Galanin inhibits leptin expression and secretion in rat adipose tissue and 3T3-L1 adipocytes

R-Y Li*1,2, H-D Song*2, W-J Shi1, S-M Hu2, Y-S Yang1, J-F Tang1, M-D Chen1 and J-L Chen1

1Ruijin Hospital, Shanghai Institute of Endocrinology, Shanghai Second Medical University, Shanghai, China 200025
2Ruijin Hospital, State Key Laboratory of Medical Genomics, Shanghai Second Medical University, Shanghai, China 200025

(Requests for offprints should be addressed to H-D Song; E-mail: huaidong_s@hotmail.com and M-D Chen; E-mail: mingdaochensh@yahoo.com)

*(R-Y Li and H-D Song contributed equally to this work)

Abstract

In addition to serving as a fat depot, adipose tissue is also considered as an important endocrine organ that synthesizes and secretes a number of factors. Leptin is an adipocyte-derived hormone that plays a vital role in energy balance. Expression of leptin is regulated by dietary status and hormones. In the present study, we report that galanin, an orexigenic peptide, inhibits leptin expression and secretion in rat adipose tissue and in 3T3-L1 adipocytes. Treatment with galanin (25 µg/animal) induced approximately 46% down-regulation of leptin secretion at 15 min, followed by 40, 37 and 47% decreases in leptin secretion at 1, 2 and 4 h respectively. Although Northern blot analysis of adipose tissue from the same animals showed that leptin mRNA expression in adipose tissue was unaffected by galanin treatment for 2 h, galanin treatment for 4 h led to decline of leptin mRNA expression in a dose-dependent manner. Meanwhile, treating the rats with galanin had no effect on leptin mRNA expression in the hypothalamus. The inhibitory action of the galanin on leptin mRNA and protein levels was also observed in vitro. When incubated with 10 nM galanin for 48 h, leptin mRNA expression and protein secretion also decreased in 3T3-L1 adipocytes. On the other hand, galanin was found not only to express in rat adipose tissue, but also to increase about 8-fold after fasting. Based on these data, we speculate that increased galanin expression in rat adipose tissue after fasting may be involved in reducing leptin expression and secretion in fasting rats.

Journal of Molecular Endocrinology (2004) 33, 11–19

Introduction

Obesity is a worldwide health problem directly linked to several disease processes such as hypertension, hyperinsulinemia and type 2 diabetes (Grundy & Barnett 1990, Friedman & Leibel 1992). Obesity initially results from a chronic minor imbalance between energy intake and energy expenditure. Leptin, an adipocyte-derived hormone, is a key factor in regulating body weight and energy expenditure and acts in rodents via hypothalamus receptors to inhibit feeding and increase thermogenesis (Zhang et al. 1994, Pelleymounter et al. 1995). After binding to receptors, leptin activates a specific signaling cascade that results in the inhibition of several orexigenic neuropeptides such as neuropeptide Y (NPY), melanin-concentrating hormone (MCH), orexin and Agouti-related peptide (Agrp) and stimulation of several anorexigenic peptides such as cocaine- and amphetamine-regulated transcript (CART), corticotropin-releasing hormone (CRH) and pro-opiominocortin (POMC) (Flier & Flier 1998, Woods et al. 1998, Schwartz et al. 2000). All these findings suggest that the critical function of leptin seems to be to inform the neurons in the hypothalamus of energy depot levels in the body. Changes in circulating leptin levels can therefore trigger an appropriate response of the central nervous system (CNS) to modulate food intake and metabolism according to the total amount of body fat (Considine et al. 1996) as well as the variations in energy balance (Boden et al. 1996).

In our recent study (Yang et al. 2003) on the gene expression profiling of human visceral adipose tissue and its secretory functions, we found using dot blot analysis that galanin and its receptors (GalR1 and GalR2), are expressed in human adipose tissue. Thus we hypothesize that galanin expressed in adipose tissue may play a role in regulating leptin expression and secretion. To address this issue, in the present study the observation that GalR1, GalR2 and galanin are expressed in rat adipose tissue was first further confirmed. Secondly, galanin was administered to rats to study its effect on leptin production in vivo. Thirdly, the effect of galanin on leptin expression and secretion in 3T3-L1 adipocytes was also observed. Finally, the expression pattern of galanin in fasting rat adipose tissue was studied to investigate the possible role of galanin-regulated leptin production in energy balance. Our results demonstrate for the first time that galanin inhibits leptin production in vivo and in vitro, and that the increased galanin expression in rat adipose tissue after fasting may play a role in the regulation of leptin expression and secretion in fasting rats.

Materials and methods

Animals

Male Sprague–Dawley rats from BK Company (Shanghai, China), each weighing 200–250 g, were used for these studies. Rats were housed in a temperature-controlled room with a ratio of 12 h light:12 h darkness. Animals were given free access to food and water and were allowed to acclimate for 7 days before the experiment.

Forty animals were assigned to weight-matched groups and each animal in the groups received a single intravenous injection of 0.3 ml saline containing 1, 5 or 25 µg rat galanin (Sigma), or 0.3 ml saline control. Blood samples were collected at 15, 60, 120 and 240 min after the injection, and kept frozen at −20 °C for RIA of leptin. The rats were killed 2 or 4 h after treatment. Samples of epididymal fat pads were quickly removed and frozen with liquid nitrogen, then stored at −80 °C until being used for Northern blot analysis.

Cell culture

3T3-L1 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) and were fed every 2 days. Two days after confluence (day 0), the medium was switched to DMEM supplemented with 10% FBS, 10 µg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine and 1 µM dexamethasone. On day 2, the medium was changed to DMEM containing 10% FBS and 10 µg/ml insulin. Beginning on day 4, the medium was changed to DMEM containing only 10% FBS, and cells were given fresh medium every 2 days until day 10. On day 10, cells were incubated in DMEM containing 0.1% BSA for 12 h. Cells were then fed fresh DMEM with 0.1% BSA containing the indicated doses of galanin and incubated for 48 h; cells were collected for real-time PCR and Western blot analysis.

Semi-quantitative reverse transcriptase-PCR (RT-PCR)

To confirm the results of our previous study, semi-quantitative RT-PCR was used. First-strand cDNAs were synthesized from total RNA (3–5 µg) of epididymal fat pads using oligo-dT in 25 µl reaction systems. Mouse GAPDH was used as an internal control. The PCR program was designed with initial melting at 95 °C followed by 28–30 cycles at 94 °C for 30 s, 56 °C for 45 s, 72 °C for 60 s, and a final extension at 72 °C for 10 min. Primer sequences were as follows: GalR1 (forward primer, 5′ TGTCTTTGTATGCCAAGGTTC 3′; reverse primer, 5′ TCGGTCTTCTTTCTTAGCATGC 3′), GalR2 (forward primer, 5′ TTCTGCTCTCTGTGGATGCC 3′; reverse primer, 5′ TCTACTGCAGGTGCAGTTG 3′), GalR3 (forward primer, 5′ TTCCTCCACTATCTCCGTGG 3′; reverse primer 5′ TCTACTGAGGCTGTGCA GTTG 3′), galanin (forward primer, 5′ GCACCG
TGCGGTAGTAGGCTTAG 3′; reverse primer, 5′ GGCGGAAGATTCCTTTAGCTTAG 3′) and GAPDH (forward primer, 5′ AAATTCACGGCAGAGTCAA 3′; reverse primer, 5′ GTCTCTGGGTTCCAGT 3′).

**In situ hybridization**

**In situ** hybridization was performed using non-isotopic DIG RNA labeling kit (SP6/T7) (Roche) according to the manufacturer’s protocol. Target fragments (galanin) were cloned into PGEM-T easy vector (Promega) and confirmed by automated sequencing. DIG-labeled probes were generated by transcription with T7 and sp6 RNA polymerase using DIG RNA labeling kit (SP6/T7) (Roche). To investigate whether the target genes were expressed in adipocytes, 3T3-L1 adipocytes were cultured on slides as mentioned above in cell culture. Then the adipocytes were fixed with 0·4% paraformaldehyde. Hybridization was performed overnight at 68 °C in a humidified chamber (100 ng digoxigenin-labeled RNA probe in 100 µl hybridization buffer per slide). The post-hybridization slides were washed in 2 × SSC/formamide at 68 °C twice, then in 0·2 × SSC at 68 °C twice (each for 30 min). The hybridization signals were detected according to the instructions of the Dig Nucleic Acid Detection kit (Roche). To terminate the color reaction, samples were rinsed several times with nuclease-free water and visualized by light microscopy.

**Real-time PCR**

To test the expression of galanin in normal and fasting rat adipose tissue, quantitative PCR was performed using TaqMan. Primer sequences were as follows: galanin primers (forward, 5′ TACCCCTGCCTGAGAGCAAT 3′; reverse, 5′ TCTCTCTGAGAGCAAT 3′) and GAPDH (forward, 5′ GTCACCAGGGCTGACCCCT 3′; reverse, 5′ GCTGCCAGAATTG3′). Each reaction was run in duplicate. Standard curves for each amplification product were generated from 10-fold dilutions of pooled cDNA to determine primer efficiency.

**Northern blot analysis**

Northern blot analysis was performed using non-isotopic DIG Northern starter kit (Roche) according to the manufacturer’s protocol. Target fragments (galanin, leptin and GAPDH) were cloned into PGEM-T easy vector and confirmed by automated sequencing. DIG-labeled probes were generated by transcription with T7 or sp6 RNA polymerase using the DIG Northern starter kit. Total RNA was isolated from rat epididymal fat pads by Trizol reagent (Gibco). Ten-microgram samples of total RNA were run in 1·2% formaldehyde–agarose denaturing gels and transferred to positively charged N membrane in 20 × SSC, the membrane was then baked at 80 °C for 2 h. Hybridization was performed at 68 °C with agitation overnight. The membrane was washed twice with 2 × SSC and 0·1% SDS for 5 min at room temperature and twice with 0·1 × SSC and 0·1% SDS for 15 min at 68 °C. According to the manufacturer’s instructions, the membrane was washed and blocked and then incubated with anti-DIG serum/alkaline phosphatase conjugate. CDP-star was used as the chemiluminescence substrate. Signals were visualized on X-ray film.

**Western blot analysis**

Cells were aspirated with a radioimmunoprecipitation (RIPA) buffer (50 nM Tris (pH 8·0), 150 nM NaCl, 0·1% SDS, 0·5% deoxycholic acid and 1% Nonindex-P-40); 120 µg protein were loaded onto a gel, subjected to SDS/PAGE (5% stacking gel, 12% running gel) and then electrophobted onto nitrocellulose membranes. Membranes were blocked in Tris–buffered saline (TBS) buffer, pH 7·6 containing 5% skimmed milk powder, at 4 °C overnight, then exposed to anti-leptin polyclonal antibody (Santa Cruz sc-842; Santa
Cruz Biotechnology, Santa Cruz, CA, USA) or anti-actin antibody (Santa Cruz sc-8432) (at a dilution of 1/200 for anti-leptin antibody and of 1/500 for anti-actin antibody) in TBS–Tween buffer containing 5% skimmed milk powder for 2 h. Membranes then were washed and incubated with anti-rabbit or anti-mouse Ig (DAKO, Carpinteria, CA, USA) conjugated to horseradish peroxidase diluted 1/1000 in the same buffer for 1 h. After a series of washes in TBS–Tween buffer, protein bands were visualized by chemiluminescence with an ECL luminescence kit (Amersham) and exposed to X-ray film.

**Leptin quantitation**

Leptin in serum was measured by RIA using a commercially available rat leptin RIA kit (Linco Research, St Charles, MO, USA) as described by the manufacturer.

**Results**

**The expression of GalR1 and GalR2 in rat adipose tissue**

The actions of galanin are mediated by at least three subtypes of receptors: GalR1, GalR2 and GalR3. These receptors have different distributions. Studies were initiated to determine whether galanin and galanin receptors are expressed in adipose tissue and 3T3-L1 adipocytes by using RT-PCR and in situ hybridization. Total RNA was extracted from rat epididymal fat pads and 3T3-L1 adipocytes. Reports to date indicate that galanin receptor mRNA is highly expressed in the hypothalamus (Waters & Krause 2000). Therefore, total RNA from rat hypothalamus was included as a positive control. As shown in Fig. 1, GalR1 and GalR2 were found to be expressed both in rat adipose tissue and in 3T3-L1 adipocytes. At the same time, galanin was detected both in rat adipose tissue and 3T3-L1 adipocytes using RT-PCR and in situ hybridization. This result suggests that galanin may exert an effect on adipocytes.

**Galanin regulates leptin gene expression and leptin protein secretion in vivo**

To analyze whether galanin has an effect on leptin gene expression and secretion in rat visceral adipose tissue in vivo, Sprague–Dawley rats were given galanin intravenously, and plasma samples were collected at 15 min, 60 min, 2 h and 4 h...
respectively for later quantitation of leptin. As shown in Fig. 2, treatment with lower doses of galanin (1 µg/animal or 5 µg/animal) showed a tendency to decrease plasma leptin levels, although no statistical significance was reached. Treatment with a higher dose of galanin (25 µg/animal) induced approximately 46% down-regulation of leptin secretion at 15 min, followed by 40, 37 and 47% decrease in leptin secretion at 1, 2 and 4 h respectively (Fig. 2D). Meanwhile, Northern blot analysis of adipose tissue revealed that leptin mRNA expressed in adipose was discovered at 4 h after treatment with galanin in a dose-dependent manner. Results are means±S.E. (n=5–6/group), *P<0.05. (C) Northern blot analysis depicting leptin expression in rat hypothalamus treated with 1, 5 and 25 µg galanin for 4 h. The results show that leptin gene expression in the hypothalamus was unaffected. (D) The effect of galanin treatment on leptin secretion. Rat plasma leptin was measured by radioimmunoassay. Results are expressed as means±S.E (n=5–9/group), *P<0.05.

Galanin regulates leptin gene expression and leptin protein secretion in 3T3-L1 adipocytes

To determine whether the in vivo changes in leptin production are the result of a direct galanin effect on adipocyte leptin expression, the effect of galanin (10 nM, 48 h) on leptin expression and secretion in 3T3-L1 adipocytes was evaluated. As shown in Fig. 3A, treatment with 10 nM galanin for 48 h induced approximately 50% decrease in leptin mRNA expression in 3T3-L1 adipocytes. Meanwhile, Western blot analysis demonstrated that leptin protein also decreased in 3T3-L1 adipocytes (Fig. 3B). These results suggest that the in vivo effects of the galanin may be due to a direct cellular effect on leptin production.

Effects of fasting on galanin and leptin expression in rat adipose tissue and on blood leptin levels

Since galanin inhibits leptin expression and secretion in vivo, additional studies were initiated to
determine whether galanin plays a role in the
regulation of leptin expression and secretion in
fasting rats. To this end, the expression of galanin
in the fed rats and in the rats after 72 h fasting was
compared; at the same time, the expression and
secretion of leptin were also examined. Sprague–
Dawley rats (200–220 g) were divided into two
groups (n=9–10/group). One group had free access
to water and food, while the other was fasted for
72 h. The rats were killed, blood samples were
taken for RIA of leptin, and epididymal fat pads
were collected for semi-quantitative RT-PCR,
Northern blot and real-time PCR. As shown in
Fig. 4, consistent with other reports (Saladin
et al. 1995), leptin secretion and leptin mRNA
expression in rat adipose tissue were decreased by
fasting, as discovered using Northern blot analysis
and radioimmunoassay (Fig. 4D and E). As
expected, the galanin mRNA probe hybridized
with a 0·9 kb transcript from rat hypothalamus
RNA, which corresponds to that previously
described for galanin (Brann et al. 1993). However,
no such hybridization signal was seen with total rat
RNA extracted from epididymal fat pads, indicating
that galanin expressed in adipose tissue was
lower than that expressed in hypothalamus (Fig.
4A). The galanin gene expression was found to
increase in fasting rat adipose tissue by using
semi-quantitative RT-PCR (Fig. 4B). To further
confirm the result, the galanin gene expressed in rat
adipose tissue was analyzed by real-time PCR. The
result showed that galanin expression increased
about 8-fold in fasting rat adipose tissue (Fig. 4C).
All of the above data suggest that increased galanin
expression in rat adipose tissue after fasting may
play a role in regulating leptin expression and
secretion in fasting rats.

**Discussion**

Adipose tissue has long been considered to serve as
a fat depot; however, research in the past decade
has demonstrated that it actually plays a vital role
in energy regulation and is now considered as a
major endocrine organ. Adipose tissue synthesizes
and secretes a number of factors, such as leptin,
TNF-α, resistin and adiponectin, etc. (Hotamisligi
et al. 1993, Zhang et al. 1994, Maeda et al. 1996,
Kim et al. 2001). Among these factors, leptin is
important because of its key roles in energy
balance.

Leptin, an ob gene product, as a sensor in
monitoring the size of adipose tissue mass, is
transported to the hypothalamus, where it binds to its receptors to regulate expression of other neuropeptides. The information is received and integrated in the CNS processing unit, and then is transmitted by the effector system, including the sympathetic nervous system, to control energy intake and energy expenditure. Furthermore, circulating leptin concentration is decreased in fasting rats and is restored to prefasting levels when rats have been refed for 4 h. The underlying mechanisms regulating leptin secretion have not been fully understood. In the present study, we first found that galanin inhibited ob gene expression and leptin protein secretion in vitro and in vivo. Interestingly, galanin was found not only to be present in rat adipose tissue, but also to increase in rat adipose tissue after fasting. Another interesting finding is that the leptin mRNA expression in rat hypothalamus was unaffected by the galanin treatment. Schwartz’s investigation showed that no significant effects were observed in rat hypothalamic levels of preprogalanin mRNA after 24 or 48 h fasting, and no relationship between changes in body weight and expression of galanin mRNA could be demonstrated (Schwartz et al. 1993). Based on these data, we speculate that increased galanin expression in rat adipose tissue after fasting may be involved in decreasing leptin expression and secretion in fasting rats.

Our results manifest that galanin inhibited leptin secretion at 15 min and 1, 2 and 4 h after injection. However, Northern blot analysis of adipose tissue from the same animals showed that leptin mRNA expressed in adipose tissue was unaffected at 2 h after treatment with galanin. These results indicated that the galanin-induced decrease in the

Figure 4 Effect of fasting on galanin and leptin expression and secretion in rat adipose tissue. Male Sprague–Dawley rats were divided into two groups. One group had free access to water and food, while the other had been fasting for 72 h. The rats were killed and blood samples and adipose tissue samples were collected for RIA of leptin and for detecting mRNA expression of these genes. Galanin expression in rat adipose tissue was detected by using Northern blot analysis. Each lane was loaded with 10 µg total RNA from control or from fasting rat adipose tissue, 10 µg total RNA from rat hypothalamus was used as a positive control. Blots were hybridized with rat galanin probe and then were stripped and reprobed with rat GAPDH probe as a control for RNA integrity and loading. (A) The result showed that the galanin mRNA probe was hybridized with a 0.9 kb transcript from rat hypothalamus RNA, however, no such hybridization signal was seen in rat adipose tissue. (B) Galanin expression after fasting was detected by semi-quantitative PCR and the result showed that galanin expression was increased in fasting rat adipose tissue. (C) To further confirm the result, galanin gene expressed in rat adipose tissue was analyzed by real-time PCR. The result showed that galanin expression was increased about 8-fold in fasting rat adipose tissue (n=14–16/group, P<0.01). Quantitation was assessed relative to a series of plasmids containing galanin fragment and normalized to GAPDH. As a positive control, leptin expression and secretion were also measured in the same animals. (D) and (E) As expected, both leptin gene expression and protein secretion were decreased after fasting, corresponding to results previously described for leptin. Plasma leptin was measured by RIA, P<0.01 (n=9–10/group).
plasma level of leptin might be mediated by inhibiting leptin release from a pre-existing intracellular pool during the early stages after treatment. Other studies also showed a similar mechanism regulating leptin secretion in rat adipocytes. Kirchgessner reported that treating cultured adipocytes with TNF-α resulted in an acute stimulation of leptin release into the media which were cycloheximide insensitive and brefeldinA sensitive, and this regulation of leptin secretion was via a secretagogue-like mechanism (Kirchgessner et al. 1997). Another positive regulator of leptin secretion, insulin, may also stimulate leptin secretion via mobilization of preformed leptin pools (Bradley & Cheatham 1999). The finding that treating rats with galanin for 4 h causes a decline in both leptin expression and secretion indicates that a sustained effect of galanin on leptin secretion seems to be involved in both transcriptional and post-transcriptional processes.

Galanin is a widely distributed transmitter in the CNS and peripheral tissues (Tatemoto et al. 1983, Merchenthaler et al. 1993). In addition to modulating food intake with a preference for fat after i.c.v. administration into hypothalamus, galanin decreases glucose-dependent insulin release in species other than man (Ahren & Lindskog 1992), stimulates growth hormone and prolactin release from the anterior pituitary (Chan et al. 1996) and induces smooth muscle contraction in the intestine (Ekblad et al. 1985). The actions of galanin are mediated by at least three G-protein-coupled receptors namely galanin-1 (GalR1), GalR2 and GalR3. GalR1 and GalR2 are highly expressed in the brain with moderate expression in other tissues such as heart, while GalR3 is widely distributed at low or moderate levels in many central and peripheral tissues. GalR1 plays an important role in feeding behavior as compared with GalR2 and GalR3 (Wang et al. 1998), therefore we deduced that the inhibitory action of galanin was probably involved in GalR1 signaling. However, this presumption needs further study.

It has been proposed that leptin production in adipocytes is regulated by changes in intracellular cAMP levels. In vivo and in vitro studies have shown that an increase in intracellular cAMP concentration induced by treatment with β-adrenergic receptor agonists results in decreased leptin expression (Mantzoros et al. 1996, Slieker et al. 1996). It is known that galanin receptors are G-protein-coupled receptors and activation of GalR1 results in inhibition of adenyl cyclase through interaction with Gαi/Gαo G-proteins (Habert-Ortoli et al. 1994, Parker et al. 1995), therefore it is tempting to speculate that galanin-repressed leptin production may be mediated by changes in intracellular cAMP level. The belief that downstream signaling cascades of galanin receptor may be involved in the regulation of leptin secretion and mRNA expression needs further investigation.

In summary, we have found galanin and its receptors, GalR1 GalR2, were expressed in rat adipose tissue and 3T3-L1 adipocytes. Leptin mRNA and protein decreased in 3T3-L1 adipocytes when incubated with 10 nM galanin. Treating rats with galanin decreased leptin protein secretion and inhibited leptin mRNA expression in visceral adipose tissues. Galanin mRNA expression was up-regulated in rat adipose tissue after fasting. These data suggest that galanin mRNA expressed in the visceral adipose tissue may be involved in the regulation of feeding and energy balance after fasting.

Funding

This work was supported in part by grants from the Foundation for the Author of National Excellent Doctoral Dissertation of People’s Republic of China (200154) (H-D S), and the National Natural Science Foundation of China (30000082 and 30270626). All the funding mentioned in this article is supported by the government. There is no conflict of interest that would prejudice the impartiality of the research or a potential conflict of interest that is not fully declared within the text of the article.

References

Leptin expression and secretion in rat adipose tissue · R-Y LI, H-D SONG and others


Received 19 February 2004
Accepted 23 March 2004