Neuroendocrine profiling in inherited stress-induced arterial hypertension rat strain with stress-sensitive arterial hypertension

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Abstract

The functions of the hypothalamic adrenal cortical and sympathetic adrenal medullary systems were studied in rats with inherited stress-induced arterial hypertension (ISIAH strain). A characteristic feature of the ISIAH strain is an increase in arterial blood pressure measured both under basal conditions and after restraint stress in particular. In the control ISIAH rats, the basal plasma ACTH concentration was slightly lower than that in the normotensive Wistar albino Glaxo (WAG) rats, and no differences were found in plasma corticosterone. However, the 0·5-h restraint stress produced higher activation of the adrenal cortex in the ISIAH rats. Gluco- and mineralocorticoid responses to the blood volume reduction stresses and ACTH and corticosterone responses to social stress were stronger in the ISIAH than in the control WAG rats. An increase in epinephrine content in adrenals in the basal state and enhanced response of the sympathetic adrenal medullary system to handling stress were observed in the ISIAH rats. Restraint stress produced significantly higher expression of genes encoding corticotropin-releasing hormone-mRNA in hypothalamus and proopiomelanocortin-mRNA in pituitary in the ISIAH than in the WAG rats. Restraint stress produced a decrease in glucocorticoid receptor (GR) gene expression (GR-mRNA) in hippocampus in the ISIAH, but not in the WAG rats. A persistent increase in tyrosine hydroxylase-mRNA in adrenals of the ISIAH rats was found. It is concluded that the ISIAH rat strain is an appropriate model of stress-sensitive hypertension with the predominant involvement of the hypothalamic adrenal cortical and sympathetic adrenal medullary systems in its pathogenesis.

Journal of Endocrinology (2007) **195,** 439–450

Introduction

There is ample evidence indicating that emotional stress may be an important contributor to the formation of the hypertensive condition (Mustacchi 1990, Boone 1991, Mancia *et al.* 1997, Light 2001, Kaushik *et al.* 2004). Support has come from extensive clinical practice (Kaplan 1978, Zimmerman & Frohlich 1990, Schneider *et al.* 2005, Šantić *et al.* 2006) and experimental research (Lawler *et al.* 1981, Anderson *et al.* 1983, Sanders & Lawler 1992, Henry *et al.* 1995, Ely *et al.* 1997, Mormede 1997, McDougall *et al.* 2004). It is common knowledge that repeated exposure to stressful situation or the chronic stress state with elevated blood pressure may lead to persistent arterial hypertension (Kulkarni *et al.* 1998, Kario *et al.* 2003, Grassi & Mancia 2004). A major role may be played by genetic predisposition to the hypertensive response to emotional stress (Friedman & Iwai 1976, Huie *et al.* 1987, Harshfield & Grim 1997, Pickering 1997). Investigation of genetic propensity to increased arterial pressure response to stressful stimulation may be helpful in the prediction of the possible development of hypertension in the time to come (Light *et al.* 1999, Carroll *et al.* 2001, 2003, Davrath *et al.* 2003). The prognostic value of the stress responses considerably increased when evaluations were based not only on the blood pressure changes but also on the state of the entire neuroendocrine system, including the pituitary–adrenocortical (Litchfield *et al.* 1998, Whitworth *et al.* 2000, Gold *et al.* 2005), sympathetic adrenal (DiBona 2004, Chou *et al.* 2005, Miyai *et al.* 2005), and renal sodium handling (Genest 2001, Grisk & Rettig 2004). All these systems are directly involved both in the stress response and arterial blood pressure regulation.

A rat strain with stress-dependent arterial hypertension was developed to study its underlying genetic and physiological mechanisms (Markel 1985, 1992, Markel *et al.* 1999). For this purpose, we used the experimental paradigm of restraint stress

DOI: 10.1677/JOE-07-0254
0022–0795/07/0195–439
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involving selection. Rats were selected from an outbred Wistar population for an increase in the response of systolic arterial blood pressure to a brief emotional stress. Rats underwent 0·5-h restraint stress by confinement in small cylindrical wire mesh cages and the blood pressure was measured by the tail–cuff method. As a result of selection, a new rat strain with enhanced blood pressure was developed. We designated it as the inherited stress-induced arterial hypertension rat strain (ISIAH). The unexpected result of this selection was an increase in blood pressure not only after stress exposure but also at the baseline resting conditions. We regarded this as evidence that the enhanced responsiveness of blood pressure to the stress challenge may be linked in a way to the development of sustained hypertension. It was assumed that certain hormonal responses to stress affecting regulatory mechanisms of cardiovascular and kidney functions may contribute largely to the link.

When comparisons were based on measurements of plasma corticosterone content, the response of the ISIAH rats to such stressors as restraint, heat stress, epinephrine injection was enhanced relative to normotensive rats (Jacobson *et al.* 1996, Petrova *et al.* 1997, Antonov *et al.* 2000). The ISIAH hypertensives were found to differ from the Wistar albino Glaxo (WAG) normotensives in the response of corticosterone to the injection of the neuromediators serotonin, noradrenaline, and agonists of the adrenergic receptors into the brain ventricle (Markel *et al.* 1999). These findings prompted us to study, along with the peripheral adrenocortical mechanisms, the central hypothalamic–pituitary stress response, and also the function of the sympathetic adrenal system, which, as indicated above, is involved in the pathogenesis of the hypertensive disease.

The objective of this study was to investigate the hypothalamic–pituitary–adrenocortical and sympathetic adrenal medullary functions in the hypertensive ISIAH rats.

Materials and Methods

Animals

The ISIAH rat strain was developed at the animal facility of the Institute of Cytology and Genetics (Novosibirsk, Russia). We proceeded on the following considerations. We have observed that the indirect tail–cuff measurement of blood pressure in conscious restrained rats placed on a heated platform is quite stressful. Hence, the criterion for selection was based on checking both the blood pressure measured in unanesthetized rats (the value corresponded to the stress-induced) and the one measured in the same but ether anesthetized rats (the value corresponded to the baseline). The ether anesthesia made it possible to exclude the effect of psychoemotional arousal on the blood pressure data. Selection was started from the outbred Wistar rat population of about 1000 animals. The selection was of three steps. At the first step (for 17 generations), we avoided closely related crosses; then at the second step (for 13 generations), we crossed rats derived from closely related families; at the third step (for more than 20 generations), the brother–sister matings were performed.

All the procedures were carried out in accordance with the International Guidelines for Animal Experimentation (the UFAW Handbook on the Care and Management of Laboratory Animals) accepted by the Institute of Cytology and Genetics of the Russian Academy of Sciences, Siberian Branch (Novosibirsk). Day 25 after birth, ratlings were weaned from their mothers. Male and female ratlings of one litter were maintained separately, 4–5 per cage. The experiments were done with males aged 4–5 months. Before starting the tests, the control and treated rats were placed in a single cage for 6–7 days to eliminate the effects of the home cage social interactions. All the rats were maintained on the standard rat chow with drinking water available *ad libitum*.

Systolic blood pressure measurement

The tail–cuff method was used to measure the systolic blood pressure. A rat restricted in a small cage was placed on a heated (37 °C) platform. A 15 mm wide cuff was put on the proximal part of the tail. The pressure inside the cuff could be gradually raised with an automated pneumatic pump. The pressure in the cuff was measured with a pressure transducer EMT33-35 (Elema Schönender, Stockholm, Sweden). The part of the tail distal to the cuff was placed in a glass cylinder coated with vaseline to make it air proof. To register the tail artery pulse, the cavity of the cylinder was connected to a high-sensitivity low-pressure transducer EMT500 (Elema Schönender). The pressure inside the cuff and the pulse curve were registered synchronously with a Mingograph 34 (Elema Schönender). The time point when the pulse oscillations disappeared was compared with the pressure level inside the cuff. This pressure level corresponded to the systolic blood pressure of a rat. Six to seven blood pressure readings were obtained for each rat and averaged.

Procedures of rat stressing

Restraint stress Each rat was restrained in a wire mesh cylinder (6 cm in diameter). After 5-, 15-, or 30-min restraint, rats were decapitated and trunk blood was sampled for subsequent measurements of adrenocorticotropic hormone (ACTH) and corticosterone concentrations. Intact WAG and ISIAH rats not subjected to stress whose blood samples were collected in the same way as from the treated rats served as controls; 10 ISIAH and 10 WAG rats were included in each experimental and control group.

Ether stress and stress of reduced blood volume A rat was confined in a glass container saturated with ether for anesthesia. Five minutes after its onset, 2·5 ml blood sample was withdrawn from the tail vein (Omaye *et al.* 1987), while anesthesia was maintained with an ether nose cone. This blood sample was used to measure the corticosterone and aldosterone responses to the 5-min ether stress. After recovery from the anesthesia, the rat was returned to its cage; it was euthanized by

decapitation 1 h later, and the second blood sample was taken to measure the hormonal response to the first 2·5 ml blood loss regarded as a blood volume reduction stress. Changes in the concentrations of aldosterone and corticosterone in plasma in response to ether and blood loss stresses were studied in the same rats of the two compared strains. The control values for hormone concentrations were measured in plasma obtained after prompt decapitation of the non-stressed WAG and ISIAH rats (separate groups); 8–10 rats of each strain were included in the experimental and control groups.

Social stress In examination of the causative factors of the hypertensive disease, much attention has been paid to social stress. A simple model of stress of this kind was reproduced by placement of six adult males of different litters in a new common cage. The time of this stress in different common cages was 15, 30, or 45 min. After that, rats were decapitated and blood samples were taken to determine corticosterone concentrations. The control values of plasma hormone concentrations were determined in intact male rats of both the ISIAH and WAG strains; 9–10 rats of each strain were included in the experimental and control groups.

Blood sampling and hormone analysis

For the ACTH, corticosterone, and aldosterone measurements, the blood samples were collected from the tail vein in anesthetized rats (in the experiment with ether stress) or after prompt decapitation. Blood samples were collected in ice-cold EDTA-coated tubes immediately centrifuged, and the plasma was stored at −70 °C until the assay was conducted. ACTH and aldosterone were measured using commercial kits ELISA-ACTH (MD Biosciences International St Paul, MN, USA) and Aldosterone RIA (Immunotech, Beckman Coulter Co., Paris, France). Plasma corticosterone concentration was measured by RIA using specific antibodies (Sigma–Aldrich) and 3H-corticosterone (Amersham).

Evaluation of the sympathetic adrenal medullary function

Assessment of the sympathetic adrenal medullary function was based on the concentrations of norepinephrine, epinephrine, and dopamine in adrenal tissues and plasma. The adrenals from intact (naive) rats (ten rats of each strain) were removed promptly after killing and weighed. Tissue was homogenized with a glass homogenizer in 1·0 ml of 0·1 M perchloric acid, containing 1 μg/ml of 3,4-dihydroxybenzilamine (DHBA) as an internal standard. After 15 min of centrifugation (7000 ***g***), the supernatant was removed and filtered (0·2 μm). The supernatant was then diluted 1/25 with 0·1 M perchloric acid; 5 μl final solution was injected into the high pressure liquid chromatograph (HPLC) system. The concentration of monoamines was expressed in nanograms per gram of wet adrenal tissue.

The blood sample for catecholamine measurements was collected with a chronic cannula implanted into the carotid artery; 9–10 rats of each strain were subjected to chronic cannulation. Rats were anesthetized with 50 mg/kg b.w. pentobarbital (i.p). A Teflon catheter (0·9 mm i.d.) filled with heparinized saline was inserted through the right common carotid artery. The catheter was tunneled subcutaneously, exteriorized at the nape, and secured to the skin. Rats were given a 1-day recovery before proceeding with the experiment. Blood samples were collected through the indwelling cannula thrice for three consecutive days. Blood collection was associated with a brief handling that caused mild stress, especially on day 1. We noted that in the subsequent days, rats rapidly habituated to this procedure, leading us to conclude that handling stress was reduced. Thus, judgments about the response of the sympathetic adrenal system to mild emotional stress can rely on the attenuation of the response of catecholamines to repeated blood samplings.

Eppendorf vials (1·5 ml) containing 50 μl of 5% EDTA were used for blood collection. After centrifugation, 0·5 ml plasma was added to 0·2 ml of 1 M Tris buffer (pH 8·6), containing 20 μl DHBA (10 ng/ml) and 10 mg alumina. Simultaneously, a standard mixture (20 μl of each working standard) was prepared in 0·5 ml of 0·1 M sodium phosphate buffer (pH 7·4) and treated in the same manner as plasma. After 15 min of shaking and centrifugation, plasma was removed by a vacuum aspirator. Alumina was washed twice with 1 ml ice-cold distilled water or 0·02 M Tris buffer. Catecholamines were extracted from alumina with 20 μl of 0·1 M perchloric acid. Five microliters of this extract were injected into the column. Five microliters of a standard mixture, treated in the same way, were also injected in an HPLC system for determination of the recovery coefficient.

Norepinephrine, epinephrine, and dopamine contents in plasma and adrenals were measured by HPLC. The system consisted of a syringe chromatographic pump Milichrom-1 and an electrochemical detector with a glassy carbon electrode (Nauchpribor Ltd, Orel, Russia). A chromatograph was equipped with stop-flow injection unit and stainless steel column of 2 mm i.d. and 65 mm in length, packed with Nucleosil C18, 5 μm (Macherey-Nagel, Düren, Germany). The mobile phase consisted of aqueous 0·05 M sodium dihydrogen phosphate, 0·05 M citric acid buffer, containing 0·5 g/l sodium octyl sulfonate (Sigma), and 60 mg/l EDTA. After buffer titration with sodium hydroxide up to pH 4·9, 15% (v/v) of freshly distilled methanol was added. The eluent was filtered (0·2 μm, millipore filter) and degassed prior to use. Eluent flow rate was 100 μl/min. The working electrode was operated at potential +0·6 V versus Ag/AgCl reference electrode. All standards and other reagents were purchased from Sigma. Stock solutions of the standards (1 mg/ml) were prepared in 0·1 M perchloric acid. Working solutions of the standards (10 ng/ml for blood and 40 ng/ml for the adrenals in 0·1 M perchloric acid) were prepared once a month and stored in a refrigerator.

Evaluation of proopiomelanocortin (POMC), corticotropin-releasing hormone (CRH), glucocorticoid receptor (GR), and tyrosine hydroxylase (TH) gene expression

To study the effect of stress on gene expression, rats were restrained for 2·5 h in the wire mesh cylinders. The rats

were promptly decapitated after the restraint stress, the tissues were sampled, frozen in liquid nitrogen, and stored at −70 °C until analysis. Tissues were obtained similarly from the control rats without preliminary stress; 6–8 rats of each strain were included in the experimental and control groups. Gene expression was studied in the following tissues: pituitary, POMC; hypothalamus, CRH; hippocampus, GR; adrenal, TH.

RNA isolation Total RNA was extracted from tissues according to the method described by Chattopadhyay *et al.* (1993) with modifications. Briefly, tissues of pituitary, hypothalamus, hippocampus, and adrenal medulla from a rat were homogenized in a mixture of water saturated phenol (10V/1V tissue) and 0·5% SDS (5V/1V tissue). Then, 2 M sodium acetate (pH 4·2) was added to 1/8 of the total volume. The homogenate was transferred into an Eppendorf tube and centrifuged for 10 min at 9000 ***g*** (Eppendorf Centrifuge 5414). Water phase was transferred into a fresh tube and purified twice by an equal volume of phenol/chloroform (1 V:1 V) followed by one cycle of extraction by an equal volume of chloroform. All the extractions were performed at 9000 ***g*** for 5 min. RNA was precipitated by 96% ice-cold ethanol at −70 °C for 30 min. The quality of RNA obtained was assessed by electrophoresis of 1 μl in 1% agarose gel.

DNA degradation Remaining traces of genomic DNA were removed from the RNA samples using DNase I ('Promega') treatment according to the manufacturer's protocol.

cDNA synthesis To obtain the cDNA, the 300–500 ng total RNA in 15 μl was mixed with 800 ng dT18 primer. After RNA denaturation (5 min at 65 °C) and primer annealing (5 min at 37 °C), we added RT buffer mix (up to 30 μl of 20 mM Tris–HCl, pH 8·3, 10 mM dithiothreitol, 100 mM KCl, 5 mM $MgCl_2$), 500 μM dNTPs, and 60 units of MoMLV reverse transcriptase (ICBFM SB RAS, Novosibirsk, Russia). We synthesized cDNA at 37 °C for 1 h, 42 °C for 30 min, and 50 °C for 10 min. The enzyme was inactivated by heating at 80 °C for 10 min. For subsequent PCR, we used from 0·5 to 2 μl cDNA.

Competitive PCR POMC-mRNA expression level was evaluated by RT-competitive PCR based on coamplification of cDNA with standard amounts of competitor DNA. To construct the competitor, we used the procedure described in Carrasco *et al.* (1997). We amplified phage T7 DNA with a pair of POMC-cDNA-specific primers at low annealing temperature and picked up a fragment whose amplification efficiency but not size were similar to cDNA. The fragment was cut out from 6% polyacrylamide gel (PAG), reamplified at specific temperature, eluted from gel again, and concentrated. A constant amount of cDNA was coamplified with a set of competitor DNA concentrations in which the competitor was diluted 1, 5, 25, and 125 times. The reaction was performed in 20 μl PCR buffer (67 mM Tris–HCl, pH 8·9; 16 mM $(NH_4)_2SO_4$; 1·5 mM $MgCl_2$; 0·01% Tween 20; 10 mM β-mercaptoethanol), containing 1 μM primers, 0·2 mM dNTPs, 1 μl cDNA, 1 μl competitor, and 1 unit of Taq-polymerase (ICBFM SB RAS) in the following conditions: 95 °C for 3 min, then 36 cycles at 94 °C for 1 min, 60 °C for 10 s, and 72 °C for 20 s. The PCR products were separated on 6% PAG and visualized with ethidium bromide. The intensities of the bands for POMC-cDNA and competitor DNA were quantified using the Scion Image software. The amount of the competitor required to give a 1:1 optical ratio of cDNA to the competitor PCR products was then determined graphically. This gave a measure of POMC-cDNA levels contained in the samples. The experiments following the same protocol were repeated twice or thrice and the results were combined for statistical analysis. To normalize the total cDNA amount in individual samples, ribosome protein RP L30 cDNA levels were detected using the same procedure and RP L30-specific competitor.

Multiplex PCR For identification of CRH-mRNA level in hypothalamus and GR-mRNA level in hippocampus, we used the RT-multiplex PCR method. After total RNA extraction from tissues and synthesis of the first cDNA strand with unspecific dT18 primer, we coamplified in the same tube the target cDNA directly with a normalizing cDNA using two pairs of specific primers. In preliminary experiments, we chose the following normalizing genes: phospholipid protein (PLP; the gene for myelin sheath lipoprotein) for CRH-cDNA and β-actin for GR-cDNA. In choice of a normalizing gene, we took into account the comparability of amplification efficiency and product size between target and normalizing cDNAs. Initially, we performed a set of PCRs using each pair of primers to determine the interval of cycle numbers when PCR products could be visualized by staining with ethidium bromide and, at the same time, PCR remained within the exponential phase. The appropriate PCR cycle (assigned *N*) to add primers for normalizing gene was identified by the 'primer-dropping' method (Wong *et al.* 1994) so that the PCR product bands for the target and normalizing genes were of similar intensity after the reaction terminated ($N=10$ for PLP and $N=8$ for β-actin). Coamplification was carried out in 15 μl PCR buffer, standard PCR buffer with 0·2 mM dNTPs, 1 μM primers, and 1 unit of Taq-polymerase in the following conditions: initial denaturation at 95 °C for 3 min, followed by *X* cycles at 94 °C for 30 s, tanneal for 10 s, and 72 °C for 30 s ($X=33$ for CRH/PLP and $X=29$ for GR/β-actin). The PCR products were electrophoresed in 8% PAG (GR/β-actin) or in 2% agarose gel (CRH/PLP) and visualized with ethidium bromide. The density and the width of each band were measured using the Gel-Pro Analyser software (Media Cybernetics Inc., Bethesda, MD, USA).

Measurement of the TH gene expression (real-time PCR)

Gene-specific TaqMan PCR primers and probes for TH were purchased from PE Applied Biosystems, each probe was synthesized with a fluorescent 5′-reporter dye (FAM: 6-carboxy-fluorescein) and a 3′-quencher dye (TAMRA: 6-carboxytetramethyl-rhodamine). Parallel PCR analysis was

run for the housekeeping gene RPL29 to normalize data for differences in mRNA quantity and integrity. The reaction mixtures contained 1× TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 1× TH-specific primer and probe mixture or 1× RPL29-specific primer and probe mixture as an endogenous control (Applied Biosystems). To quantify TH expression, an amount of cDNA corresponding to 8 ng reverse-transcribed RNA was used in a 20 μl reaction volume.

Relative copy numbers of TH transcripts and RPL29 cDNA were determined using calibration curves generated with standards of known concentrations. The procedure used for the PCRs was: 94 °C for 10 min (activation of hot-start DNA polymerase), and then 40 cycles at 94 °C for 30 s followed by 72 °C for 2 min, as recommended by Applied Biosystems. A no-template control with water was performed in parallel in all the experiments. Each series of experiments was performed twice.

Statistical analysis

Analysis of interstrain differences in adrenal catecholamines content was performed using paired Student's *t*-test. Statistical comparisons of two phenotype groups subjected to different stress exposures were assessed with two-way ANOVA. The *post hoc* comparison of means was performed with Tukey's honestly significant difference (HSD) test. The null hypothesis was rejected at $P<0{\cdot}05$. Data in the figures are shown as mean ± S.E.M.

Results

Arterial blood pressure

The basal levels of systolic blood pressure measured by the indirect tail–cuff method in the ether anesthetized male rats were 128±3 ($n=27$) mmHg in the WAG and 167±3 ($n=27$) mmHg in the ISIAH strains. The stress-induced levels of systolic blood pressure measured by the same tail–cuff method in the conscious rats restrained in small wire mesh cylinders for 0·5 h were 137±3 ($n=27$) mmHg in the WAG rats and 198±4 ($n=27$) mmHg in the ISIAH rats. The blood pressure values were significantly dependent on rat strain ($F_{1,104}=259$; $P<0{\cdot}00001$) and the restraint stress ($F_{1,104}=39$; $P<0{\cdot}00001$). Also, strain–stress interaction reached significance ($F_{1,104}=12{\cdot}8$; $P<0{\cdot}00053$). This interaction was produced by the very different responses of the ISIAH and the WAG rats to stress: the increment in blood pressure was 30 mmHg in the ISIAH and 8 mmHg only in the WAG rats. *Post hoc* Tukey's HSD test revealed highly significant differences between all the compared blood pressure values.

Hormonal response of the pituitary–adrenocortical system to stress

The response to the 30-min restraint stress Restraint stress was accompanied by a rapid increase in plasma ACTH concentration in both the ISIAH and WAG strains, but it was more pronounced in the WAG rats (Fig. 1, upper panel). After the rats

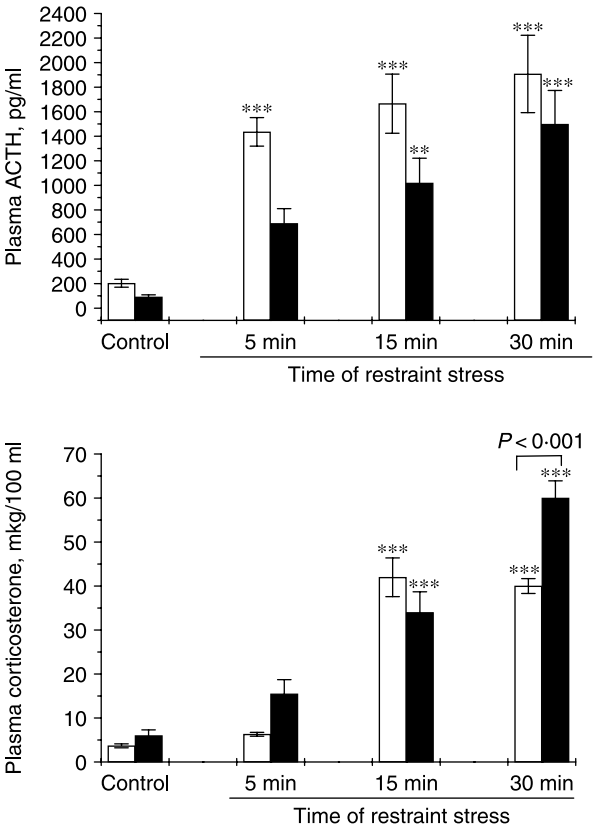

Figure 1 Effect of 30-min restraint stress on ACTH (upper panel) and corticosterone (lower panel) plasma concentrations (mean ± S.E.M., $n=10$ in each group) in the WAG (white columns) and the ISIAH (black columns) rat strains. Significant differences between experimental and control groups are shown by asterisks: $^{**}P<0{\cdot}010$, $^{***}P<0{\cdot}001$. Significant interstrain differences are shown by *P* values above the bars. Data were compared by two-way ANOVA with Tukey's HSD test.

experienced restraint for 30 min, plasma ACTH concentration became virtually the same in both strains. Significant effect of strain ($F_{1,72}=12{\cdot}04$; $P<0{\cdot}001$) and stress ($F_{3,72}=23{\cdot}33$; $P<0{\cdot}0001$) on plasma ACTH changes during restraint was observed. No effect of the stress–strain interaction was found. A *post hoc* pairwise Tukey's comparison revealed significant differences between the control levels of ACTH and subsequent stress induced hormone concentrations measured on min 15 ($P<0{\cdot}01$) and 30 ($P<0{\cdot}001$) of stress in the ISIAH rats and on min 5 ($P<0{\cdot}001$), 15 ($P<0{\cdot}001$), and 30 ($P<0{\cdot}001$) of stress in the WAG rats. The interstrain differences between the ACTH concentrations both in control and under stress were not significant.

As for plasma corticosterone, the pattern was quite different (Fig. 1, lower panel). Effects of strain ($F_{1,72}=7{\cdot}63$; $P<0{\cdot}01$) and stress ($F_{3,72}=106$; $P<0{\cdot}0001$) on the corticosterone changes were statistically significant, like the effect of interaction of these two main factors ($F_{3,72}=7{\cdot}62$; $P<0{\cdot}0001$). Restraint stress resulted in significant plasma corticosterone rise relative to the control levels (Tukey's *post hoc* test) in both rat strains on min 15

($P<0{\cdot}001$) and 30 ($P<0{\cdot}001$) of stress and at the end of stress, on min 30, plasma corticosterone concentration in the ISIAH was much higher than that in the WAG rats ($P<0{\cdot}001$).

Gluco- and mineralocorticoid responses to the ether and blood volume reduction stress

Strain had no significant effect on aldosterone level in plasma. Stress revealed the major influence on the plasma aldosterone dynamics ($F_{2,53}=57{\cdot}2$; $P<0{\cdot}0001$; Fig. 2, upper panel). A significant increase (Tukey's *post hoc* test) in plasma aldosterone relative to the control levels was observed in both the WAG ($P<0{\cdot}001$) and the ISIAH ($P<0{\cdot}001$) rats only after blood loss stress. This increase was more pronounced in the ISIAH than in the WAG rats, and this was responsible for the stress–strain interaction effect ($F_{2,53}=5{\cdot}62$; $P<0{\cdot}01$). Ether stress had no influence on plasma aldosterone concentration relative to the control in both strains.

Both main factors, rat strain ($F_{1,54}=45{\cdot}8$; $P<0{\cdot}0001$) and stress ($F_{2,54}=73{\cdot}87$; $P<0{\cdot}0001$), significantly affected corticosterone dynamics in this experiment (Fig. 2, lower panel). Two types of stress, ether and blood loss, were followed by significant increase in plasma corticosterone in both rat strains when compared with the control groups ($P<0{\cdot}001$ for the both stressors and both rat strains; Tukey's *post hoc* test), but corticosterone level after the blood loss was significantly higher in the ISIAH than in the WAG rats ($P<0{\cdot}0001$, Tukey's *post hoc* test). In ISIAH, but not in the WAG rats, blood loss stress resulted in significantly higher plasma corticosterone concentration than the ether stress ($P<0{\cdot}001$, Tukey's *post hoc* test). This interstrain difference in response to blood loss stress resulted in significant effect of strain–stress interaction ($F_{2,54}=28{\cdot}29$; $P<0{\cdot}0001$).

Effect of social stress

The patterns of ACTH increase induced by social stress were different in the ISIAH and WAG rats (Fig. 3, upper panel). In the WAG rats, the highest concentration of ACTH in plasma was reached rapidly on min 15 of stress and then it tended to

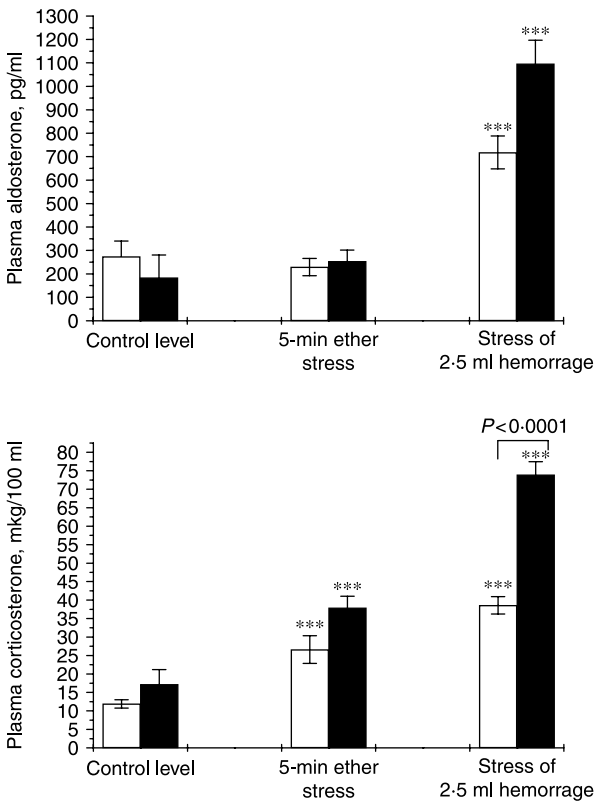

Figure 2 Effect of the 5-min ether stress and stress of 2·5 ml blood loss on aldosterone (upper panel) and corticosterone (lower panel) plasma concentrations (mean±S.E.M., $n=8$–10 in each group) in the WAG (white columns) and the ISIAH (black columns) rat strains. Significant differences between experimental and control groups are shown by asterisks: ***$P<0{\cdot}001$. Significant interstrain differences are shown by P values above the bars. Data were compared by two-way ANOVA with Tukey's HSD test.

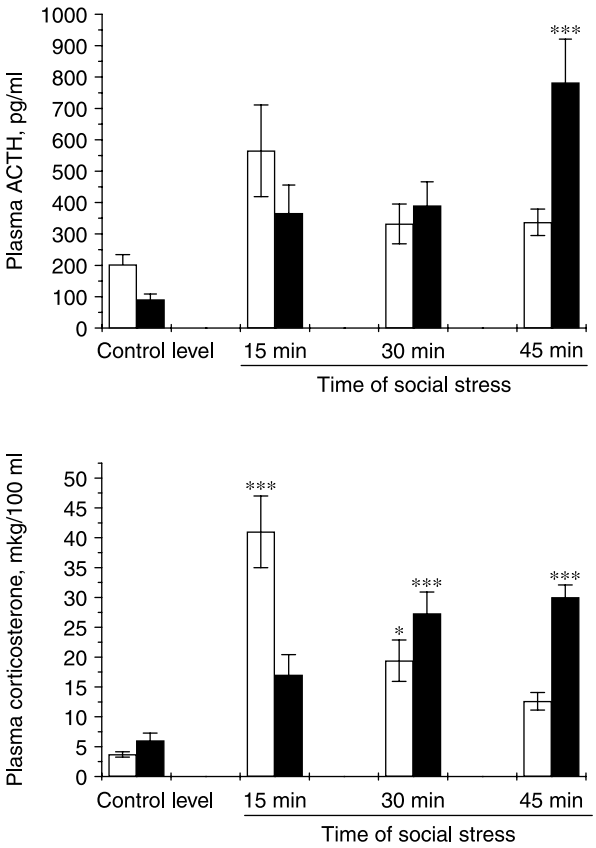

Figure 3 Effect of social stress on ACTH (upper panel) and corticosterone (lower panel) plasma concentrations (mean±S.E.M., $n=9$–10 in each group) in the WAG (white columns) and ISIAH (black columns) rat strains. Significant differences between experimental and control groups are shown by asterisks: *$P<0{\cdot}05$, ***$P<0{\cdot}001$. Data were compared by two-way ANOVA with Tukey's HSD test.

decrease. In the ISIAH rats, plasma ACTH concentration rose slowly and reached the highest level by the end of stress on min 45. This intersection of the time courses of plasma ACTH in ISIAH and WAG rats made significant the effect of strain–stress interaction ($F_{3,68}=5{\cdot}677$; $P<0{\cdot}001$). No significant effect of strain on plasma ACTH changes in socially stressed rats was demonstrated, but the effect of stress was significant ($F_{3,68}=9{\cdot}227$; $P<0{\cdot}0001$). In the ISIAH rats, plasma ACTH concentration on min 45 of stress was higher than in the control group ($P<0{\cdot}001$, Tukey's *post hoc* test).

The course of changes in plasma corticosterone in the socially stressed rats (Fig. 3, lower panel) was the same as that for the ACTH changes. The effect of strain was insignificant and that of social stress was very prominent ($F_{3,66}=23{\cdot}399$; $P<0{\cdot}0001$). Like in the case of ACTH, the highest value of plasma corticosterone relative to control level was reached on min 15 of stress by the WAG rats ($P<0{\cdot}001$, Tukey's *post hoc* test) and on min 45 of stress by the ISIAH rats ($P<0{\cdot}001$, Tukey's *post hoc* test). This different dynamics of corticosterone response to social stress in the ISIAH and WAG rats underlay the significance of the strain–stress interaction effect ($F_{3,66}=16{\cdot}464$; $P<0{\cdot}0001$).

Reactivity of the sympathetic adrenal medullary system

The norepinephrine and dopamine contents measured in adrenals of control unstressed rats were lower in the ISIAH than in the WAG rats ($P<0{\cdot}001$ for norepinephrine and $P<0{\cdot}05$ for dopamine interstrain differences, Student's *t*-test; Fig. 4). However, the value of the main adrenal catecholamine epinephrine (the concentration of epinephrine in adrenal medulla exceeded about tenfold that of norepinephrine) was significantly increased in the adrenals of the hypertensive rats ($P<0{\cdot}001$, Student's *t*-test).

The sympathetic adrenal medullary activity was further clarified by measuring the catecholamine concentration in the blood sampled at three consecutive days through a chronically implanted arterial cannula (Fig. 5). Both the main factors, strain and sampling day, did not reveal significant effects on norepinephrine and dopamine concentrations in peripheral blood. As for plasma epinephrine concentration, the influence of strain ($F_{1,67}=7{\cdot}1038$; $P<0{\cdot}01$) and day of blood collection ($F_{2,67}=6{\cdot}1620$; $P<0{\cdot}01$) reached significance. In the ISIAH rats, the epinephrine response to handling associated with the blood sampling was distinctly higher on days 1 ($P<0{\cdot}01$) and 2 ($P<0{\cdot}01$, Tukey's *post hoc* test) as was compared with the epinephrine response on day 3 of sampling. In the WAG rats, plasma epinephrine concentration was low and it did not respond to the handling stress associated with the first days of blood sampling.

Expression of the key genes for the function of the hypothalamic–pituitary–adrenocortical and sympathetic adrenomedullary systems

The expression of the four genes encoding CRH in the hypothalamus (CRH-mRNA), POMC in pituitary (POMC-mRNA), GR in hippocampus (GR-mRNA), and TH in adrenal medullary tissue (TH-mRNA) was studied (Fig. 6).

CRH gene expression Effect of 2·5-h restraint stress on the CRH-mRNA content in hypothalamus was significant ($F_{1,25}=4{\cdot}56$; $P<0{\cdot}05$). This effect appeared due to significant elevation of the CRH-mRNA content in hypothalamus of the ISIAH rats compared with the control level ($P<0{\cdot}05$, Tukey's *post hoc* test). The control levels of the CRH gene expression in the ISIAH and WAG rats were virtually the same and the CRH-mRNA content in hypothalamus of the WAG rats remained unchanged after 2·5 h of restraint stress.

POMC gene expression Two main factors, rat strain and stress, and also their interaction significantly affected the levels of the POMC-mRNA in pituitary ($F_{1,17}=21{\cdot}88$; $P<0{\cdot}001$ for strain; $F_{1,20}=14{\cdot}28$; $P<0{\cdot}01$ for stress; $F_{1,20}=5{\cdot}16$; $P<0{\cdot}05$ for strain–stress interaction). The POMC gene expression in pituitaries of the ISIAH rats was augmented when compared with that of the WAG normotensives both in the control ($P<0{\cdot}01$, Tukey's *post hoc* test) and stress ($P<0{\cdot}05$, Tukey's *post hoc* test) conditions. In the ISIAH rats, stress resulted in a considerable increase in the level of the POMC-mRNA, when compared with the control level ($P<0{\cdot}01$, Tukey's *post hoc* test). The WAG rats had a relatively low POMC gene expression in the control conditions, and they did not respond to the restraint stress exposure by a significant increase in the POMC-mRNA above the control level.

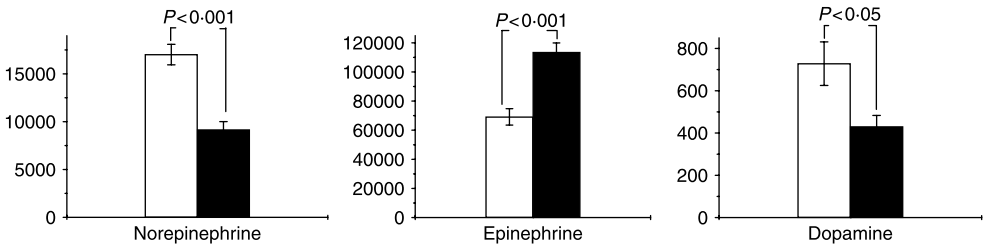

Figure 4 Adrenal catecholamine content (nanograms per gram of tissue from both adrenals, mean ± S.E.M., $n=10$ in each group) in the WAG (white columns) and the ISIAH (black columns) rat strains. Significance of interstrain differences was calculated by Student's *t*-test.

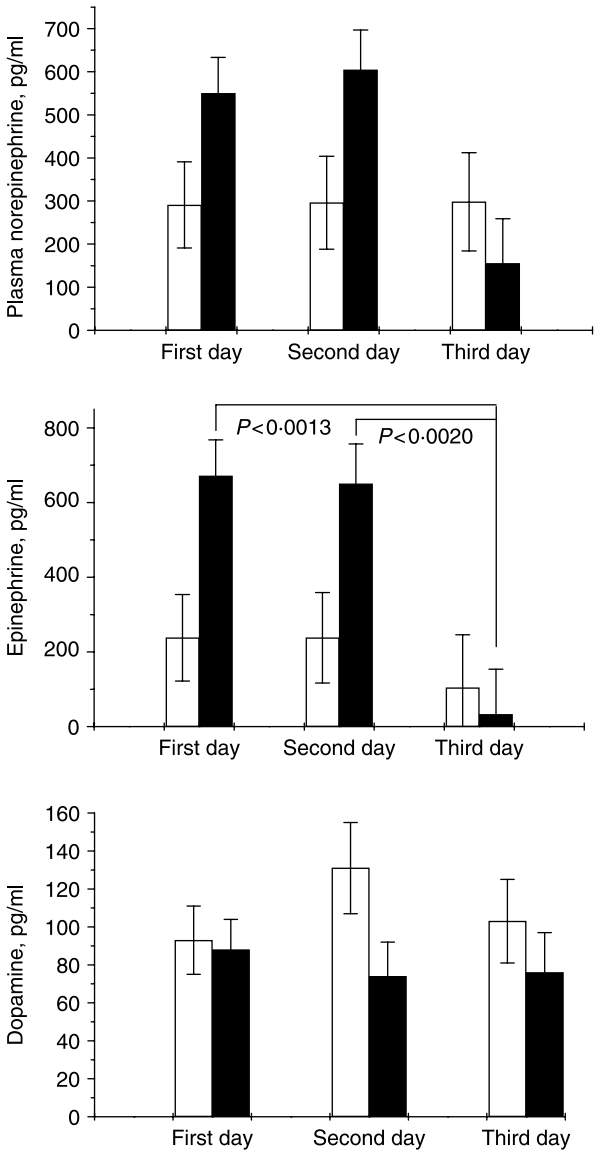

Figure 5 Concentration of norepinephrine, epinephrine, and dopamine in the blood plasma sampled for three consecutive days through a cannula indwelling in the carotid artery of the WAG (white columns) and the ISIAH (black columns) rats (mean ± S.E.M., $n = 9$–10 in each group). Data were compared by two-way ANOVA with Tukey's HSD test.

GR gene expression Stress was the only main factor affecting the GR-mRNA content in hippocampus ($F_{1,21} = 10{\cdot}16$, $P < 0{\cdot}01$). Expression of the GR gene in hippocampus was decreased after the 2·5-h restraint stress in the ISIAH hypertensives when compared with the control level ($P < 0{\cdot}001$, Tukey's *post hoc* test), yet remaining unaltered in the WAG normotensives.

TH gene expression A true effect of the stress procedure ($F_{1,25} = 4{\cdot}67$; $P < 0{\cdot}05$) and significant strain–stress interaction ($F_{1,25} = 8{\cdot}98$; $P < 0{\cdot}01$) were demonstrated for the TH-mRNA changes in adrenal medulla. After stress, the TH-mRNA in the WAG rats increased ($P < 0{\cdot}05$; Tukey's *post hoc* test), and reached the level found in the control ISIAH rats. In the ISIAH rats, this stress did not produce further elevation in the TH-mRNA in comparison with the control level.

Discussion

Rat strains with increased blood pressure have been developed worldwide in attempts to reproduce human hypertension (Yamori 1984, Rapp 2000). The hallmark feature of all the rat strains is increased blood pressure, but numerous comparative studies revealed that different genetic and physiological mechanisms underlie the hypertension in the strains (Ferrari & Bianchi 1995, Kurtz 1995). This supported the heterogeneous nature of the human hypertensive disease. As for the ISIAH hypertensive rats, it is a reasonable inference that their enhanced hypothalamic–pituitary–adrenocortical and sympathetic adrenal medullary responsiveness to stress may have a crucial influence on their hypertension formation.

Indeed, the current study showed that the activity of the sympathetic adrenomedullary system in the ISIAH rats was enhanced when compared with the WAG normotensives. Elevated content of epinephrine in adrenals, also an increase in the expression of the gene encoding the key enzyme of catecholamine synthesis TH were evident in the ISIAH rats under control conditions. Epinephrine concentrations in the blood sampled through a chronically implanted arterial cannula were significantly higher in the ISIAH rats on days 1 and 2 of blood sampling than on day 3. We suggested that the first two days of blood sampling in the ISIAH rats was associated with a mild emotional stress (handling) and the ISIAH rats responded to this stress with an increase in epinephrine synthesis. In the WAG rats, no response of catecholamines to this kind of stress was observed. All this indicated that the sensitivity of the sympathetic adrenal system to the mild emotional stress and to the 'stress of life' (the control conditions) was increased in the ISIAH rats.

Analysis of the activity of the hypothalamic–pituitary–adrenocortical system revealed also significant changes in the ISIAH rats. They concerned the function of both the central (hypothalamus, pituitary) and the peripheral (adrenal cortex) links of the system. Restraint stress produced a significant enhancement of the expression of the CRH and POMC genes in the ISIAH rats, whereas the WAG rats showed no significant changes in their expression. In addition, a decrease in GR-mRNA was observed in the ISIAH rats after the restraint stress. This may be indicative of an attenuation of the negative feedback link. This may explain, at least partly, why the stress response in ISIAH rats was continued and significantly higher than in WAG rats. In addition, comparisons of the ACTH and corticosterone plasma levels in the course of restraint stress in the ISIAH and WAG rats

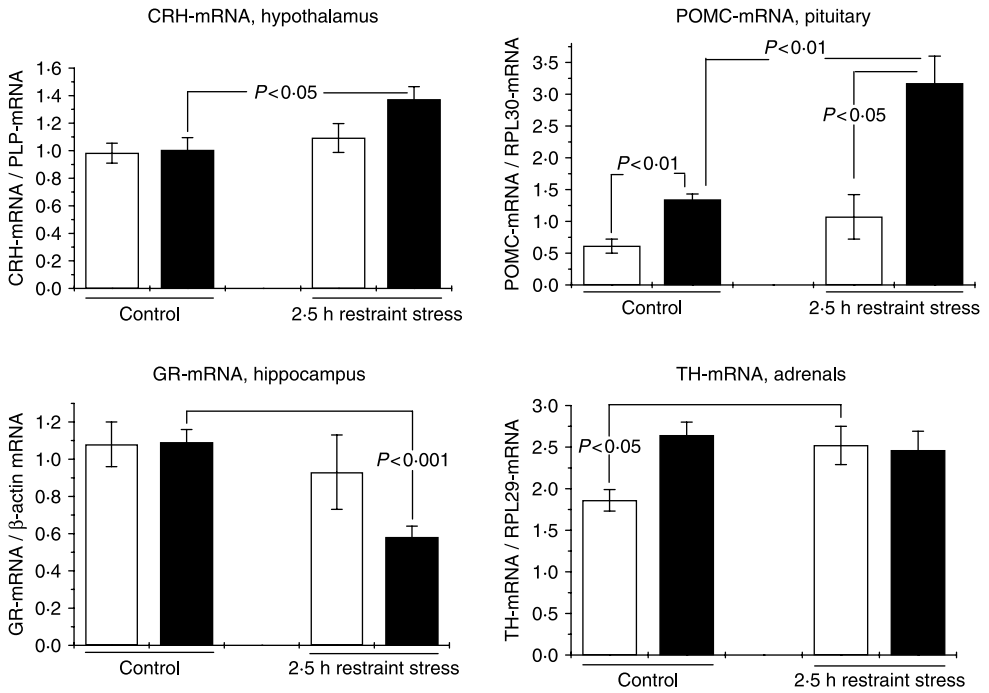

Figure 6 Gene expression in the WAG (white columns) and the ISIAH (black columns) rat strains (mean ± S.E.M., $n=6–8$ in each group). Data were compared by two-way ANOVA with Tukey's HSD test.

suggested higher sensitivity of the adrenal cortex of the ISIAH rats to the stimulating effect of ACTH.

It was found that the glucocorticoid response of the adrenal cortex in the ISIAH and WAG rats was dependent on the stress type. The dynamics of the hormonal response to the social stress was different from that to the restraint stress and was entirely different in the two strains. In the WAG rats, the response developed rapidly and was followed by its rapid extinction. In the hypertensives, the response was gradual and much faster than in the WAG rats by the end of stress on min 45.

The ISIAH rats displayed a dramatic increase in both the gluco- and mineralocorticoid responses caused by blood loss stress. It may be supposed that brain and kidneys of ISIAH rats became habituated to high-pressure blood perfusion and lowering of high pressure after the blood loss produced strong hormonal responses directed to water and sodium retention to restore the blood volume and the high blood pressure. In the control condition, the plasma aldosterone and corticosterone concentrations in the ISIAH and WAG rats were practically the same.

Putting all together, it appears that the function of the sympathetic adrenomedullary and hypothalamic–pituitary–adrenocortical systems are considerably modified in the ISIAH hypertensives. Changes in stress responsiveness and in the function of these hormonal regulatory mechanisms may be involved in development of different psychological and cardiovascular pathological states (Chrousos & Gold 1998, de Kloet *et al.* 2005). Taking into account that stress is the main cause of arterial hypertension in the ISIAH rats, it appears that the observed changes in their hormonal responses to stress may underlie the hypertension development.

The general pattern of changes in the neuroendocrine profiling of the ISIAH rats resembles that of the much more widespread spontaneously hypertensive rat (SHR) strain. In both strains, a considerable increase in the sympathetic adrenal medullary activity was observed (for the SHRs, see Judy *et al.* 1976, Korner *et al.* 1993, O'Connor *et al.* 1999, Cabassi *et al.* 2002, Reja *et al.* 2002, Kuo *et al.* 2004). Emotional stress caused by air jet induced a significantly threefold greater tachycardia and a tenfold greater activation of the sympathetic adrenal system in the SHR than in the Wistar Kyoto (WKY) rats (Zhang & Thoren 1998). Enhanced response to stimulation of SHR adrenals isolated *in vitro* was found, electrical stimulation increased the norepinephrine output from the SHR adrenals significantly greater than from those of the normotensive WKY rats (Nagayama *et al.* 1999). Stimulation of cholinergic receptors of isolated *in vitro* adrenals of the SHR produced a more prominent catecholamine release than from those of the WKY rats (Lim *et al.* 2002). The role of hyperactivity of the sympathetic adrenal system in sustained hypertension has been confirmed by experiments with its inhibition; long-term inhibition of the sympathetic adrenal system in young but mature SHR by rilmenidine, a centrally active antihypertensive agent interacting with imidazoline receptors, not only reduced blood pressure to normotensive levels, but had beneficial effects on cardiovascular structure, potentially reducing risk factors for

cardiac and renal abnormalities frequently seen in long-term hypertension (Bobik *et al.* 1998). A more modern approach to treatment of hypertension in the SHRs has been reported; the effect of antisense oligodeoxynucleotides against TH on hypertension and sympathetic nervous system activity has been demonstrated: systolic blood pressure in the treated SHRs became significantly lower than that in the control SHRs, epinephrine and norepinephrine levels, TH activity, and TH protein levels in the adrenal medulla of the treated SHRs were reduced concomitantly with changes in systolic blood pressure (Kumai *et al.* 2001).

Nevertheless, some discrepancies between the data obtained for the ISIAH and SHRs should be noted. Moura *et al.* (2005) demonstrated that, in contrast to increased norepinephrine content in both plasma and tail artery wall, which corresponds to higher peripheral sympathetic activity, the basal TH activity and catecholamine content in the adrenals of SHRs were markedly reduced before, during, and after the development of hypertension. Our findings revealed increase in the level of the TH-mRNA and the epinephrine adrenal content in the control ISIAH rats.

Certain differences between the ISIAH and SHRs were observed in the functional features of the hypothalamic–pituitary–adrenocortical system. The adrenal cortical responsiveness to stressful stimulation was increased in the SHRs, too, although, differences from the ISIAH rats were found in their responses to different stressors. The SHR strain is more sensitive to handling and less to restraint stress when compared with the normotensive controls (Hausler *et al.* 1983, Roman *et al.* 2004). Moreover, the increased responsiveness of the SHRs was not associated with an increase in the reactivity of the central hypothalamic–pituitary link. The effects of chronic stress on the hypothalamic–pituitary–adrenocortical axis were studied in five inbred rat strains: Brown Norway, Fischer, Lewis, SHR, and WKY (Gomez *et al.* 1996). In rats under basal conditions (in the morning), there were no differences among strains in adrenal weight, plasma ACTH and corticosterone levels, CRH-mRNA in the hypothalamic paraventricular nucleus (PVN), and the hippocampal glucocorticoid and mineralocorticoid receptor (GR- and MR-) mRNAs. Although the chronic stress increased the corticotrophin releasing factor (CRF) mRNA content in the PVN, the responses were similar in all the compared strains. Chronic immobilization stress down-regulated the GR-mRNA in hippocampus and slightly up-regulated the CRF-mRNA in the hypothalamic PVN, and these changes were similar in all the strains (Gomez *et al.* 1996).

Clearly, our ISIAH rat model for stress-induced arterial hypertension differs in heavier involvement of the hypothalamic–pituitary–adrenocortical system in the pathogenesis of the hypertensive disease from the SHR model for spontaneous arterial hypertension.

As for the renin system, our previous study showed no evidence for increased endocrine function of the renin–angiotensin system in the ISIAH rats (Amstislavsky *et al.* 2005). In the SHRs, the function of the endocrine renin–angiotensin system, assessed by either plasma renin activity and plasma angiotensin-II concentration, or kidney renin content, was not enhanced, too (Koletsky *et al.* 1972, Dietz *et al.* 1984, Iwai *et al.* 1996, Hlavacova *et al.* 2006).

It should be noted that, despite the general resemblance of the phenotypic changes in the neuroendocrine profiling between the two rat hypertensive models, the quantitative trait loci (QTLs) associated with an increase in blood pressure are, at least partly, different in the SHR and ISIAH rats (Redina *et al.* 2006). From this observation, it follows that a specificity of the genetic and certain of the pathophysiological backgrounds of arterial hypertension may be masked by the similar patterns of phenotypic expression of these partly different mechanisms underlying hypertension. Many researchers have described the involvement of the neuroendocrine, particularly of the sympathetic adrenal medullary mechanisms, in the formation of arterial hypertension in human (Mancia *et al.* 1997, Williams 1997, Sharma *et al.* 1998, DeQuattro & Feng 2002, DiBona 2004). The sympathetic-dependent changes in the kidney function would contribute additionally to the pathogenesis and progression of the hypertensive disease (Schneider *et al.* 2001, Aileru *et al.* 2004, Grisk 2004, Schlaich *et al.* 2004, Huang *et al.* 2005).

In conclusion, the ISIAH rat strain may be regarded as a good model of the human hypertensive disease with the predominant involvement of the neuroendocrine hypothalamic–pituitary–adrenocortical and sympathetic adrenal medullary systems during the disease development. The characteristic feature of the ISIAH strain is the genetically determined enhanced responsiveness to stressful stimulation.

Acknowledgements

This work was supported by grants from the Russian Foundation of Basic Research and from the Russian Academy of Sciences. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 25 August 2007
Accepted 1 October 2007
Made available online as an Accepted Preprint
1 October 2007