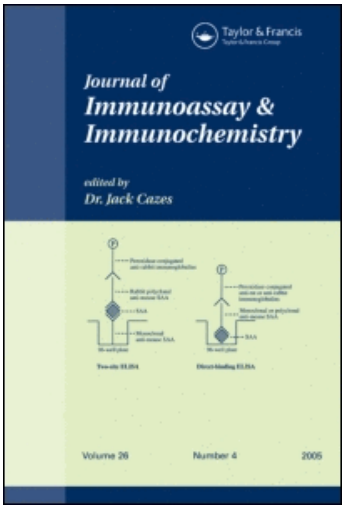

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Establishment and Primary Application of a Highly-Sensitive Orexin-A Radioimmunoassay

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ABSTRACT

Orexin-A was labeled by ^{125}I using the chloramine-T method, and was purified with a Sephadex G-25 chromatographic column. The reaction between antigen and antibody was carried out by a one-step balance method and was incubated at 4°C for 24 hours, then bonded and free antigen were separated by PR reagent. The detection range of this RIA is 21–2000 pg/mL; the lowest detection level is 21 pg/mL. The intra-assay and inter-assay variations were 7.8% and 9.7%, respectively. Plasma orexin-A levels of 30 normal individuals and 30 patients with hyperlipidemia (serum triglyceride >1.7 mmol/L and serum total cholesterol >5.7 mmol/L) were detected by this RIA, while orexin-A

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levels of plasma and hypothalamus in rat intestinal ischemia-reperfusion injury model were also measured. Plasma orexin-A levels of normal individuals was 338.48 ± 20.24 pg/mL, while those of patients with hyperlipidemia were 343.51 ± 15.49 pg/mL; there were no significant differences between these two groups $t = -0.1976$; $P = 0.8441$. We also found that orexin-A levels of rat plasma and hypothalamus did not express a significant change during the early stages of intestinal ischemia-reperfusion injury. These results have shown that this orexin-A radioimmunoassay is stable, simple, and specific, being sensitive enough to test orexin-A levels in human plasma, rat plasma, and hypothalamus.

Key Words: Orexin-A; Radioimmunoassay; Plasma; Hypothalamus.

INTRODUCTION

Orexin-A, also called hypocretin-1, is a novel neuropeptide which is secreted by specific neurons in the lateral hypothalamus. It consists of 33 amino-acids, and has a molecular weight of 3562 Da.[1] Orexin-A is C-terminally amidated and contains two intramolecular disulfide bonds that connect cysteine residues from positions 6–12 and 7–14, respectively. The structure of orexin-A is conserved among human, rat, mouse, and cow.[2] Recent findings suggest that orexin-A provides a critical link between the peripheral energy balance and central nervous system mechanisms that coordinate sleep–wakefulness and motivated behaviors such as food seeking, especially in the physiological state of fasting stress.[3,4]

Ischemia-reperfusion injury is a classical model which is known well enough to reflect acute stress status and inflammatory response.[5] We established a highly-sensitive and concise orexin-A radioimmunoassay and used it to explore the relationship between ischemia-reperfusion injury and orexin-A levels. This work provided great help in finding out the determinant role which orexin-A plays in acute inflammatory response.

EXPERIMENTAL

Materials

Twenty-seven male Sprague–Dawley rats (weight, ~250 g) were supplied by the experimental animal center of our hospital. Rats were maintained in a room at 22–25°C under a constant day/night rhythm and given food and water, ad libitum. All animal experiments were carried out in accordance with the NIH Guide for Care and Use of Laboratory Animals and was approved by

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the animal care and use committee at our hospital. Hollow plasma of 30 normal individuals and 30 patients with hyperlipidemia (serum triglyceride >1.7 mmol/L and serum total cholesterol >5.7 mmol/L), were supplied by the Clinical Biochemistry Department of our hospital. Each group consists of 15 men and 15 women, ages 20–60.

Human orexin-A, rabbit-anti-human orexin-A antibody, Sephadex G-25, BSA, endothelin (ET), adrenocorticotropin hormone (ACTH), ubiquitin, interleukin-8 (IL-8), epidermal growth factor (EGF) and calcitonin gene related protein (CGRP) were purchased from Sigma, St. Louis, MO. Sodium iodide ($Na^{125}I$) was purchased from Amersham Biosciences, UK. Parafilm was purchased from National Can, USA. PR reagent was supplied by Northern Bio-tech Company, China. Other reagents were purchased locally and were of analytically pure grade.

Iodination of Antigen

Three micrograms orexin-A was dissolved in 12 μL of 0.1 mol/L, pH 7.4, phosphate buffer; 5 μL of $Na^{125}I$ (18.5 MBq) was added, following 10 μg chloramines-T in 10 μL to start the reaction for 30 s. The iodination was terminated by adding 20 μg sodium metabisulfite in 10 μL solution. The reaction solution was loaded onto a Sephadex G-25 gel column which had been equilibrated with 0.1 mol/L, pH 7.4, phosphate buffer, then eluted with the same buffer solution. The eluate was collected at the rate of one tube per minute; the whole process of elution is shown in Fig. 1. After reacting with anti-orexin-A, those iodinated orexin-A with high specific binding rate and low fault binding rate were taken as successfully iodinated orexin-A, mixed with an equal volume of 1.5% BSA, and stored at -20°C.[6]

Radioimmunoassay

Two hundred microlitre standard orexin-A (21, 62.5, 125, 250, 500, 1000, and 2000 pg/mL) and samples were mixed with 50 μL antibody (1 : 1000) as well as 50 μL ^{125}I-orexin-A (about 20,000 cpm) respectively, and allowed to react at 4°C for 24 hr. After adding 500 μL PR reagent (combined reagent of polyethylene glycol and donkey-anti-rabbit IgG antibody), it was reacted for 15 min at room temperature, all tubes were centrifuged for 20 min at 4°C, at 2830 rpm. Then, the supernatant was discarded and the radioactivity of the precipitate in the tubes was detected. All data were treated by radioimmunoassay software and the binding rate of each point was calculated; sample concentrations were obtained from a standard curve.[6]

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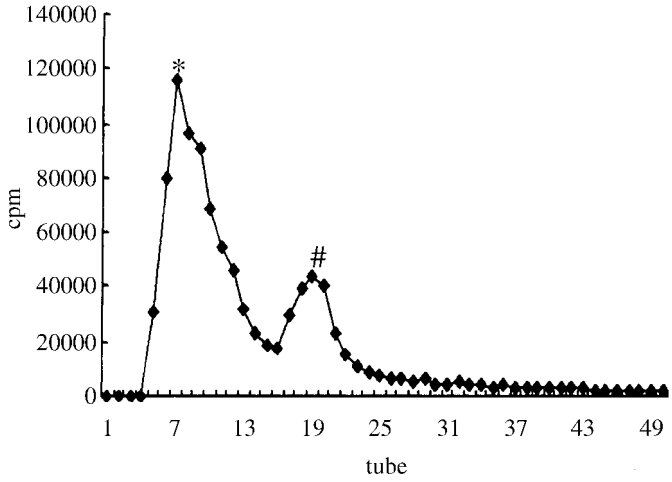

Figure 1. Elution profile of radioiodinated orexin-A on Sephadex G-25.

Optimizing Analysis Conditions

The incubation times at 3–6 h at 37°C and 16–24 h at 4°C were studied for optimized reaction conditions. Three different concentrations of iodinated marker were compared with optimized binding rate, and with no specific binding, for the best standard curve shape.

Measurement of Plasma Orexin-A in Normal Individuals and Hyperlipidemia Patients

Orexin-A level in the plasma of 30 normal individuals and 30 patients with hyperlipidemia were measured. Two milliliters of blood from each group was drawn from the median cubital vein; plasma was isolated by centrifugation and was stored, for testing, at −20°C.

Establishment of Rat Intestinal Ischemia-Reperfusion Injury Model

Twenty-seven rats were divided randomly into three groups; each group contained nine rats. The first group was set for 60 min ischemia–30 min reperfusion (I60′R30′), the second group was set for I60′R90′, and the third

group was set for I60′R150′. Establishment of rat intestinal ischemia-reperfusion injury model was referred to Turnage's method.[7] Two milliliters of blood was taken from the tail vein of each rat before injury, as a self control; during the experiment, blood was drawn from rats' hearts.

Preparation of Rat Plasma and Hypothalamus Tissue Samples

Separated rat plasma, according to Arihara's method,[8] extracted orexin-A from rat hypothalamus tissue according to Sakura's method.[9] One milliliter of cold normal saline was added to 200 mg brain sample, then the mixture was homogenized at 4°C, 20,000 rpm, for 30 s (for three times) it was stopped for 10 s after each time. All the homogenized fluid was collected and centrifuged at 4°C, 12,000 rpm, for 20 min; the median supernatant was stored at −20°C.

Measurement of Orexin-A Levels in Rat Plasma and Hypothalamus

Samples preparation method for radioimmunoassay is shown in Table 1. Before testing, 10 μL hypothalamus homogenized fluid was taken and diluted

Table 1. Plasma and hypothalamus orexin-A radioimmunoassay samples preparation method.

	Addition sequence (μL)				
	0.02 M pH 7.4 PB	Standard orexin-A	Sample[a]	Anti-orexin-A	^{125}I-labeled orexin-A
T	—	—	—	—	50
N	250	—	—	—	50
S_0	200	—	—	50	50
S_1	—	200	—	50	50
S_2	—	200	—	50	50
S_3	—	200	—	50	50
S_4	—	200	—	50	50
S_5	—	200	—	50	50
S_6	—	200	—	50	50
S_7	—	200	—	50	50
Sample	—	—	200	50	50

[a]When testing hypothalamus orexin-A, add 10 μL homogenized sample and dilute to 200 μL in 0.02 M pH 7.4 PB.

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to 500 μL in PB (0.02 mol/L, pH 7.4, containing 0.01 mol/L KCl, 0.01 mol/L EDTA, and 0.1% BSA). Then the Coomassie Brilliant Blue G-250 method was used to check the total protein in the mixed fluid, and compared with orexin-A level in 100 μg total protein for each tissue sample.

Statistical Analysis

We used Stata 7.0 software to process our data. For those data which agreed with normal levels and showed no difference in standard deviation, we used parametric statistical analysis (Student's *t* test or one way analysis of variance); for those data which did not agree with normal levels or showed differences in standard deviation, we used non-parametric statistical analysis (Wilcoxon signed rank test). A probability level of less than 0.05 was chosen as a threshold for statistical significance; all data are shown as mean ± SEM.

RESULTS

Standard Curve and Antibody Dilution

The best curve shape had been achieved at 4°C for 24 h incubation through the testing of 3–6 h at 37°C and 16–24 h at 4°C. Adding 20,000 cpm of ^{125}I-labeled orexin-A could yield a suitable binding rate (39–47%) and non-specific binding (2.3–2.6%). Final antibody dilution was 1 : 3000, and a good binding curve had been obtained in the standard field of 21–2000 pg/mL, as Fig. 2 shows.

Sensitivity and Precision

Calculated as $B_0 \pm 2S$, the minimum measurable value of orexin-A is 21 pg/mL. Moreover, three rat plasma samples had been tested at the same time for five times, respectively; one sample had been tested five times and it was demonstrated that the intra-assay and inter-assay variations were about 7.8% and 9.7%, respectively.

Specificity and Validity

There were no cross reactions among orexin-A antibody with ET, ACTH, ubiquitin, IL-8, EGF, and CGRP in the 6.25–200 ng/mL range. The recovery rates ranged from 96% to 102% when adding 100, 500, and 1000 pg/mL of

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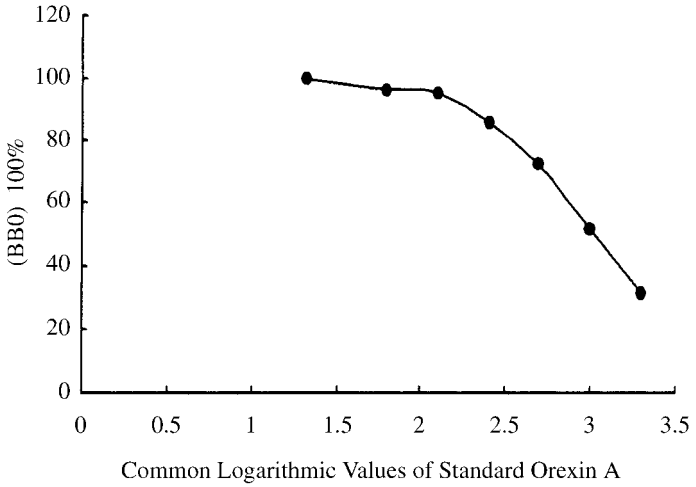

Figure 2. Competition suppression curve of orexin-A radioimmunoassay.

orexin-A standards to 200 μL normal rat plasma. There was good linearity between the tested values and dilution multiples when a rat plasma sample with high orexin-A concentration was diluted from 1 : 2 to 1 : 16 times.

Plasma Orexin-A in Normal Individuals and Hyperlipidemia Patients

Plasma orexin-A levels of 30 normal individuals were 338.48 ± 20.24 pg/mL, while those of 30 patients with hyperlipidemia were 343.51 ± 15.49 pg/mL; there was no significant difference between these two groups ($t = -0.1976$, $P = 0.8441$).

Orexin-A Levels in Rat Plasma and Hypothalamus

Plasma orexin-A levels of 27 male Sprague–Dawley rats, before ischemia-reperfusion injury was 676.81 ± 27.36 pg/mL. Plasma and hypothalamus orexin-A levels of each experimental group are shown in Table 2. There were no significant differences of plasma orexin-A levels between self control and IR groups. Among different IR groups, plasma and hypothalamus

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Table 2. Effect of intestinal ischemia-reperfusion injury on rat plasma/tissue orexin-A level.

Groups	Cases	Self control plasma value (pg/mL)	IR plasma value (pg/mL)	IR tissue value[a] (pg/mL)
I60′R30′	9	648.82 ± 40.53	720.39 ± 39.39	562.08 ± 75.76
I60′R90′	9	703.62 ± 56.81	719.99 ± 60.61	504.29 ± 66.38
I60′R150′	9	720.42 ± 33.07	742.09 ± 53.71	517.36 ± 92.16

[a]Comparing tissue orexin-A levels pg in 100 μg total protein of homogenized hypothalamus tissue samples.

orexin-A levels also expressed no significant differences, but their variational trends showed some consistency.

DISCUSSION

Orexins, also called hypocretins, are a pair of neuropeptides (orexin-A and Orexin B, also called hypocretin-1 and hypocretin-2) expressed by a specific population of neurons in the lateral hypothalamic area, a region of the brain implicated in feeding, arousal, and motivated behavior. Orexin neurons interact with feeding centers in the hypothalamus, arousal, and sleep-wakefulness centers in the brain stem, sympathetic and parasympathetic nuclei, and the limbic system.[3,10,11] Among these two Orexins, orexin-A has been the focus of researches in recent years. Besides its existence in the central nervous system, orexin-A was also detected in the kidney, adrenal gland, pancreas, placenta, stomach, ileum, colon, and colorectal epithelial cells. These results suggest the production of orexin-A in various peripheral tissues and orexin-A may also play important roles in some peripheral organs.[12]

Intestinal ischemia-reperfusion injury is a classical model for trauma, infection, and stress. As a result of the superior mesenteric artery (SMA) being clamped, animals' intestines bleed and putrefy; meanwhile, large amounts of endotoxin and oxygen free radicals are released into the circulation and activate monocytes, phagocytes, and neutrophils, which leads to severe systemic inflammatory response (SIRS). At the same time, sympathetic and parasympathetic nervous systems are activated and cause sharp increases of catecholamines, adrenocorticoids, and glucose. Through cooperation of the central nervous system and the endocrine system, the body maintains endo-homeostasis and enhances functions and metabolism of each organ. It has been reported that orexin-A has a certain relationship with catecholamines, gluco-corticoids, glucose, and irritability of the sympathetic nervous system;[13–16]

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so, we hypothesized that orexin-A level may change during ischemia-reperfusion injury. But there are no published articles citing orexin-A and ischemia-reperfusion injury, or other kinds of acute inflammatory responses. If we can reveal the determinate role orexin-A plays in ischemia-reperfusion injury, it will be greatly helpful for the clinical emergent aid, such as for trauma, infection, and systemic inflammatory responses.

We have established a highly-sensitive and highly-specific orexin-A radioimmunoassay; using this assay can lead to good experimental results. The ^{125}I-labeled orexin-A can be stored for 2 months at -20°C after lyophilization; standard orexin-A and orexin-A antibody can be stored for a longer time at -20°C after lyophilization. Moreover, during the storage life, the modality of the standard curve was valid, and other indices, such as intra-/inter-assay variances, recovery, and specificity also reached the requirements of radioimmunoassay. We used sheep-anti-rabbit orexin-A IgG antibody and 15% polyethylene glycol as separation reagents. The non-specific binding (NSB/T) was about 2.48–2.67%. Although our assay is not the first orexin-A radioimmunoassay, our assay is simpler and more feasible than Arihara's[8] or Mitsuma's[17] methods. We do not have to use organic reagents such as acid-acetone, acetonitrile, methanol, or trifluoroacetic acid in iodination or measurement of orexin-A. In addition, the methods we used to extract orexin-A from homogenized hypothalamus tissue and to prepare radioimmunoassay buffer are simpler and could achieve good results paralleling to those using Sep Pak C_{18} cartridges in iodination.

As the structure of human orexin-A is almost the same as that of rat or mouse, we used this radioimmnuassay to explore the effect of rat intestinal ischemia-reperfusion injury on plasma and hypothalamus orexin-A levels. Results showed that there was no significant difference in orexin-A levels of three groups (I60′R30′, I60′R90′, I60′R150′) before and after ischemia-reperfusion injury, but orexin-A level of the I60′R30′ group, after injury, expressed a trend of increase compared with the self control. This result may attest to the insufficiency of cases of experimental animals and time of reperfusion, which caused insufficient response of orexin-A to the stimuli. However, ischemia-reperfusion injury caused a similar mutative trend of orexin-A levels in plasma and hypothalamus. All of these results may be explained by the following reasons: (1) in the early stage of ischemia-reperfusion injury, the high metabolic rate changed the body into a situation mimicking hunger. As plasma orexin-A increases during the hungry status,[18] plasma orexin-A level of the I60′R30′ group after injury seemed to be higher than the self control; (2) in the ischemia-reperfusion injury, neutrophils produce large amounts of free radicals through "respiratory burst" and the free radicals destroy all proteins, including orexin-A, which caused the decrease of orexin-A; (3) as time passed, the hypothalamus expressed more orexin-A to

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compensate for the decrease of plasma orexin-A, and the high metabolic status recovered, so orexin-A level increased step by step in the median and late stage of ischemia-reperfusion injury. Although the change of orexin-A levels in our experiment did not express a significant difference, it could be inferred that orexin-A might have a delayed response to inflammatory stimuli. We will develop further researches in this area.

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