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The Effects of Intra-Ductal Mammary Administration of Orexin-A Antagonist (SB-334867) on the *PPARγ* and *SREBP1c* Gene Expression and Serum Adiponectin Levels in Lactating Rats

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Abstract

Introduction: Orexin A and adiponectin are involved in controlling metabolism and energy distribution in the body during lactation. Lactation is a process with high demand for lipid synthesis. Peroxisome proliferator-activated receptor gamma (PPARγ) and sterol regulatory element-binding protein 1 (SREBP1c) are two transcription factors that regulate milk lipid synthesis. The goal of this study was to assess the effects of mammary administration of the orexin A receptor antagonist on the expression of *PPARγ* and *SREBP1c* genes as well as serum adiponectin levels in the lactating rats.

Methods: Orexin A receptor antagonist (SB-334867) was injected intraductal into the mammary glands of lactating female rats at three doses of 1, 2 and 4 µg/kg BW in a solvent volume of 50 µL. The gene expressions of *PPARγ* and *SREBP1c* were measured using real-time polymerase chain reaction (PCR), along with serum levels of adiponectin using ELISA.

Results: The results of quantitative RT-PCR showed a significant decrease in the relative expression of *PPARγ* and *SREBP1c* genes compared to that of the control group. The rate of reduction at a dose of 4 µg/kg BW SB was greater than the doses of 1 and 2 µg/kg BW SB-334867. The serum levels of adiponectin significantly decreased in the 4 µg/kg BW SB group compared to that of the control group.

Conclusion: These results indicated that intra-mammary administration of 4 µg/kg orexin-A antagonist in lactating rats decreased the gene expression of two transcription factors involved in milk fat synthesis, accompanying by a reduction in serum adiponectin level.

Keywords: Orexin receptor antagonist, PPARγ, SREBP1, Adiponectin, Lactation

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Introduction

Orexins are neuropeptides that are involved in feeding, sleep-wakefulness, homeostasis, autonomic regulation and play an important role in the regulation of the metabolic rate.[1] Orexin A also plays a critical role in regulating appetite, energy consumption and metabolism.[2] Conversely, orexin B does not have these effects.[3] In addition, intraventricular injection of orexin A promotes feeding-related behaviors, while injecting orexin B had not provided similar effects in rats. In addition, Lipogenic effects of orexin A have been observed in adipose tissue cells.[4] Therefore, orexin A is more potent than orexin B as a stimulator. Orexin A receptors are found in the hypothalamus and the peripheral tissues and their biological role in the hypothalamus-pituitary axis, gastrointestinal tract, endocrine pancreas, gonadal tissues and other peripheral tissues was revealed, [1] but anyway their role in the mammary glands is not studied yet. The receptors of orexins (OXs) are OX1R and OX2R. Orexin A receptor (OXR1) is highly selective for orexin A but OXR2 binds both orexin A and B with the similar affinity.[1] Orexin A receptor antagonist (SB-334867), is a selective OX1R antagonist which Selectively inhibits the function of orexin A, it has been found to inhibit feeding behavior and food intake and elevate the onset of behavioral satiety.[3] Therefore, it can be used as a useful tool to study the effects of orexin A. In lactating rats, providing

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milk for litters is associated with a dramatic increase in caloric intake, a reduction in energy expenditure, and changes in the pattern of energy utilization as well as storage.[5] The mammary gland during lactation represents one of the most metabolically active tissues.[6] and increase in food intake occurs to satisfy the increased metabolic needs of the active mammary gland.[7] Orexin A is involved in regulating energy metabolism like these changes but its role in the mammary gland is not clear.

Given this background, we hypothesized that orexin A as an important metabolic factor can probably affect the milk synthesis process, including the production of milk fat. Milk fat plays an important role in determining the quality and energy composition of dairy products[8] and milk lipid synthesis is a tightly regulated process.[9] Milk fatty acids are obtained from two sources: extraction from the bloodstream and de novo synthesis within mammary epithelial cells.[10] previous research has also shown that Peroxisome proliferator-activated receptor gamma (PPARγ) plays an important role in regulating the de novo synthesis of the milk fatty acid as well as the synthesis and secretion of triglyceride glycerol.[11] Also various studies suggested that sterol regulatory element binding protein 1c (SREBP1c) is a critical regulator of fatty acid de novo synthesis in the mammary epithelium during lactation.[12] Therefore, we selected these two important factors in the milk fat synthesis for our study.

On the other hand, adiponectin is another abundant protein in serum secreted from the adipose tissue[13] and several studies have shown that adiponectin, like orexin A, plays a critical role in appetite regulation, energy expenditure, and metabolism. This hormone maintains systemic energy balance.[14] Researchers have found that there are interactions between orexin A and adiponectin and the interaction between these peptides has been investigated in different dietary conditions, but still the exact interaction is not clearly understood.[2] Accordingly, our first goal in the present study was to investigate the possible physiological role of orexin A in the mammary glands of the lactating rats, and to clarify this issue, we investigated the effects of mammary administration of the orexin A antagonist (SB-334867) on the expression of two main transcription factors (PPARγ and SREBP1c) involved in milk lipid synthesis. The second goal was to investigate whether or not the injection of an orexin A antagonist was effective on the alterations of serum adiponectin in lactating rats.

Materials and Methods

Animals

Twenty females lactating Wistar albino strain of *Rattus norvegicus* (weighing 250-300 g) were divided into four groups (n = 5). During the study period, the animals were kept under controlled conditions with a temperature of 22 ± 2°C, in a 12-hour light/dark cycle and a relative humidity of about 50%. During the experiment, they had free access to water and food. The rodents were euthanized at the end of experiment under stress-free conditions. The ethics committee of Shahid Beheshti University, Tehran also supervised and approved the process in all stages.

Materials

Orexin A receptor antagonist (SB-334867) was purchased from Tocris Co., USA; and was diluted by a solvent with a combination of 50% physiological normal saline and 50% dimethylsulfoxide (DMSO) solution. For animal anesthesia, inhalational anesthetic isoflurane (produced by Halocarbon, USA) was used. TAE Buffer (Tris-acetate-EDTA) (50X) (Thermo Scientific, USA, Catalog Number: B49), 2% agarose gel Thermo Scientific, USA, CN: G442002) and 50 bp DNA Ladder (Thermo Scientific, USA, CN: FERSM0371) were used for electrophoresis the PCR products.

General Procedures and Surgical Methods

Animals were randomly divided into four groups of five rats including the control group that received 50 µL of solvent (combination of 50% physiological normal saline and 50% DMSO). One experimental group received SB-334867 at a dose of 1 µg/kg BW in 50 µL of solvent; another group received SB-334867 at a dose of 2 µg/kg BW in 50 µL solvent, and the last group received SB-334867 at a dose of 4 µg/kg BW in 50 µL solvent. The doses were selected based on previous studies.[15,16] All rats were injected on the twentieth day of lactation. We first anesthetized the animals with an inhalational anesthetic isoflurane and removed the hair around the nipple. Next, we checked the opening of the nipple and if there was a creatine cap, we would carefully remove it and would disinfect the injection site with 70% ethanol. The injections were performed under a dissecting microscope. All injections were carried out at 9 am, and after 8 hours which gives sufficient time for milk synthesis in rats[17] at 5:00 pm (17:00). The animals were put under deep anesthesia using a solution of ketamine and xylazine in proportion to body weight, and their breast tissue was removed. The breast tissues after separation, were transferred into a nitrogen tank immediately and were stored in the freezer (- 80°C) until RNA extraction. While under deep anesthesia, the rats were decapitated by a decapitator.

Serum Collection and Hormone Assay

Blood samples were collected from the necks of the animals, and the serum of the blood samples were separated using a centrifuge (Hermle Co, Germany) and stored until further analysis at -20 ° C. Serum levels of adiponectin were measured using the Rat Adiponectin ELISA kit (RayBiotech, Inc., Korea) according to the manufacturer's instructions (Kit's sensitivity as well as intra-assay and inter-assay coefficients of variation were

0.8 ng/ml, < 10% and < 12%, respectively).

RNA Extraction and Gene Expression

Total RNA extraction from breast tissue samples was performed using an RNA extraction kit (Pars Tous, Mashhad, Iran) according to the company's instructions. To remove possible DNA contamination, total RNA was treated with DNase I (Thermo Scientific, USA) and then used for cDNA synthesis by the EasyTM cDNA Synthesis Kit (Pars Tous, Mashhad, Iran). All steps were performed according to the instructions provided by the manufacturer. The mRNA expression of *PPARγ*, *SREBP1c*, transcription factors, and β-actin, as the housekeeping gene for normalization, was measured using specific primers (Table 1) and by the real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) method. Relative gene expression for each sample was performed by a Real-time PCR 2x Master Mix kit (SYBR® Green, Pars Tous., Mashhad, Iran) and using the Corbett-RG 6000 X device (Corbett Research, Australia). The PCR mixture was placed in the machine according to the kit's instructions. For each given transcript, PCR was conducted in a duplicate manner with a final volume of 20µl based on the temperature cycles program: 10 minutes at 95 ° C, 35 cycles of 94° C for 15 seconds, 57° C for 30 seconds and 72° C for 30 seconds, then 5 min at 72 ° C. The calculation method of formula $2^{-\Delta\Delta Ct}$ was used to analyze PCR data,[18] and the relative expression of target genes was obtained following the normalization with *β-actin* reference gene. PCR products were electrophoresis on 2% TAE agarose gel mixed with DNA safe stain dye for 45 minutes, then gels were examined under UV light to determine the bands (Figure 1).

Data Analysis

Data were analyzed using GraphPad Prism software, version 9. All data were reported as means ± SEM. The normality assumption was confirmed through Shapiro-Wilk test. To compare the results of this study, one-way analysis of variance was used and Tukey post hoc test was adopted to compare the pairs. Data differences were considered significant with $P < 0.05$.

Results

Effect of Intra-ductal Injection of SB-334867 on Serum Adiponectin Level

No significant difference in serum adiponectin level was observed between the groups that received SB at doses of

Table 1. Forward and Reverse Primer Sequence Used for qRT-PCR

Gene Name	NCBI Accession Number	Primer Sequence (5′-3′)	Amplicon size(bp)	Tm (°C)
Pparγ	NM_013124.3	F- ATGGGTGAAACTCTGGGAGAT	70	58.43
		R- TCATAGGCAGTGCATCAGCG		60.53
SREBP1c	NM_001276707.1	F- ATCATGACCGTGGCAACTCT	89	60.9
		R- CAGCCATAATGACAACGGACT		60.1
β-Actin	NM_031144.3	F- CTGACCCTGAAGTACCCCAT	55	59.5
		R- CCATATCGTCCCAGTTGGTG		59.4

Pparγ: Peroxisome proliferator-activated receptor gamma; *SREBP1c*: sterol regulatory element-binding protein 1c; F: Forward; R: Reverse.

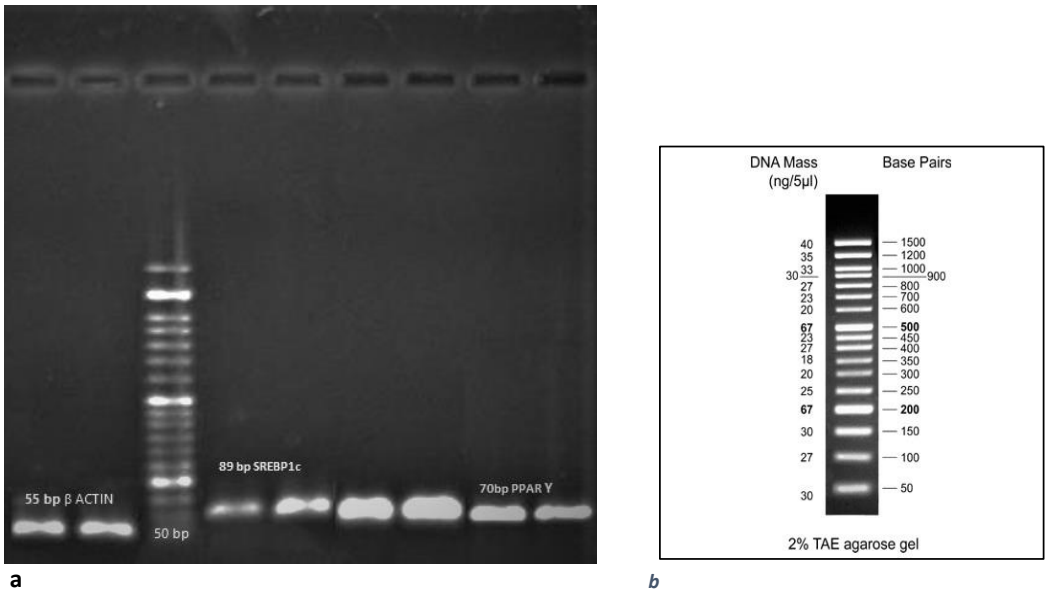

Figure 1. Gel Electrophoresis of qRT-PCR Products. Two samples of each gene were loaded on the gel, and the bands were observed (a). 50 bp DNA Ladder (b) was used to determine the position of the bands.

1 and 2 µg/kg BW and the control group. However, the injection of 4 µg/kg BW SB was associated with a significant reduction ($P=0.001$) in the serum levels of adiponectin in these animals compared to the control group. Moreover, a pairwise comparison amongst the groups by Tukey post hoc test revealed a significant difference between the SB group with a dose of 1 µg/kg BW compared with SB with a dose of 4 µg/kg BW ($P=0.006$; Figure 2)

Effect of Intra-ductal Injection of SB-334867 on PPARγ Gene Expression

The analyses of RT-PCR data exhibited that the gene expression of *PPARγ* transcription factor significantly reduced in the experimental groups that were injected with different doses 1 and 2 and 4 µg/kg BW SB compared to the control group that was injected with the solvent. The decrease in the dose of 4 µg/kg SB compared to control group ($P=0.001$) was greater than the decrease in the dose of 2 µg/kg SB compared to the group of control ($P=0.03$). There was no significant difference between the doses of 1, 2, and 4 µg/kg SB (Figure 3).

Effect of Intra-ductal Injection of SB-334867 on SREBP1c Gene Expression

The injection of SB at doses of 1 and 2 µg/kg BW did not lead to a significant change in the expression of *SREBP1c* compared to the control group. However, injection of SB at a dose of 4 µg/kg BW significantly reduced the expression of *SREBP1c* compared to the control group ($P=0.001$). Moreover, the Tukey post hoc test showed that there was a significant difference between the groups receiving 1 µg/kg and 4 µg/kg SB ($P=0.002$; Figure 4).

Discussion

In the present study, assuming that orexin A plays a physiological role in breast metabolism during lactation, we used its selective antagonist to see if blocking its function could alter the gene expression of the transcription factors involved in milk fat synthesis. The results of this study unveiled that the intra-ductal injection at the dose of 4 µg/kg BW of a selective orexin A antagonist to lactating rats reduced the expression of the genes involved in lipogenesis in mammary gland epithelium, as well as serum adiponectin level. To our knowledge, no study has yet been done on the role of this receptor and the effects of orexin A on lactation.

Previous studies have reported that orexin A has important roles during pregnancy and lactation periods in interaction with other hormones.[19] Adiponectin is one of these hormones. Studies have also shown that orexin A increases lipogenesis and inhibits lipolysis and stimulates the secretion of adiponectin and these effects are conferred to PPARγ.[20] It has been reported that orexin A in association with PPARγ is involved in controlling energy homeostasis in dairy cows. PPARγ may be involved in the transmission and translation of orexin messages.[21]

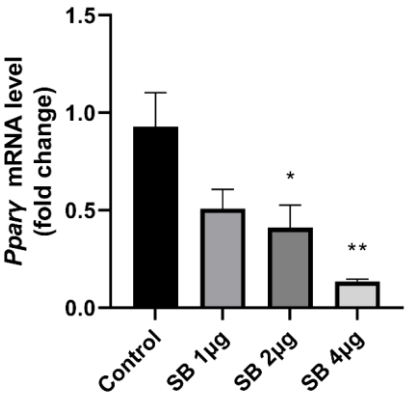

Figure 3. Relative Expression of *PPARγ* mRNA in the Mammary Gland of Lactating Rats After Injection of 1, 2, and 4 µg/kg BW SB (n = 5 in each group). Data are shown as Mean ± SEM. * indicates a significant difference at $P<0.05$ compared with the control group; ** indicates a significant difference at $P<0.01$ versus the control group.

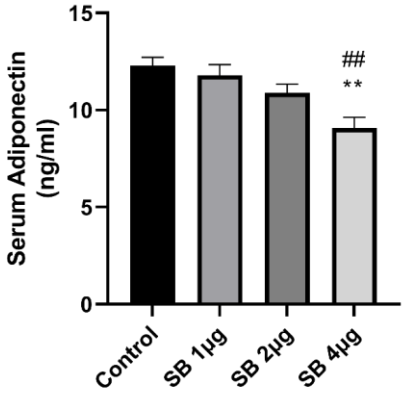

Figure 2. Serum Adiponectin Levels (ng/mL) 8 Hours After the Injection of 1, 2, or 4 µg/kg BW SB Compared to the Control Group (Solvent Injection) (n = 5 in each group). Results in each group are represented as Mean ± SEM. Symbols: ** indicates a significant difference at $P<0.01$ compared with control group, ## indicates a significant difference at $P<0.01$ compared with the SB 1µg/kg BW group.

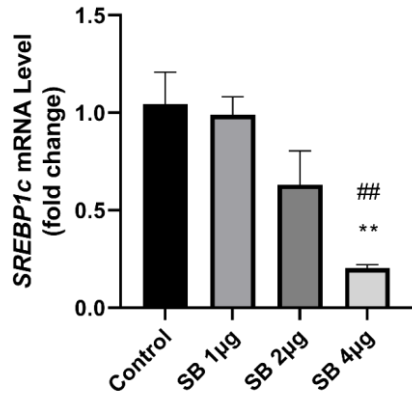

Figure 4. Relative Expression of *SREBP1c* mRNA in the MAMMARY GLAND of Lactating Rats After Injection of 1, 2, and 4 µg/kg BW SB (n = 5 in each group). Data are shown as mean ± SEM. ** indicates a significant difference at $P<0.01$ versus the control group; ## indicates a significant difference at $P<0.01$ versus the SB 1 µg/kg group.

So it is probable that orexin A antagonist administration will be able to inverse these effects. Decrease the gene expression of two important and main transcription factors at lipogenesis namely PPARγ and SREBP1c in our experiment at dose 4 μg/kg BW SB, which consequently decrease lipogenesis, as well as decrease of adiponectin serum, which is agreeable with this idea. The reason for the significant decline at the dose of 4 μg/kg compared to the two lower doses can probably be the stronger antagonism effects on the function of orexin A, significantly reducing the gene expression of these transcription factors, and in parallel, serum adiponectin levels.

Reduced levels of serum adiponectin are probably associated with some events. Adiponectin is an adipokine and is produced by the adipose tissue. A large amount of adipose tissue in the breast leads to the hypothesis that adipokines are also produced in mammary glands.[22] Previous studies have reported that lipogenic transcription factors, PPARγ and SREBP1c, are involved in the expression regulation and secretion of adiponectin.[23] Research has also shown that SREBP1c controls the expression of the adiponectin gene in differentiated adipocytes of the rats.[24] In addition, it has been reported that the mechanism of circulating adiponectin decline in obesity, unlike most adipokines that increase in obesity, probably occurs under the influence of SREBP1c factor. It has been witnessed that the expression of SREBP1c in adipose tissue has been reduced in obese individuals.[23] It has also been acknowledged that the possible mechanism of action of adiponectin in homeostasis involves the activation of PPARγ.[14] The idea that there is an important physiological relationship between PPARγ and adiponectin is basically based on the fact that in many cases, changes in PPARγ activity are related to changes in adiponectin levels.[25] Studies to date have strongly supported the notion that PPARγ activation increases the levels of circulating adiponectin[25] Therefore, both transcription factors have an inducing effect on the expression and secretion of adiponectin. However, no reports of adverse effects of these factors on adiponectin have been identified. Adiponectin is also produced in mammary gland, and it may potentially have an impact on the levels of serum adiponectin concentration. Thus, it is probable low serum adiponectin levels are associated with the decreased adiponectin production in breast fat tissues due to decreased expression of these transcription factors. These apart, researchers observed that orexin A, in the isolated adipocytes of rats, induced adiponectin secretion by a mechanism regulated via PPARγ.[4]

The results of our study are consistent with these reports. In other words, in our experiment, a decrease in the expression of PPARγ and a blockade of the induction effect of orexin A on adiponectin secretion at dose of 4 μg/kg BW SB may have reduced serum adiponectin levels. Probably, orexin A antagonist SB could be absorbed into systemic circulation and have reduced adiponectin secretion from the fat tissue. Furthermore, it is probable that the interaction of SB with other receptors or factors in the breast (whose nature is probably unknown) could affect adipose tissue biogenesis and adiponectin secretion. However, the exact mechanism is not known, and more research is needed to determine the precise mechanisms.

We faced substantial financial and time constraints with the present experiment. It was not possible to take into account some other parameters such as preparing and injecting an orexin A agonist too, or measuring the amount of produced fat in the milk. However, for the first time, we did manage to block the hypothetical function of orexin A in lactation using breast intra-ductal administration of its selective antagonist, and observed changes suggest a possible physiological role for orexin A in the breast. It is recommended that future studies focus on identifying orexin A receptor in the breast.

Conclusion

The present study, for the first time revealed that the intra-ductal injection of a selective antagonist of orexin A (SB-334867) to lactating female rats reduced the gene expression of two main transcription factors involved in milk lipid synthesis and serum adiponectin levels. These findings can propose some physiological roles for orexin A in breast through a receptor (which is yet to be identified) to stimulate milk fat synthesis. These results can be useful to modulate the gene expression of main lipogenic factors in breast to increase milk fat levels and improve the quality of milk and dairy products.

Acknowledgements

The present study was conducted in the research laboratory of the Regional Department of Animal Sciences and Marine Biology, Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, Tehran, Iran.

Authors' Contributions

SBG and HK contributed to the initial conception and design. SBG carried out all experiments, statistical analysis and interpretation of data. HK supervised the research plan. SBG wrote the manuscript and HK revised it. All authors read and approved the last version of the manuscript.

Competing Interests

The authors confirm that there is no conflict of interests in the present research.

Ethical Approval

All experimental procedures were done according to the guidance of the ethical committee of the Shahid Beheshti University (IR.SBU.REC.1400.006).

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