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Individual variation in response to simulated hypoxic stress of rats

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With an aim to categorize the animals exposed to simulated hypobaric hypoxia and to evaluate the hormonal profile responsible for individual variation in response to hypoxic stress, degree of tolerance to hypobaric hypoxia was measured by exposing the animals to a simulated altitude of 10,668 m at 32 °C and animals were categorized as low and high tolerant groups based on their gasping time. The hormonal profiles of these groups were evaluated just after exposure to the test. The results showed a distinct individual difference in response to hypoxic tolerance test. There was a significant increase in plasma norepinephrine concentration in high tolerant group than low tolerant rats. After hypoxic tolerance test, total circulating corticosterone (CORT) level also increased but this was not significant in high tolerant rats as compared to low tolerant rats. Corticosteroid binding globulin (CBG) concentration differ significantly between high and low tolerant groups of rats resulting in significant changes in circulating free corticosterone that in turn may be responsible for individual differences in hypoxic gasping time. Significant differences were also observed in prolactin and testosterone levels of both the groups. The results established the method of differentiating the animals according their response to hypoxic tolerance test. These data indicate that multiple components rather than only plasma glucocorticoid of the stress response are providing a basis for individual differences in physiological responses to hypoxic stress.

Keywords: ACTH, CBG, Catecholamine, Corticosterone, Hypoxic stress, Testosterone

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. The occurrence of high altitude illness is dependent upon elevation, the rate of ascent and individual susceptibility[1]. 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[2-4], 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. 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[5-8]. Wood *et al*.[9] studied the individual differences in reactivity to social stress and suggested that inherent differences in stress reactivity, perhaps as a result of differences in CRF regulation, may predict long-term consequences of social stress and vulnerability to depressive-like symptoms.

The hypothalamic-pituitary-adrenal (HPA) axis, the efferent sympathetic-adrenomedullary system and components of the parasympathetic system are the principal neuroendocrine systems responsible for physiological adaptation to stress and interactions between the two systems would be produced already at the initial stages of corticosteroid signaling[10,11]. Although regulatory and adaptive process of autonomic nervous system (ANS) and hypothalamic pituitary adrenal axis play the major role in circumventing the stressful environment, the endocrine response to same environmental stress in a given population is not always uniform. In similar stressful situation two animals behave differently with reference to their adaptive neuroendocrine responses. But the origins of differences in stress responses among the same populations of the animal were not studied adequately.

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In the present study, an attempt has been made to categorize the animals when exposed to a simulated hypobaric hypoxia condition and detect possible differences in hormonal status between animals with high and low tolerant to hypoxia by measuring blood levels of catecholamines, corticosterone, ACTH, prolactin and testosterone because these are the markers of adaptive response to stress. The present study clearly demonstrated differences in endocrine responses between high and low tolerant rats in the HPA axis after hypoxic tolerance test.

Materials and Methods

Animals—Sprague–Dawley rats (170±10 g; 120) maintained in the Institute's animal house were used for experiments (screening of rats). 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 humanly and experiments were carried out according to the guidelines of the ethical committee of the Institute.

Determination of hypoxic tolerance—Hypoxic tolerance was determined by measuring the gasping time (GT) while exposing animals one at a time to simulated hypobaric hypoxia of 10,668 m in an animal decompression chamber at 32 °C (Decibel Instruments, Delhi, India) coupled to a mercury barometer[12]. The airflow in the chamber was 2 L/min, while the relative humidity was maintained at 40- 50%. The time taken for appearance of the first sign of gasping was recorded using an electronic stopwatch. Then the animals were divided into three groups according to their gasping time as follows:

High tolerant	:	Gasping time above 25 min
Normal	:	Gasping time between 5-25 min
Low tolerant	:	Gasping time below 5 min

Rats were sacrificed immediately after hypoxic tolerance test and blood sampling. Tissue samples were collected and stored at -80 °C until estimation. The blood samples were centrifuged immediately at 3500 rpm for 20 min at 4 °C. The plasma samples were then separated and stored at -80 °C.

Plasma catecholamine—The plasma catecholamines were analyzed by HPLC (Waters) after preparation of the samples using kit supplied by M/s Chromsystems (USA)[13]. The concentrations of the catecholamines were expressed in terms of pg/mL of plasma.

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 0.46 pg/mL. The intra and inter-assay coefficients of variations were 3.1 and 5.8% respectively.

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

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

Testosterone in plasma—Testosterone in plasma samples were estimated using commercially available ELISA kits (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.

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. Diluted samples (100 µL) were used for the assay. The sensitivity of CBG assay was 0.25 µg/mL. The intra and inter-assay coefficients of variations were 3.9 and 5.5% respectively.

Free corticosterone in plasma—CBG-bound and unbound, *i.e.*, free, corticosterone was calculated using mass equation of Barsano and Baumann[14] :

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

where 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 analyzed and presented as mean ± SE. Level of significance of all the parameters was determined using Student's t-test using SPSS Vr 14.

Results

Hypoxic gasping time—Matched animals were divided into following three groups according to their gasping time:

Low tolerant	-	29.27% (35 out of 120)
Normal	-	48.78% (58 out of 120)
High tolerant	-	21.95% (26 out of 120)

Adrenal gland weight—The weight of adrenal gland (expressed in mg/100 g body weight) in HT and LT groups was 16.35 ± 0.92 and 12.22 ± 0.83 respectively. It was significantly higher in HT rats than LT (P<0.05) where as in normal rats it was 15.53±0.54.

Catecholamine in plasma—There was significant difference in norepinephrine concentration between high and low tolerant rats (P<0.05) and plasma epinephrine level was more in high tolerant rats than the low tolerant rats but not significantly (Table 1).

ACTH, prolactin, corticosterone and testosterone in plasma—ACTH concentration in plasma was significantly higher in HT rats than LT rats. Plasma prolactin level was significantly lower in HT rats when compared with LT rats. Plasma testosterone level was also significantly higher in HT rats when compared to LT rats (Table 1). There was no significant difference in plasma concentration of Total corticosterone between HT and LT rats (Fig. 1).

Concentrations of corticosteroid binding globulin (CBG) and free corticosterone in plasma—Plasma CBG concentration was significantly lower in HT rats when compared with LT rats (Fig. 1). Once CBG capacity and total CORT are known, free CORT titers can be estimated using the mass action-based equation of Barsano and Baumann[14]. Total CORT levels and CBG capacity were used to estimate a mean for free CORT (Fig. 1). Error bars for free CORT were estimated by putting total CORT mean ± SE and CBG mean ± SE into the Barsano and Baumann[14] equation .

Discussion

This is the first study to categorize the rats as high and low tolerant according to their response time using hypoxic tolerance test. The principal finding of this study is that there was a distinct individual difference as shown in terms of gasping time or sensitivity to hypoxia and that may be mainly due to varying reactions occurring at the level of regulatory systems, the hormonal system in particular. Hypoxic stress stimulates several adaptive hormonal responses. These are the catecholamine, epinephrine and

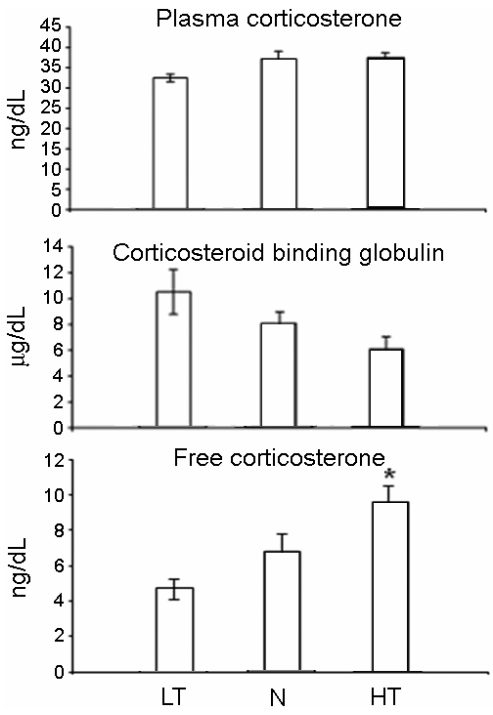

Fig. 1—Plasma level of total corticosterone corticosteroid binding globulin and free corticosterone in low tolerant (LT) , normal (N) and high tolerant (HT) groups of rats. [values are mean ± SE. *P<0.05 when compared with LT]

Table 1—Plasma ACTH, prolactin, testosterone, epinephrine and norepinephrine concentration of low (LT) and high tolerant (HT) rats exposed to hypoxic tolerance test
[Values are mean ± SE]

Groups	ACTH (pg/mL)	Prolactin (ng/dL)	Testosterone (ng/mL)	Epinephrine (pg/mL)	Norepinephrine (pg/mL)
Low tolerant (LT) (n=18)	28.82 ± 6.19	0.85 ± 0.090	2.72 ± 0.19	210.17 ± 8.5	262.62 ± 10.1
Normal (N) (n=20)	40.9 ± 3.01	0.69 ± 0.054	3.1 ± 0.5	225.7 ± 10.6	349.5 ± 14.3
High tolerant (HT) (n=14)	38.05 ± 2.60*	0.6 ± 0.060*	4.64 ± 0.36*	241.3 ± 10.8	414.66 ± 15.26*

*P<0.05 when compared with LT rats

norepinephrine from the adrenal medulla, corticosteroids from the adrenal cortex and adrenocorticotropin from the anterior pituitary[15,16]. Epinephrine and norepinephrine are released almost immediately from the sympathetic nervous system in response to a stressor. In the present study, plasma norepinephrine was significantly increased in high tolerant rats when compared to low tolerant animals. Significant increase of norepinephrine helps the high tolerant group to cope up with the stress more significantly than low tolerant group.

Individual differences have been reported in hormonal responses to different stresses and also in adrenal gland weight[17-21]. Present results also revealed the similar observation where the adrenal gland weight was significantly different in high and low tolerant rats.

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[22]. However, there was significant difference of adrenocorticotropic hormone (ACTH) between the two groups which further influence the level of corticosterone in rats. Comparative studies of the stress response also have focused primarily on the adrenocortical response to stress, in particular the measurement of plasma levels of glucocorticoids[23]. Present results 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[24,25], it was thought 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[26,27]. 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[28]. The link between stressor and CBG binding capacity has been illustrated. Thus, the amount of CORT that is taken up by target tissue is approximated by the non-CBG bound fraction of total circulating steroid[29,30].

In the present study, though there was no significant difference in total circulating level of corticosterone between HT and LT rats, 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[31]. Apart 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[31,32]. Present study also showed the similar type of observation where high tolerant rats had significantly low level of plasma prolactin than the low tolerant group.

Knol[33] 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 may 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 HT rats than LT 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 their gasping time and shows that multiple components of the stress

response (and not only plasma glucocorticoid), provide a basis for individual differences in physiological responses to hypoxic stress. Two individuals may or may not show the expected differences in glucocorticoid response but the plasticity in stress responsiveness may depend on various endocrine factors at multiple levels. The role of CBG as an active component of the stress response warrants further study.

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