Original Article

Breast Intra-Ductal Injection of Orexin-A receptor antagonist (SB-334867-A) Decreases Gene Expression of Mammary Lipogenic Enzymes and Insulin Serum Levels in the Lactating rats

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Abstract

Background: Orexins regulate the body's energy balance during lactation. Also, Orexin A has been reported to have lipogenic effects on adipose tissue, but its role in the mammary glands is unclear. Acetyl CoA carboxylase (ACC) and glucose 6-phosphate dehydrogenase (G6PD) is critical lipogenic enzymes that catalyze the rate-limiting steps in milk fatty acid synthesis. This study was performed for the first time to investigate the effects of blocking the function of orexin-A in lactating female rats on the gene expression of mammary lipogenic enzymes and possible interaction with serum insulin hormone.

Materials and Methods: Orexin A receptor antagonist (SB-334867-A) was injected at three doses of 1, 2, and 4 µg/kg BW, in a volume of 50 µl solvent by intra-ductal method into the mammary glands of lactating rats and breast tissues were taken after eight hours. Serum insulin levels using ELISA and the relative expression of mammary *ACC* and *G6PD* were measured.

Results: Quantitative PCR results showed that by injecting 4 µg/kg BW of SB intra-ductally, a parallel decrease in the gene expression of *ACC* and *G6PD* and serum insulin levels was observed compared with the control group.

Conclusion: Blocking the function of orexin-A in the lactating rats decreased the expression of lipogenic mammary enzymes and serum insulin.

Keywords: Lactation, Orexin A, SB-334867-A, Insulin, Acetyl-CoA Carboxylase, Glucose-6-Phosphate Dehydrogenase

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Introduction

Orexin peptides control energy balance, reward, nutrition, regulate sleep, and wake rhythms[1]. In addition to central regulatory functions, orexin-A has been produced in several peripheral tissues to maintain energy and glucose homeostasis through hormone-like actions[2]. Orexin-A and its receptors have been detected in the brain and the peripheral tissues, including the adrenal glands, gonadal tissues, endocrine islets, and gut[3,4]. Still, the physiological role of orexin-A in the mammary gland has not been reported. Orexins act through G-protein coupled receptors called receptor type 1 (OX1R) and receptor type 2 (OX2R). The OX1R binds to orexin-A with high selectivity, while the OX2R binds to both orexins with

the same affinity[5]. Lactation is an endergonic process in the female reproductive cycle.

Undoubtedly, a complex endocrine system regulates the distribution of energy in the body at different times of the reproductive cycle. Orexins have been claimed to be part of this complex system. Food intake increases in lactation[6]. Orexins stimulate appetite and food intake in rats, and orexin-A had a stronger and longer-lasting effect on appetite than orexin-B[7]. Researchers have found that the OXA receptor is involved in appetite, food intake, energy expenditure, and reproductive functions, while the OXB receptor regulates sleep-wake cycles[8]. Also, previous studies reported that orexin-A stimulates lipogenesis in cultured adipocytes [3]. Therefore, we hypothesized that orexin-A probably has physiological effects in the mammary gland during lactation, including an impact on milk lipid synthesis.

Orexin-A receptor antagonist SB-334867 selectively inhibits the function of orexin-A[9,] and it can be helpful to check out the possible physiological effect of orexin-A in the breast. On the other hand, researchers have reported that orexin-A is involved in the secretion of the insulin hormone[2], an essential lactogenic hormone in the lactation period[10]. Insulin, similar to orexin-A, regulates energy intake and metabolism[11]. Preliminary studies showed insulin-stimulated lipid synthesis in breast incisions taken from lactating ruminants[12]. Numerous studies have shown that the deficiency or excess of insulin in vivo strongly affects the fatty acids de novo synthesis in the mammary gland[10]. Several lipogenic enzymes have been observed in the lactating mammary gland, such as acetyl CoA carboxylase (ACC) and glucose 6-phosphate dehydrogenase (G6PD), regulated by insulin[10]. Several factors and conditions alter milk fatty acids' composition or lipid secretion[10].

Along with insulin, orexin-A may be one of these factors. Milk fat is essential because it is the primary energy component in milk and represents many of the nutritional quality characteristics of milk and dairy products[13]. Lipogenic enzymes, through fatty acid synthesis, play an essential role in the supply of milk triglycerides (98% milk fat)[14]. The conversion of acetyl CoA to malonyl CoA in the mammary epithelial cells is the first and rate-limiting step of the fatty acid synthesis, which is catalyzed by the vital enzyme acetyl CoA carboxylase. Malonyl CoA provides dual carbon units for synthesizing long-chain fatty acids. Each cycle requires two NADPH molecules[10]. Glucose oxidation by the pentose phosphate shunt pathway is thought to be the main source of NADPH for fatty acid synthesis in the mammary gland epithelial cells of rats[15]. The first step in the pentose pathway is a rate-limiting step catalyzed by the glucose 6-phosphate dehydrogenase (G6PD), involving the conversion of glucose 6-phosphate to 6-phosphogluconate[10]. Against this background, the first intention of the present study was to probe the effects of the orexin-A receptor antagonist on the expression of two major lipogenic enzymes. They are involved in the de novo synthesis of fatty acids in the lactating rat breast tissue to find the physiological role of orexin-A in the mammary gland. The second goal was to investigate the possible changes in serum insulin concentration. The significance of this study was that it might be suggested that orexin-A probably has a physiological role in the breast and may play a cooperative role with insulin in affecting milk fat synthesis in lactating rats.

Methods

Chemicals: Orexin-A receptor antagonist (SB-334867) was prepared from Tocris Co., USA; and was diluted by a solvent with a combination of 50% physiological normal saline and 50% dimethylsulfoxide (DMSO) solution (ThermoFisher, USA). Inhalational anesthetic isoflurane (produced by Halocarbon., USA) was used for animal anesthesia.

Animals: Twenty randomly selected female lactating rats (weighing 250-300 g) of the Wistar albino strain of *Rattus norvegicus* for this study. During the study period, the temperature of the animal care environment was 22±2°C, and the relative humidity was about 50%. The circadian cycle consists of 12 hours of light and 12 hours of darkness. During the experiment, animals had free access to food and water. All experiments were performed following the International Code of working with animals, and the Ethics Committee of Shahid Beheshti University approved all stages of this study.

Table 1: Forward and Reverse Primer Sequence.

Name	Accession number	Sequence (5′-3′)	Amplicon size(bp)	Annealing Temperature(° C)
ACC	NM_022193	F- GACTTAAGCAGCTCAACCAC R- TTTACCTGAAAACCTCCGAGA	53	51
G6PD	NM_017006.2	F- ATCATGACCGTGGCAACTCT R- CAGCCATAATGACAACGGACT	74	55.1
β-Actin	NM_031144.3	F- CTGACCCTGAAGTACCCCAT R- CCATATCGTCCCAGTTGGTG	55	52.7

ACC: Acetyl CoA carboxylase; *G6PD*: Glucose 6-phosphate dehydrogenase; F: Forward, R: Reverse

Intra-Ductal injection and breast tissue sampling: Lactating rats were categorized randomly into four groups (n=5 in each group):

1) the control group received 50 µl of solvent. 2) the group received SB-334867 at a dose of 1 µg/kg BW in 50 µl of solvent. 3) the group receiving SB-334867 at a dose of 2 µg/kg BW per 50 µl solvent, and 4) the group receiving SB-334867 at a dose of 4 µg/kg BW per 50 microliters solvent. Doses were selected according to previous studies[16,17]. First, the animals were anesthetized with inhalational anesthetic isoflurane. Hairs around the nipples were removed. Suitable nipples for the injection were located, and dead skin covering the nipple was removed using tweezers. The injection site was disinfected with 70% ethanol. 50 µl of injection solution was Loaded into an insulin syringe with a 31 Gauge needle (Pic Solution, Farir Teb). The nipple was kept with fine tweezers and lifted slightly to make it available for injection. Then under a diskette microscope, the solution was carefully injected and gently with an insulin syringe. The prepared solutions were injected slowly and carefully to avoid damaging the nipple due to rapid fluid movement or shaking of the needle tip and not create a bulge around the nipple. Thus a successful injection was performed. All injections were carried out on the twentieth day of lactation at 9 a.m. After 8 hours which gives sufficient time for milk synthesis in rats [18], the animals were put under deep anesthesia using a solution of 80 mg/kg ketamine and 10 mg/kg xylazine. Their breast tissues were removed entirely. The breast tissues were immediately transferred into liquid Nitrogen and stored at minus 80°C until RNA extraction.

Hormonal Assay: After the mammary gland sampling in deep anesthesia, the rats were decapitated by a decapitator, and blood samples were taken from the necks of the animals. The serum of the blood samples was separated using a centrifuge (Hermle Co, Germany) and stored until further analysis at -20°C. Serum insulin levels were measured using an ELISA kit (Ray Biotech, Inc. Korea) following the instructions provided by the manufacturer (Kit's sensitivity, as well as intra-assay and inter-assay coefficient of variation, were 5µIU/ml, <10%, and <12%, respectively).

Gene Expression Measurement by Quantitative Real-time PCR (qRT- PCR): Total RNA was extracted from mammary gland tissue samples using an RNA extraction kit (Pars Tous., Mashhad) according to the company's instructions. Total RNA was treated with DNase I (Thermo Scientific, USA). Then cDNA was synthesized by the EasyTM cDNA Synthesis Kit (Pars tous., Mashhad) utilizing the one µg extracted RNA. Beta-actin was used as the reference gene. All steps were performed according to the manufacturer's protocol. The mRNA expression of *ACC*, *G6PD*, and *β-Actin* was measured based on their specific primers using the RT-qPCR method (Table 1). Relative gene expression for each sample was performed using a Real-time PCR 2x Master Mix kit (SYBR® Green, Pars tous., Mashhad) and a Corbett-RG 6000 X device (Corbett Research, Australia). PCR was duplicated for each transcript at a final volume of 20 µl. The amplification conditions were as follows: 10 minutes at 94°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. Real-time PCR analysis was performed by the $2^{-\Delta\Delta Ct}$ method[19], and the relative expression of target genes

was obtained following the normalization with a *β-Actin* reference gene.

Statistic: All values were expressed as means ± SEM. Data were normalized through the Shapiro-Wilk test and were tested by one-way ANOVA using graph pad prism software version 9. Tukey post hoc test was adopted to compare the pairs. Data differences were considered significant with $p<0.05$.

The ethical committee of Shahid Beheshti University approved all experimental procedures (IR.SBU-.REC.1400.006).

Results

Results of measuring serum insulin levels: According to the results of the ELISA kit, no significant change in serum insulin was observed between the groups that received SB at doses of 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 ($p=0.006$) in the serum levels of insulin in these animals compared to the control group. The pairwise comparison amongst the groups by the Tukey post hoc test didn't reveal a significant difference between groups (Figure 1).

Results of *ACC* Gene Expression: The PCR data indicated that the Injection of SB at doses of 1 and 2 µg/kg BW did not cause a significant change in the expression of *ACC* compared to the control group. However, Injection of SB at a dose of 4 micrograms significantly reduced the expression of *ACC* compared to that of the control group ($p=0.01$). There was no significant difference between the doses of 1, 2, and 4 µg/kg BW SB (Figure 2).

Results of *G6PD* Gene Expression: The analyses of RT-PCR data exhibited that the expression of the *G6PD*

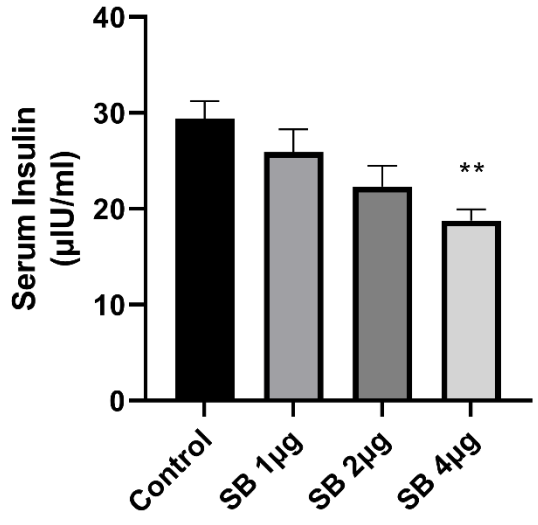

Figure 1. Serum insulin Levels (µIU/ml) 8 hours after the injection of 1,2,4 µg/kg BW SB compared to control group with solvent injection. Results from each group are represented as mean ± standard error of the mean (SEM) of five rats per group. **P<0.01 compared with Control group.

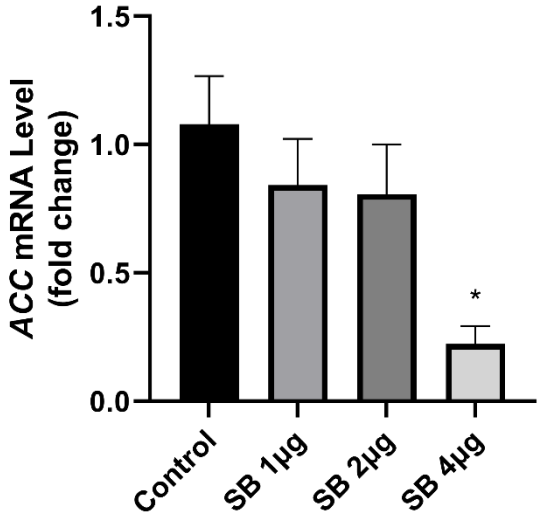

Figure 2. Relative expression of *ACC* mRNA in the mammary gland of the lactating rats after injection of 1,2,4 µg/kg BW SB, compared with the control group. Data are shown as mean ± standard error of the mean (SEM) of five rats per group. * P <0.05 , versus Control group.

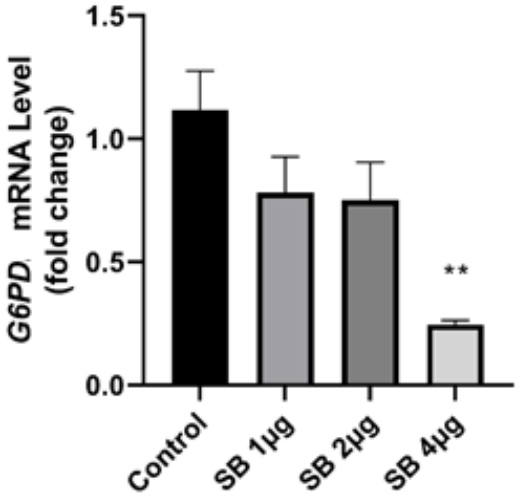

Figure 3. Relative expression of *G6PD* mRNA in the mammary gland of the lactating rats after injection of 1,2,4 µg/kg BW SB, compared with the control group. Data are shown as mean ± standard error of the mean (SEM) of five rats per group. ** P<0.01 compared with Control group.

gene was significantly reduced in the experimental group that was injected with 4 µg/kg BW SB compared with the control group that was injected with solvent (p=0.001). The doses of 1 and 2 µg/kg BW SB didn't cause a significant change in the expression of *G6PD* compared with the control group. Moreover, comparing the groups in pairs via the Tukey post hoc test showed no significant difference between the doses of 1, 2, and 4 µg/kg BW SB (Figure 3).

Discussion

The present research was a preliminary study on the effects of orexin-A antagonist on milk fat synthesis enzymes in the breast, which was done for the first time. The hypothesis was that orexin-A affects the lipogenic enzymes in the breast cells. Orexin-A antagonist (SB-334867) was injected to block orexin-A function during lactation and then investigated the gene expression of important enzymes in the milk fat synthesis. Following mammary intra-ductal Injection of selective SB-334867, at three doses of 1, 2, and 4 µg/kg BW, after eight hours, serum insulin levels and gene expression of mammary lipogenic enzymes, *ACC*, and *G6PD* decreased in the lactating rats' experimental group with 4 µg/kg BW SB. Previous studies reported that orexin-A increased glucose uptake and lipogenesis. It also reduced lipolysis in the cultured rodent adipocytes. It led to an accumulation of triacylglycerols[3]. Orexins are originally characterized as orexigenic hypothalamic neuropeptides in mammals. Various studies have reported the stimulation effects of orexin-A on lipogenesis and lipid accumulation. Still, reports about orexin-A effects on ACC and G6PD enzymes didn't found. However, more recently, some research on the avian (non-mammalian) species also revealed that the orexin system is expressed in chicken liver and avian hepatocyte cell lines. That orexin-A administration modulates the expression of lipogenic genes. In addition, treatment with recombinant orexin-A activated acetyl-CoA carboxylase (ACC) in the avian hepatocyte cell culture[20].

The enzymes involved in fat milk synthesis (ACC and G6PD) increase after parturition. Since the significant regulation of fatty acid synthesis occurs at the gene expression level, the increase or decrease in lipogenic enzyme activity is expected to reflect the increase or decrease in enzyme mRNA[10]. In the present experiment, administration of orexin-A antagonist at a 4 µg/kg BW dose led to decreased *ACC* and *G6PD* expression, indicating a decrease in enzyme activity and lipogenesis activity. It means that SB-334867 has blocked the lipogenic effects of orexin-A in the mammary glands. On the other hand, various researches have demonstrated the effects of orexin-A on insulin levels[21,22]. Orexin A has been reported to stimulate insulin secretion from pancreatic cells in vivo and in-vitro[22]. Also, one nmol subcutaneous injection of orexin-A resulted in increased blood glucose and insulin levels in rats under non-fasting conditions[21].

Another study reported that orexin-A induced a dose-dependent increase of glucose-stimulated insulin secretion (GSIS) in the perfused pancreas of rats, and pretreatment with the SB-334867, blocked the excitatory effects of orexin-A on insulin secretion[22]. In the present research, mammary Injection of the SB-334867 was associated with decreased serum insulin levels in lactating rats. Probably orexin-A antagonist having absorbed into the systemic circulation, and having reduced the insulin secretion from pancreatic cells, which in the following has caused the decrease of mammary lipogenic enzymes expression. Although in some studies, orexin-A inhibited insulin secretion[3]. Therefore, the effects of orexin-A on insulin levels may vary depending on the animal's physiological conditions, experimental conditions, and duration of treatment. These reports suggest that peripheral orexin-A may modulate the insulin hormone. In this study, other factors may have played a role. 4-microgram SB injection has activated another factor by interacting with the different receptors or hormones in the breast. This factor, which has entered into the systemic circulation, has reduced insulin secretion.

On the other hand, many reports indicate that insulin impacts lipogenic enzymes in the liver, adipose tissue, and mammary glands. Insulin has been reported to stimulate the activity or expression of lipogenic enzymes. In one research, lipogenesis in insulin-treated rats increased 5-fold in the liver and 20-fold in white adipose tissue[23]. Some studies focused on the effects of insulin on the mammary glands, often with a high-fat or starvation diet, to alter endogenous insulin levels. These treatments reduced de novo synthesis of fatty acid, and

in both cases, insulin administration restored fatty acid synthesis to some extent by restoring ACC activity to normal levels[12]. It has been suggested that insulin is the major effector of the hepatic glucose 6-phosphate dehydrogenase (G6PD) enzyme, which provides NADPH for the reaction[24]. Most likely, in the present research, decreased expression of *ACC* and *G6PD* mammary lipogenic enzymes have been associated with reduced serum insulin levels. These findings are consistent with the reported results on the stimulatory effects of insulin on lipogenic enzymes and lipogenesis. It is reported that insulin is likely to affect important metabolic functions in secretory cells of rat breast, in addition to its potential effects on membrane transport. In particular, it stimulates fatty acid synthesis and affects or moderates cellular electron transfer processes or energy metabolism[25]. In any case, the simultaneous observation of decreased insulin levels and the relative expression of mammary lipogenic enzymes following the injection of 4 micrograms of the orexin-A antagonist may indicate interactions between orexin-A and insulin in affecting mammary lipid synthesis enzymes during lactation.

Nonetheless, the exact mechanisms are not known. More experiments will be needed to shed more light on the mechanisms, including injecting several doses of orexin-A, its agonist and antagonists, and insulin and observing the effects or testing in conditions such as starvation or high-fat diets, and analyzing the results, which were not possible to be investigated in the present study due to financial and time limitations. For the first time, the study checked out the effect of the orexin-A antagonist on the milk lipid synthesis enzymes. The intra-ductal injection method was used for the non-invasive treatment of lactating rats, which allows examining the desired injection's effects on essential enzymes involved in the synthesis of milk fat without disrupting the milk production mechanism. It is suggested that in further studies, further experiments will be performed to identify the receptor and the exact physiological roles of orexin-A in the mammary glands during lactation, and the synthesis of fat, protein, and lactose in milk be measured and examined.

Conclusion

The results of this study revealed that mammary administration of orexin-A antagonist by the intra-ductal method has significantly reduced the gene expression of main milk fat synthesis enzymes and insulin serum levels in lactating rats. Thus, these results can indicate the physiological roles of orexin-A in the mammary gland tissue, which directly or indirectly, through interaction with other factors such as insulin, has essential functions in milk fat synthesis. Present findings can be helpful in management measures to improve the quality of dairy production.

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