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www.wjgnet.com
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World J Gastroenterol 2005;11(7):1000-1004
World Journal of Gastroenterology ISSN 1007-9327
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• *BASIC RESEARCH* •

Effect of intestinal ischemia-reperfusion injury on protein levels of leptin and orexin-A in peripheral blood and central secretory tissues

Ji Lin, Guang-Tao Yan, Xiu-Hua Hao, Lu-Huan Wang, Kai Zhang, Hui Xue

Ji Lin, Guang-Tao Yan, Xiu-Hua Hao, Lu-Huan Wang, Kai Zhang, Hui Xue, Research Laboratory of Biochemistry, Basic Medical Institute, General Hospital of PLA, 28 Fuxing Road, Beijing 100853, China
Supported by the National Natural Science Foundation of China, No.39970717
Correspondence to: Professor Guang-Tao Yan, Research Laboratory of Biochemistry, Basic Medical Institute, General Hospital of PLA, 28 Fuxing Road, Beijing 100853, China. yan301@263.net
Telephone: +86-10-66937072 Fax: +86-10-66937521
Received: 2004-07-12 Accepted: 2004-09-04

Abstract

AIM: To explore the effect of intestinal ischemia-reperfusion injury on protein levels of leptin and orexin-A in peripheral blood and their central secretory tissues and to find out the role leptin and orexin-A play in acute inflammatory responses.

METHODS: An intestinal ischemia-reperfusion (I/R) injury model of rats was established and rats were divided randomly into six groups: sham-operation group, 60 min ischemia/30 min reperfusion group (I60′R30′), I60′R90′, I60′R150′, I60′R240′ and I60′R360′, 9 rats each group. Two highly-sensitive radioimmunoassays for leptin and orexin-A were established and used to check the change of their concentrations in peripheral blood and central secretory tissues before and after intestinal I/R injury.

RESULTS: Compared with the serum leptin level before injury, it decreased significantly in I60′R30′ group and increased significantly in I60′R360′ group; compared to sham-operation group after injury, serum leptin level increased significantly in I60′R360′ group; compared to sham-operation group after injury, adipose leptin levels decreased significantly in I60′R30′ and I60′R90′ groups, while increased significantly in I60′R360′ group. There was no significant difference between the expression levels of orexin-A before and after I/R injury.

CONCLUSION: Leptin has a time-dependent response and orexin-A has a delayed response to acute inflammatory stimuli such as intestinal I/R injury and they may participate in metabolic disorders in injury as inflammatory cytokines.

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Key words: Ischemia-reperfusion; Intestinal; Leptin; Orexin-A; Radioimmunoassay; Inflammation; Acute; Cytokine

Lin J, Yan GT, Hao XH, Wang LH, Zhang K, Xue H. Effect of intestinal ischemia-reperfusion injury on protein levels of leptin and orexin-A in peripheral blood and central secretory tissues. *World J Gastroenterol* 2005; 11(7): 1000-1004
http://www.wjgnet.com/1007-9327/11/1000.asp

INTRODUCTION

Leptin is an active protein specifically secreted by adipose tissue in mice, rats, and humans, which consists of 167 amino acids and has a molecular weight of 16 ku. It is a product of *obese* gene and has a feedback regulation by neuroendocrine system. Recent studies have found that leptin plays a role in food-consuming behavior, energy metabolism and weight balance, primarily reducing food-intake and promoting energy expenditure through binding to its receptor in hypothalamus[1]. Moreover, leptin also participates in hematopoiesis[2], thermogenesis[3], reproduction, angiogenesis, and immune homeostasis[4-7].

Orexin-A, also known as hypocretin-1, is a novel neuropeptide secreted by specific neurons in lateral hypothalamus. It consists of 33 amino acids and has a molecular weight of 3 562 u[8]. Orexin-A is C-terminally amidated and contains 2 intra-molecular 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[9]. 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 physiological state of fasting stress[10,11].

Intestinal ischemia-reperfusion (I/R) injury is a classical traumatic model well- reflecting acute stress and inflammatory responses, when the body suffers severe energy metabolic impediment and out-of-control inflammation[12]. As leptin and orexin-A are a couple of active mediators closely-related with energy metabolism and their concentrations change inversely in previous experiments[13-15], we hypothesize that leptin and orexin-A might undergo an inverse fluctuation during severe metabolic impediment of acute inflammation as I/R injury, and play a role as inflammatory cytokines.

We established highly-sensitive and simple radioimmunoassays for murine leptin and human/rat/mouse orexin-A (as the structure of human orexin-A is almost the same as that of rat or mouse)[9] respectively, and used these assays to detect the changes of leptin and orexin-A levels in peripheral

blood (serum and plasma) and central secretory tissues (adipose tissue and hypothalamus) in rat intestinal I/R injury.

MATERIALS AND METHODS

Animals and reagents

Three male New Zealand white rabbits (weighing 1.2 kg) and 54 male Sprague-Dawley rats (weighing 250 g) were supplied by the Experimental Animal Center of our hospital. Animals were maintained at 22-25 ℃ 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 approved by the Animal Care and Use Committee at our hospital.

Recombinant murine leptin was purchased from PeproTech Inc. (London, UK). Complete and incomplete Freund's adjuvant were purchased from Gibco/BRL® (Gaithersburg, USA). Human orexin-A, rabbit-anti-human orexin-A antibody, Sephadex G-25 and bovine serum albumin (BSA) were purchased from Sigma® (St. Louis, USA). Sodium iodide ($Na^{125}I$) was purchased from Amersham® Biosciences (Piscataway, USA). PR reagent was supplied by Northern Biotech Company (Beijing, China). Other reagents purchased were of analytically pure grade.

Preparation of antibody

New Zealand white rabbits were immunized subcutaneously with an emulsion of 120 μg leptin in 3 mL complete Freund's adjuvant, each rabbit was given 1 mL solution. Two weeks later, rabbits were intensively immunized 4 times using incomplete Freund's adjuvant at intervals of 4 wk. One week after the last injection, blood was withdrawn via the right internal carotid artery under conscious condition. Serum containing rabbit anti-rat leptin antibody was separated by centrifugation at 3 000 r/min for 15 min, and stored in -80 ℃.

Iodination of antigen

Antigen was iodinized as previously described[16,17]. The eluate of Sephadex G-25 chromatography column was collected at the rate of one tube per minute, and the tubes containing the first elution peak were reserved. After reaction with anti-body, iodinized leptin and orexin-A with a high specific binding rate and a low fault binding rate were taken as successfully iodinized and mixed with an equal volume of 15 g/L BSA, then stored at -20 ℃.

Intestinal I/R injury model

Fifty-four rats were divided randomly into six groups, nine rats each group. Group 1 served as sham-operation group, group 2 as 60 min ischemia/30 min reperfusion group (I60'R30'), groups 3-6 as I60'R90', I60'R150', I60'R240' and I60'R360' groups, respectively. The abdominal cavity of rats anesthetized with pentobarbital sodium (60 mg/kg, IP) was opened, the superior mesenteric artery was separated and clipped with a microvessel clip for 60 min ischemia, then released for reperfusion[18].

Blood sample collection

Two milliliter blood was taken from tail vein of each rat, three days before I/R injury. During experiment, blood was drawn from the heart of rats. Rat serum was separated from whole blood by incubating blood samples in 37 ℃ water for 20 min and centrifuging at 3 000 *g* for 10 min at 4 ℃. Rat plasma was separated from whole blood as previously described[19]. Serum and plasma samples were used for detecting leptin and orexin-A levels in peripheral blood by radioimmunoassay.

Tissue sample collection

Epididymal fat pads (a kind of white adipose tissue) on the left side[20] and hypothalamus tissue[21] of each rat were separated, tissue samples were snap-frozen in liquid nitrogen and stored at -80 ℃. One milliliter cold normal saline was added to each 200 mg tissue sample, samples were homogenized by centrifugation at 20 000 r/min for 30 s at 4 ℃, and repeated twice at intervals of 10 s. Then the homogenized fluid was centrifuged at 12 000 r/min for 20 min at 4 ℃, the supernatant was collected and stored at -20 ℃. Homogenized fluid samples were used for detecting leptin and orexin-A levels in central secretory tissue by radioimmunoassay.

Radioimmunoassay

The standard points set for serum samples were 1.1, 3.3, 11, 33, 100 and 300 μg/L, while those for adipose samples were 0.55, 1.65, 5.5, 16.5, 50 and 150 μg/L. The standard points set for plasma and hypothalamus samples were 21, 62.5, 125, 250, 500, 1 000, and 2 000 ng/L. One hundred microliter serum sample, 200 μL adipose sample, 200 μL plasma sample or hypothalamus sample were added for testing. Radioimmunoassay was carried out as previously described[16,17]. Before testing, 50 μL of each adipose sample or 10 μL of each hypothalamus sample was taken and diluted to 500 μL in ddH_2O. Then Coomassie Brilliant Blue G-250 method[22] was used to check the total protein concentration in this diluted fluid, and adipose leptin levels (ng) or hypothalamus orexin-A levels (pg) were compared in 100 μg total protein of each tissue sample.

Statistical analysis

We used Stata 7.0 software to process our data. For the data, which accorded with normal distribution and showed no difference in standard deviation, we used parametric statistic analysis (Student's *t* test or one-way analysis of variance); for the data that did not accord with normal distribution or showed difference in standard deviation, we used non-parametric statistic analysis (Wilcoxon signed-rank test). *P* value less than 0.05 was considered statistically significant. All data were expressed as mean±SE.

RESULTS

Standard curve and antibody dilution

The best curve shape was achieved at 4 ℃ after 24 h incubation with a suitable binding rate of 36-40% and a non-specific binding rate of 2.3-2.6%. The final antibody dilutions were 1:3 000 (serum), 1:4 000 (adipose) and 1:6 000 (plasma and hypothalamus). Good binding curves were obtained in the standard field of 1.1-300 μg/L for serum samples, 0.55-150 μg/L for adipose samples (which paralleled well

with the former), and 21-2 000 ng/L for plasma and hypothalamus samples (Figure 1).

Leptin levels before and after intestinal I/R injury

Serum levels *vs* self-control Compared to self-control (before injury), serum leptin level decreased significantly in I60'R30' group (t = 2.3891, P<0.05), expressed a trend to increase in I60'R150' group (t = -0.7176, P = 0.4834) and increased significantly in I60'R360' group (t = -2.3437, P<0.05), as shown in Figure 2A.

Serum and adipose levels *vs* sham-operation group Compared to sham-operation group after injury, serum leptin level expressed a trend to increase in I60'R240' group (t = -2.0327, P = 0.0590) and increased significantly in I60'R360' group (t = -2.8085, P<0.05); while adipose leptin levels decreased significantly in I60'R30' and I60'R90' groups (t = 2.2804 and 2.5170, respectively, P<0.05), and increased significantly in I60'R360' group (t = -2.8401, P<0.05), as shown in Figure 2B.

Orexin-A levels before and after intestinal I/R injury

Compared to self-control (before injury), plasma orexin-A expression level in each group after injury had no significant difference (P>0.05). Compared to sham-operation group after injury, plasma orexin-A expression level or hypothalamus orexin-A expression level in each group had no significant difference (P>0.05), as shown in Table 1.

Table 1 Plasma and hypothalamus orexin-A levels of each group before and after I/R injury (mean±SE)

Groups	Cases	Plasma level (ng/L)		Hypothalamus level (pg)[1]
		Before injury	After injury	
Sham	9	587.17±99.63	834.33±156.3	419.08±27.94
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
I60′R240′	9	675.23±100.1	808.98±168.4	486.76±31.54
I60′R360′	9	678.38±72.08	570.18±80.43	480.16±29.30

[1]Calculated in 100 μg total protein of hypothalamus tissue homogenized fluids.

DISCUSSION

Association of intestinal I/R injury with leptin and orexin-A

Intestinal I/R injury causes intestinal bleeding and necrosis. Large amounts of endotoxin and oxygenic free radicals are released into blood circulation while monocytes, phagocytes and neutrophils are activated, leading to severe systemic inflammatory responses and metabolic disorders. At the same time, sympathetic and parasympathetic nervous systems are activated, causing sharp increases of catecholamines, adrenocorticoids and glucose in blood circulation[23,24]. It has been reported that leptin and orexin-A have a certain relationship with catecholamine[25,26], glucocorticoids[27,28], glucose[29,30] and sympathetic nervous system[31,32]. Moreover, leptin can activate monocytes and T lymphocytes[33,34], and interact with inflammatory mediators such as IL-1β, TNF-α and C-reactive protein[35-37]. All these results present a possible link between leptin and orexin-A and systemic metabolic disorders, suggesting that they play a potential role as inflammatory cytokines in I/R injury.

Highly sensitive and simple radioimmunoassay

Our methods for detecting leptin and orexin-A are highly sensitive and simple, and can achieve good experimental

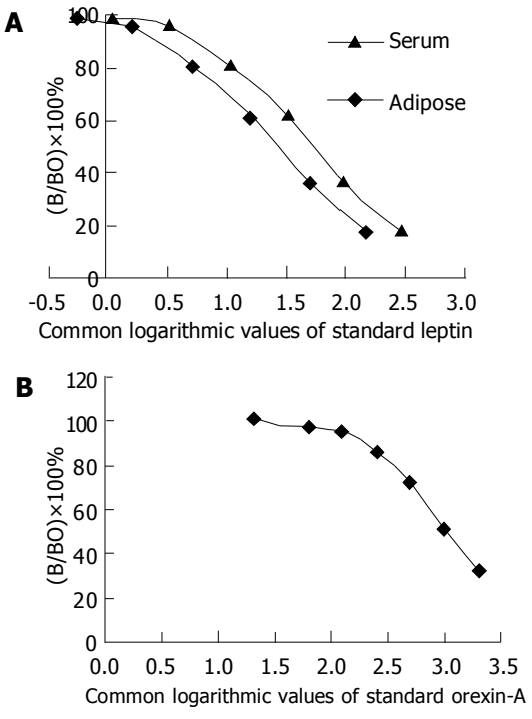

Figure 1 Standard curves of leptin (A) and orexin-A (B) radioimmunoassay.

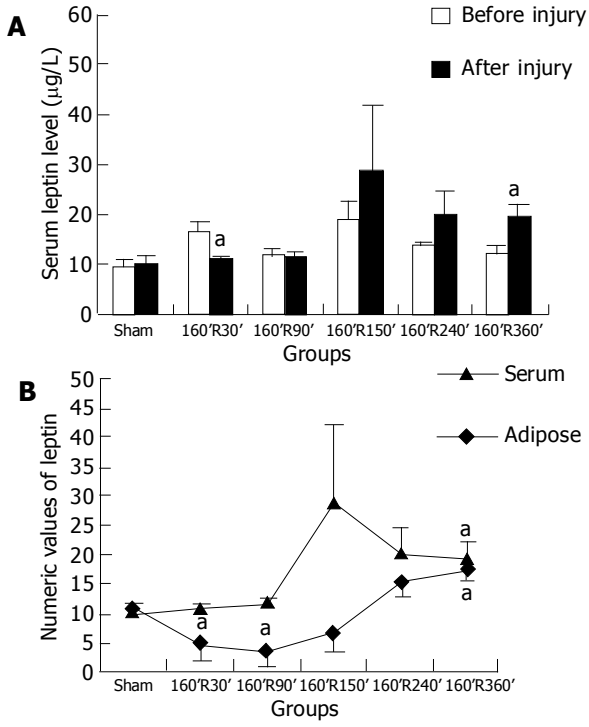

Figure 2 Serum leptin (A) and leptin (B) levels before and after intestinal I/R injury [a]P<0.05, *vs* before injury and sham-operation group.

results. ^{125}I-labeled leptin or orexin-A can be stored for 2 mo at -20 ℃ after lyophilization. Standard leptin or orexin-A and their antibody can be stored for 6 mo at room temperature or for 2 years at -20 ℃ after lyophilization. Moreover, during the storage, the modality of standard curve is good and other indexes such as intra/inter-assay variances, recovery rate or specificity also meet the request of radioimmunoassay. Although our assays are not the first radioimmunoassay for leptin and orexin-A, they are more simple and feasible than others[38,19]. The methods we used to extract leptin or orexin-A from homogenized tissues and prepare buffer for radioimmunoassay are more simple. In addition, we need not use organic reagents such as acid-acetone, acetonitrile, methanol or trifluoroacetic acid in iodination or measurement of orexin-A, and can also achieve good results.

Leptin fluctuates as inflammatory cytokines

In the early stage of I/R injury, the high metabolic status can change the body into a situation similar to hunger. As leptin decreases significantly during hungry status through the action of neuroendocrine system[39], leptin level in the early stage of I/R injury (I60'R30') is significantly lower than that before injury. As time goes on, adipose tissue expresses more leptin to compensate for the decrease of serum leptin, and the high metabolic status recovers, thus increasing the serum leptin level step-by-step (from I60'R90' to I60'R240') which is finally higher (I60'R360') than self-control. In adipose tissue, as serum leptin level declines significantly in the early stage (I60'R30'), adipose tissue expresses more leptin for compensation due to a negative feedback mechanism. Since the trans-membrane secretion of leptin from intra-adipocytes to extra-adipocytes and blood circulation is quite active, leptin level in adipose tissue declines significantly than that in sham-operation group (from I60'R30' to I60'R90'). Then, serum leptin level begins to increase and the activity of trans-membrane secretion of leptin is inhibited due to less concentration difference between two sides of adipocyte membrane. Therefore, leptin level in adipose tissue begins to increase step-by-step. When reperfusion is prolonged (from I60'R240' to I60'R360'), serum leptin level remains slightly higher than in sham-operation group. Due to a potentially positive feedback mechanism protecting the body[40], adipose tissue expresses more leptin, causing a significant increase of leptin level.

Orexin-A has a delayed response to inflammatory stimuli

Results showed that there was no significant difference between plasma orexin-A levels of each group before and after injury. The results may be attributed to two counteractive factors (one increased orexin-A level and the other suppressed it) and the limited number of experimental animals, which caused the delayed response of orexin-A to inflammatory stimuli. In the early stage of I/R injury, the high metabolic level changes the body into a situation like hunger. As plasma orexin-A increases during hungry status through the action of neuroendocrine system, plasma orexin-A levels in the early and median stages of I/R injury (from I60'R30' to I60'R150') should be higher than self-control. But as orexin-A is secreted by hypothalamus and its level may be greatly influenced by inhibition of central nervous system (e.g., anesthesia), the increasing trend of orexin-A level caused by hungry status is suppressed by anesthesia. When reperfusion is prolonged (from I60'R240' to I60'R360'), the high metabolic status recovers step-by-step and the anesthetized inhibition decreases, the trend of orexin-A to decrease is counteracted. In hypothalamus tissue, the change of orexin-A level is also affected by the two counteractive factors. Therefore, during the whole process of our experiment, no significant change was found in expression of orexin-A. If we increase the number of animals and/or prolong reperfusion time, we would find new positive results.

In conclusion, leptin and orexin-A undergo a certain change in intestinal I/R injury. When reperfusion is prolonged, protein levels of leptin in central secretory tissue and peripheral blood fluctuates correspondingly, while protein levels of orexin-A in central secretory tissue and peripheral blood showed no significant change. Leptin has a time-dependent response and orexin-A has a delayed response to acute inflammatory stimuli such as intestinal I/R injury, and they may participate in metabolic disorders as inflammatory cytokines.

ACKNOWLEDGEMENTS

The authors thank Miss Xiao-Ning Gao, Mrs Na-Qing Zhang and Miss He-Dan Lu for their generous assistance during the experiment.

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