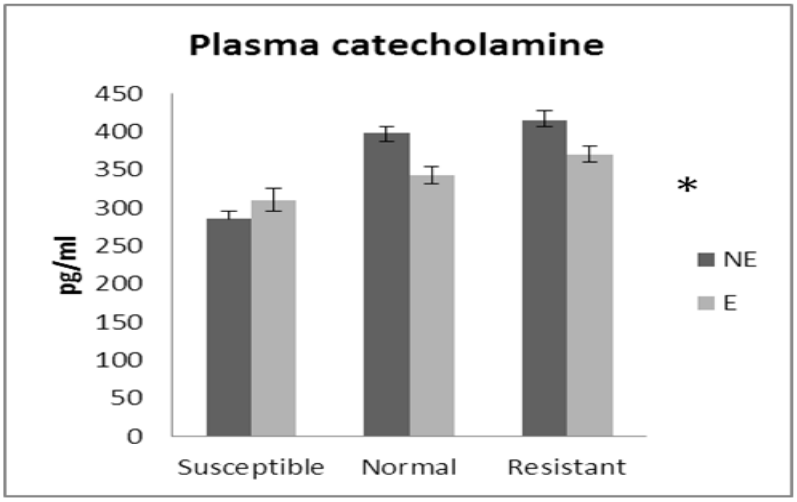

Fig. 1.

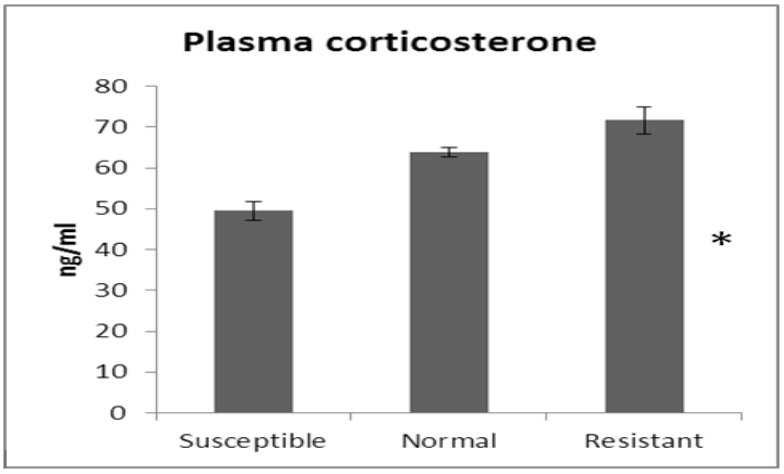

Fig. 2.

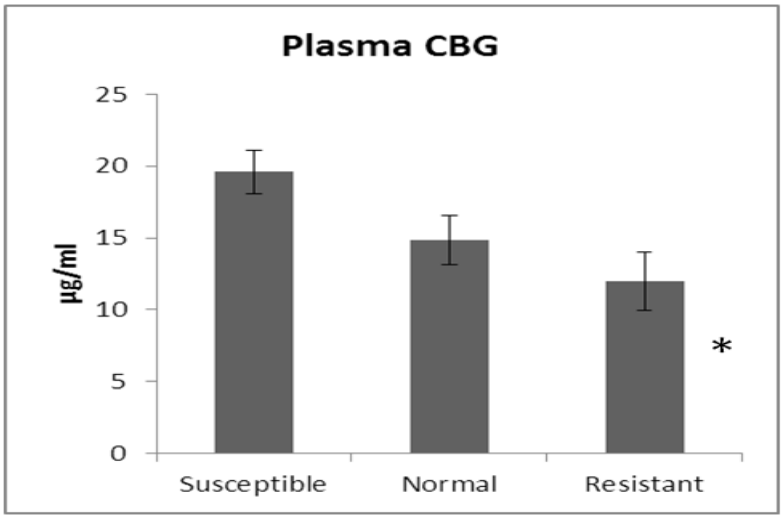

Fig. 3.

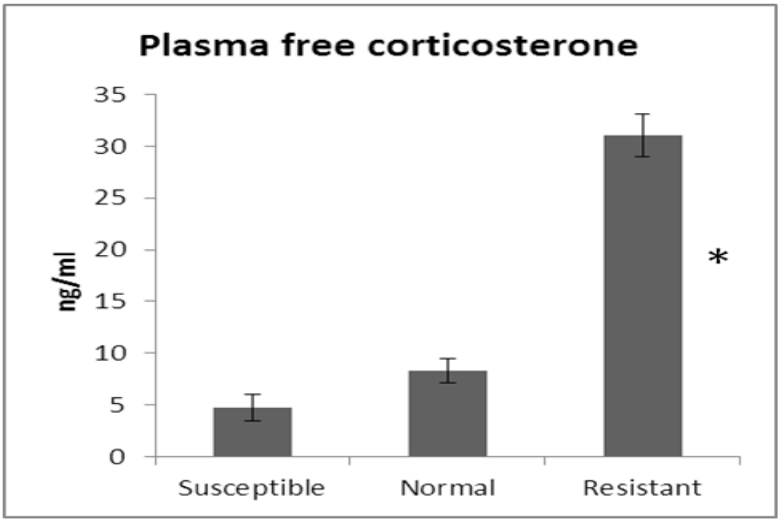

Fig. 4.

DISCUSSION

Previously Cold-Hypoxia-Restrain (C-H-R) stress was followed to evaluate the adaptogenic activity of herbal extracts.[11,25] In the present study, first time we have used the C-H-R stress to differentiate the rats as the susceptible or resistant according to their time taken to attain T_{rec} 23^0C. Stress is defined as an event resulting in increased activity of the HPA axis. In response to any stressful stimuli, the hypothalamic-pituitary-adrenal (HPA) axis and autonomic nervous system are activated

and play a central role in adaptation to the stressful conditions.[8] One of the classic characteristics of the mammalian stress response is the wide inter-individual variations. Although the same event is perceived by two individuals, the each responds is quite variable. One may show little or no effect while the other may begin the process required to re-establish lost equilibrium. In the present study, time taken to attain Trec 23oC of rats were recorded to and they were differentiated as susceptible and resistant rats accordingly. There was a distinct individual difference as shown in the time to attain t rec 23oC or sensitivity to cold and hypoxia and that may be mainly due to varying reactions occurring at the level of regulatory systems, the hormonal system in particular. A number of complex interactions are involved in the regulation of these hormones.

When faced with a stressor, there is an associated upregulation in hypothalamic-pituitary- adrenal (HPA) axis activity; the ultimate result of this is a rise in plasma corticosteroid (CORT) levels (1). However, there was no significant difference of adrenocorticotropic hormone (ACTH) found between the two groups. Comparative studies of the stress response also have focused primarily on the adrenocortical response to stress, in particular the measurement of plasma levels of glucocorticoid.[26] Present study revealed that, there was no significant difference in plasma total corticosterone level between the high and low tolerant groups of rats. As more than 90% of circulating corticosteroids in rats and humans are bound to a specific binding protein, corticosteroid binding globulin (CBG) and CBG binds corticosteroid with high affinity and thus regulates availability of free hormone to target tissues or alter CORT clearance rates[14,30], we wanted to study whether there is any individual variation in CBG concentration in different rats and the resultant free corticosteroid level which is ultimately regulating the coping style of an organism. There was significant difference in CBG concentration between the high and low tolerant groups.

According to the free hormone hypothesis, steroid bound to plasma binding globulin is unavailable to tissue; the free (unbound) hormone is the biologically active fraction, able to enter cells, activate intercellular or membrane receptors, and also be available for metabolism in the liver. In this hypothesis, the primary role of CBG is to regulate the bioavailability and metabolic clearance of glucocorticoids.[15,22] CBG provides a reservoir of CORT in the plasma (CORT is hydrophobic; binding to CBG increases the solubility of CORT in plasma). However, higher CBG capacity means a larger pool of CORT is available in the blood if it is needed (5). A number of studies have illustrated the link between stressor and CBG binding capacity. Thus, the amount of CORT that is taken up by target tissue is approximated by the non-CBG bound fraction of total circulating steroid.[23,29] In the present study, though there was no significant difference in total circulating level of corticosterone between resistant and succeptible rats but significant difference was observed in free corticosterone level between the two groups because of significant difference in CBG level.

The prolactin (PRL) release from the pituitary gland is a very sensitive marker of both physical and psychological stress in mammals.[10] Aside from its actions on reproductive processes, prolactin plays a role in maintaining the constancy of the internal environment by regulation of the immune system, osmotic balance, and angiogenesis. Adaptation to the stress seems to be accompanied by a diminished response of prolactin.[10] Present study also showed the similar type of observation where resistant rats had significantly low level of plasma prolactin than the succeptible group.

Knol (1991)[18] proposed that stressors generally induce depression of hypothalamo-pituitary-testis system, mediated by activated hypothalamo-pituitary-adrenocortical system, resulting in fall in plasma testosterone levels. Plasma CBG levels also varied negatively as a function of testosterone concentration and thus testosterone dependent decrease in CBG synthesis might be associated with an enhanced nuclear uptake of CORT, which permit greater access of CORT to its receptors for physiological function and also enhance glucocorticoid feedback regulation of ACTH release. Plasma testosterone level was significantly higher in resistant rats than susceptible rats, which was responsible for decreased synthesis of CBG in HT group which in turn regulate the free CORT level in the circulation.

CONCLUSION

The present study establishes a hypoxic tolerance test differentiating rats according to theirgasping time and shows that multiple components of the stress response (and not only plasmaglucocorticoid), are providing a basis for individual differences in physiological responses tohypoxic stress. Two individuals may or may not show the expected differences in glucocorticoidresponse but the plasticity in stress responsiveness may depend on various endocrine factors atmultiple levels. The role of CBG as an active component of the stress response warrants furtherstudy.

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