Legal Medicine 16 (2014) 193–196

Contents lists available at ScienceDirect

Legal Medicine

journal homepage: www.elsevier.com/locate/legalmed

Temporal changes of the adrenal endocrine system in a restraint stressed mouse and possibility of postmortem indicators of prolonged psychological stress

Takahito Hayashi [a,*], Kazuya Ikematsu [b], Yuki Abe [c], Yoko Ihama [d], Kazutoshi Ago [a], Mihoko Ago [a], Tetsuji Miyazaki [d], Mamoru Ogata [a]

[a] Department of Legal Medicine, Graduate School of Medical and Dental Sciences, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8544, Japan
[b] Division of Forensic Pathology and Science, Unit of Social Medicine, Course of Medical and Dental Sciences, Graduate School of Biomedical Sciences, Nagasaki University School of Medicine, 1-12-4 Sakamoto, Nagasaki City, Nagasaki 852-8523, Japan
[c] School of Medicine, Nagasaki University, 1-12-4 Sakamoto, Nagasaki City, Nagasaki 852-8523, Japan
[d] Department of Forensic Medicine, Graduate School of Medicine, University of the Ryukyus, 207 Uehara, Nishihara, Okinawa 903-0215, Japan

ARTICLE INFO

Article history:
Received 12 December 2013
Received in revised form 12 March 2014
Accepted 21 March 2014
Available online 2 April 2014

Keywords:
Restraint stress
Adrenal endocrine systems
Temporal changes
Prolonged psychological stress
Practical application

ABSTRACT

We investigated temporal changes of adrenal endocrine systems through the hypothalamic–pituitary–adrenal (HPA) and sympathetic–adrenomedullary (SA) axis in restraint stressed mice. Restraint stress for 1 day to 3 weeks caused a significant increase in serum levels of ACTH and glucocorticoids accompanied with an increase in adrenal weights, indicating activation of the HPA axis. Reflecting the overproduction of glucocorticoids, adrenal cholesterol content decreased. Moreover, adrenal gene expression involved in cholesterol supply, including scavenger receptor-class B type I, HMG-CoA reductase, and hormone-sensitive lipase, was increased over the same period. After 4 weeks stress, all of these changes returned to control levels. In contrast, adrenal gene expression of chromogranin A, which is cosecreted with catecholamine via the SA axis, was increased with 1 day to 2 weeks of stress, and decreased with 3–4 weeks of stress. Our results suggest that analyses of adrenal endocrine systems based on the combination of several markers examined here would be useful for not only proving prolonged psychological stress experience but also determining its duration.

1. Introduction

Prolonged psychological stress potentially exerts harmful influences on various internal organs [1]. Appropriate biomechanical responses to stress must be driven in order to maintain an internal homeostasis of the organs. The hypothalamic–pituitary–adrenal (HPA) axis and the sympathetic–adrenomedullary (SA) axis are the common systems that are responsible for playing these roles during a stress state, with the adrenal gland being an essential organ common to both systems [2–4]. In the HPA axis [2,4], adrenocorticotropic hormone (ACTH), which is secreted from the anterior lobe of pituitary through corticotropin-releasing hormone (CRH), acts on the zona fasciculata of the adrenal cortex to produce the "anti-stress hormones", glucocorticoids, from intracellular cholesterol in the adrenal gland. The adrenal cholesterol is supplied from several sources such as (1) receptor-mediated cellular uptake of lipoprotein including high-density lipoprotein (HDL) or low-density lipoprotein (LDL), (2) *de novo* intracellular synthesis consisting of a series of reactions catalyzed by numerous enzymes, and (3) the mobilization of stored cholesteryl esters [4]. In the SA axis [2,3], neural activation of the sympathetic nervous system acts on the chromaffin cells in the adrenal medulla to release catecholamines.

Using a murine model of prolonged psychological stress, we examined temporal changes in the HPA and SA axis after stress. In the present study, we investigated whether these changes may represent basal evidence proving chronic stress and of estimating its duration.

2. Materials and methods

2.1. Animals

Pathogen-free 7 week old male BALB/c mice were obtained from SLC (Shizuoka, Japan). All mice were bred and housed in a temperature-controlled (23 ± 2 °C) environment with a 12 h light/

* Corresponding author. Tel.: +81 99 275 5313; fax: +81 99 275 5315.
E-mail address: takahito@m2.kufm.kagoshima-u.ac.jp (T. Hayashi).

http://dx.doi.org/10.1016/j.legalmed.2014.03.005
1344-6223/

12 h dark cycle. They were fed with standard feed and given water *ad libitum*. All animal experiments were approved by the Animal Care Committee of Nagasaki University.

2.2. Restraint stress

A widely reported model of psychological stress, a restraint stressed mouse [5,6], was used in this study. Mice were restrained in a 50 ml polypropylene centrifuge tube with air holes for 1 h in the early morning for either 1 day or 1, 2, 3, or 4 weeks ($n = 8$ in each group). Mice were sacrificed by rapid decapitation at 1 h after the last stress procedure, with blood and both adrenal glands being collected immediately. Control samples were collected from duration-matched non-restrained mice ($n = 8$ in each group). The adrenal glands were weighed after the surrounding fatty tissue was removed completely. The left one was immersed in RNA Later (Perkin–Elmer Applied Biosystems, Foster City, USA) and stored at −80°C for the extraction of RNA. The right one was fixed in 4% formaldehyde buffered with phosphate-buffered saline (PBS; pH 7.2) for the extraction of adrenal cholesterol. Collected blood was centrifuged at 4000 rpm for 15 min. Serum was then rapidly frozen and stored at −80°C for hormone assays.

2.3. Hormone assays

Serum levels of ACTH were measured using the EIA kit, ACTH 1–39 (Peninsula Laboratories, Inc., San Carlos, California, USA), according to the manufacturer's instructions. The assay sensitivity was approximately 0.62 ng/ml, and the detection range was 0–25 ng/ml. Serum levels of corticosterone and cortisol were measured using the EIA kits (Cayman Chemical Company, Ann Arbor, Michigan, USA) according to the manufacturer's instructions. The sensitivity of these assays were approximately 0.15 ng/ml and 0.18 ng/ml, respectively, and their detection limits were 30 pg/ml and 35 pg/ml, respectively.

2.4. Extraction of adrenal cholesterol and quantitation of cholesterol contents

The cholesterol content of adrenal glands was measured using the Calbiochem® Cholesterol/Cholesteryl Ester Quantitation Kit (EMD Biosciences, Inc., Darmstadt, Germany). Ten milligrams of adrenal cortex was homogenized in 200 µl chloroform with 1% Triton X-100 detergent, and centrifuged at 14,000 rpm for 10 min. The organic phase was transferred, and dried at 50 °C for 20 min, followed by vacuum-drying for 30 min. After drying, the remaining lipids were dissolved in 200 µl of chloroform with 1% Triton X-100 detergent. One microliter of the dissolved sample was adjusted to 50 µl with Cholesterol Reaction Buffer and added to 50 µl of Reaction Mix in the well of a plate, and incubated at 37°C for 1 h in darkness. After incubation, fluorescence was measured at an excitation wavelength of ∼535 nm and emission wavelength of ∼590 nm by a fluorimeter. The total cholesterol concentration of each sample was calculated based on the standard curve. The detection range was 0.02–1 µg/well.

2.5. Extraction of total RNA, reverse transcription and quantitative real-time PCR

To examine the gene expression of chromogranin A (CgA) and molecules involved in cholesterol supply such as scavenger receptor-class B type I (SR-BI), low density lipoprotein receptor (LDLR), HMG-CoA reductase (HMGR), and hormone-sensitive lipase (HSL) in adrenal glands, quantitative real-time PCR analyses were performed as described previously [7,8]. Total RNA was extracted from the adrenal gland samples using ISOGEN (Nippon Gene, Toyama, Japan). One microgram of total RNA was reverse transcribed into cDNA using PrimeScript™ RT Reagent Kit (Takara Bio Inc., Otsu, Japan). Thereafter, the generated cDNA was subjected to real-time PCR analysis using SYBR Premix Ex Taq (Takara Bio Inc.) with specific primer sets (Table 1). The expression levels of target transcripts are given as the ratio of the target normalized against the endogenous reference (18Rps).

2.6. Statistical analysis

The means and standard deviations (SDs) were calculated for all data. Statistical analyses concerning differences of the two groups were performed using the Mann–Whitney's *U* test. $P < 0.05$ was accepted as significant.

3. Results and discussion

In control mice, there was no significant temporal change in any markers within the experimental periods (Figs. 1–4, open bars).

In restraint stressed mice, the weight of the adrenal gland showed a significant increase at 1 week and 2 weeks in comparison to that of control mice (Fig. 1). Serum levels of ACTH showed a significant increase with 1 day to 3 weeks of stress (Fig. 2a). Corresponding to the change of ACTH, serum levels of corticosterone and cortisol showed a significant increase with 1 day to 2 weeks, and 3 weeks of stress, respectively (Fig. 2b and c). In contrast, the adrenal cholesterol content showed a significant decrease with 1 week of stress (Fig. 2d). All of these changes returned to control levels after 4 weeks. According to "Selye's stress theory" [2] and recent investigators [9,10], the adrenal glands are enlarged due to overproduction of glucocorticoids through activation of the HPA axis in the early phase of chronic stress, which Selye termed the "alarming reaction (A-R) stage". Subsequently, in the later phase, which Selye termed the "resistance stage", the production of glucocorticoids is reduced to the lowest levels for resisting the stress. Therefore, our results suggest that restraint stress for 1 day to 3 weeks and for 4 weeks may correspond to the "A-R stage" and "resistance stage". These temporal changes may be useful markers for not only proving prolonged psychological stress experience but also for the estimation of stress duration.

As a practical application of these results to forensic casework, an initial evaluation of adrenal weights would be appropriate because of its simple and routine examination. In contrast, the diagnostic assessment of postmortem serum hormone levels is controversial [11–15]. Therefore, we performed a quantitative analysis of adrenal cholesterol, a starting material for glucocorticoids. Actually, the cholesterol was decreased significantly in mice restrained for 1 week in our study (Fig. 2d). Supportingly, intracellular lipid-droplet depletion in the zona fasciculata, implying an exhaustion of cholesterol, has been observed in the early phase

Table 1
Sequences of primers used for real time-PCR.

Transcript	Sequence	Product size, (bp)
SR-BI	F: 5′-TTGCCAACGGGTCCGTCTA-3′ R: 5′-AACACAGGGTCGGCGTTGTAA-3′	139
LDLR	F: 5′-TGACCTTCATCCCAGAGCCTTC -3′ R: 5′-AAGGCATGAGCGGGTATCCA-3′	138
HMGR	F: 5′-CTCGTTAGCACTGGTCCAGGAA-3′ R: 5′-GGCTCCATCATTGGCTCTGTAAG-3′	85
HSL	F: 5′-TCCTGGAACTAAGTGGACGCAAG-3′ R: 5′-CAGACACACTCCTGCGCATAGAC-3′	93
CgA	F: 5′-CCACTGCAGCATCCAGTTCC-3′ R: 5′-CTCCATCCACTGCCTGAGAGTC-3′	90
18Rps	F: 5′-TTCTGGCCAACGGTCTAGACAA-3′ R: 5′-CCAGTGGTCTTGGTGTGCTGA-3′	127

F, forward primer; R, reverse primer.