Current Trends in Immunology
Vol. 16, 2015

Original Communication

Role of endogenous orexin-A in carrageenan-induced inflammation in rats

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ABSTRACT

Orexins/hypocretins are novel neuropeptides originally discovered in neurons in the lateral hypothalamus and are effective in a wide variety of physiological functions such as food intake, sleep/awake cycle and neuroendocrinological response. The aim of this study was to investigate the role of endogenous orexin-A in the inflammatory response. Male Wistar albino rats were divided randomly into three groups, namely saline, carrageenan and carrageenan+SB-334867. The orexin type 1 receptor (OX1R) antagonist SB-334867 was administered to rats to block the effect of orexin-A through OX1R. The carrageenan (1%) was injected into the dorsal air pouches of the rats to induce an inflammatory reaction. Plasma orexin-A level increased significantly due to carrageenan-induced inflammation. The inflammation index, the chemotactic activity of exudate cells and the concentration of pro-inflammatory mediators in exudate increased in response to carrageenan injection. The inflammation index and TNF-α concentration decreased significantly in the group treated with SB-334867 compared to the carrageenan group. OX1R antagonist did not alter the production of PGE2 and NO, the expression of COX-2 and iNOS and the activities of chemotaxis and phagocytosis of exudate cells. These results indicate that orexin-A is involved in inflammation and that the inflammatory effect of orexin-A is independent of NO and PGE2.

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KEYWORDS: orexin-A, air pouch, inflammation, exudate cells, chemotaxis, phagocytosis

INTRODUCTION

Orexins are neuropeptides which were originally discovered from the rat brain and consist of two peptides orexin-A and orexin-B, both of which are derived from the same precursor prepro-orexin by proteolytic reactions [1, 2]. Orexins exert their effects by targeting two G protein-coupled receptors, namely orexin type 1 (OX1R) and orexin type 2 (OX2R) receptors. Though orexin-A and orexin-B have very common pharmacological effects, some effects observed for orexin-A has not been reported for orexin-B. Since orexin-A binds with higher affinity to the OX1R than orexin-B and both izoforms bind with apparently equal affinity to the OX2R, many investigators have narrowed their attention to the pharmacological effects of orexin-A [1, 3]. Previous studies have shown that orexin-containing neurons are present in the lateral hypothalamic area and have projections from hypothalamus to various brain regions for regulating brain functions such as feeding and sleeping [4, 5]. Moreover, the presence of orexin-A has been reported in plasma [6]. Orexins and their receptors have been detected in a wide range of tissues including the intestine, pancreas, adrenals, kidney, adipose tissue and reproductive tract [7-9]. For many tissues, the precise functions of orexins still remain unclear.

Inflammation is an important response to harmful environmental factors. Inflammation process is

characterized by an increased blood flow to the inflammation site, which leads to cellular infiltration and accumulation of exudate. Migrated phagocytic cells play a crucial role in the progression of inflammation by producing pro-inflammatory mediators such as NO and PGE_2 [10]. NO is a signal molecule in inflammation and iNOS is a key molecule for inducing the production of NO during inflammation. PGE_2 is produced by COX-2 during inflammation. It is known that iNOS and COX-2 are expressed in many cells such as macrophages, endothelial and epithelial cells and transcriptionally activated by NF-κB, a transcription factor regulating the immune response. Recently, it has been shown that the expression of orexin-A mRNA in various organs is associated with a time dependent response to acute inflammatory stimuli such as ischemia-reperfusion damage [11]. Yan *et al.* reported that orexin-A expression was especially high in the stomach, lung and kidney of rats exposed to ischemia/reperfusion [11]. The results suggest that the variation in the expression of orexin-A may be a novel marker for acute inflammation.

The role of endogenous orexin-A in acute inflammatory response has not been studied adequately. The aim of the present study was to investigate whether endogenous orexin-A plays a role in the response to inflammatory stimuli.

MATERIALS AND METHODS

Experimental design

Male Wistar rats, weighing 170-200 g, used in this study were provided by Akdeniz University, Faculty of Medicine, Experimental Animals Care and Production Unit. Rats were housed in a temperature controlled environment (23 ± 1 ºC) and on a 12-h light/dark cycle, with free access to rat chow and tap water *ad libitum* and allowed to remain for at least 7 days prior to the experiments to get adapted to the conditions. Rats were randomly divided into three groups; saline, carrageenan and carrageenan+SB-334867. OX1R antagonist, SB-334867 (Tocris, 1960) was dissolved in 10% (wt/vol) dimethyl sulfoxide (DMSO)/sterile water. In the carrageenan+SB-334867 group, the rats were injected SB-334867 (30 mg/kg, intraperitoneally) just before injection of carrageenan. All the experimental procedures were performed in accordance with mandated standards of human care and were approved by Animal Care and Use Committee of Akdeniz University.

Induction of air-pouch type inflammation in rats using carrageenan

Rats were lightly anesthetized with diethyl ether and the air-pouch was formed by an injection of 20 ml of sterile air into the subcutaneous tissue of interscapular region. After 3 and 6 days, the pouches were re-inflated with 10 ml of sterile air. On day 7, inflammation was induced in the inflammation groups (the carrageenan and carrageenan+SB-334867 groups) by injection of 2 ml of a 1% (w/v) suspension of carrageenan (Sigma Aldrich, C-1013) in saline into the air-pouch under light diethyl ether anaesthesia.

Determination of pouch fluid volume and cell infiltration in the pouch fluid

Six h after the injection of carrageenan solution, the rats were anesthetized with diethyl ether inhalation. The pouches were flushed with 2 ml of phosphate buffered saline (PBS, pH 7.4) containing 0.1% ethylenediaminetetraacetic acid (EDTA) and vigorously massaged for 30 s. Then, they were opened with a small incision and the exudates were carefully collected using a sterilized Pasteur pipette [12]. The total volume of the pouch fluid was measured and the exudate volume was obtained by subtracting 2 ml from the total volume. The total number of cells was counted by an electronic hematology analyzer (Micros, ABX Co., France).

Determination of TNFα concentration in the pouch fluid

The pouch fluid was centrifuged at 2,500 rpm for 10 min to remove infiltrating cells. The levels of TNFα were measured in supernatants using commercial enzyme linked immunosorbent assay kit (ELISA) (Pierce-ERTNFA Co., Rockford, USA) [13]. Samples were applied to ELISA kit according to the manufacturer's instructions. The results are expressed as pg/mL.

Determination of nitrate/nitrite concentration in the pouch fluid

Nitrate/nitrite (NOx) concentration in supernatants of the exudates was determined using a colorimetric assay kit (Cayman Chemical Co., Ann Arbor, MI 48108, USA, 780001) [14]. In brief, exudates were centrifuged at 2,500 rpm for 10 min. The supernatant of the exudate was passed through a 30 kDa filter by centrifugation at 15,000 rpm for 60 min. The filtrate was incubated with nitrate reductase and cofactor mixtures for 3 h at room temperature to

convert nitrate in the sample to nitrite. The optical density of the wells was measured by microplate reader (Biotek, ELx800, Highland Park, USA) at 540 nm, and the concentration of total nitrite was calculated by using a standard nitrite curve after the reaction with Griess reagent. The results are expressed as μM.

Determination of PGE_2 concentration in the pouch fluid

PGE_2 assay in supernatants of the exudates was performed using the PGE_2 Enzyme Immunoassay Kit (Cayman Chemical Co., Ann Arbor, MI 48108, USA, 514010) according to the manufacturer's protocol [15]. In brief, 0.5 ml of water:ethanol (1:4 v/v) solution was added to 0.5 ml of exudate. 10 μl of glacial acetic acid was added to each sample followed by 5 min incubation at room temperature. Samples were centrifuged at 2,500 g for 2 min. The supernatants were applied to C18 minicolumns (Amersham Pharmacia Biotech, UK) and the columns were washed with distilled water and hexane. PGE_2 was eluted with ethyl acetate. This fraction was lyophilized to dryness and stored at -80 °C. According to the protocol, samples were dissolved in assay buffer at a proportion of 1:500 and PGE_2 levels were assayed in 96-well plates. The results are expressed as pg/ml.

Determination of phagocytic and chemotactic activities of the exudate cells

The cells were separated by centrifugation and the isolated cells were resuspended in Roswell Park Memorial Institute medium (RPMI)-1640 medium and adjusted to 2×10^6 viable cells/ml for assaying phagocytic activity, % phagocytose, chemotactic activity and cell viability. To determine the phagocytic activity, 100 μl of activated charcoal suspension (1%) was added to 100 μl cell suspension (2×10^6 cell/ml) and incubated for 1 h at 37 °C. After the incubation, the number of particles phagocytosed by macrophages was counted and average of 100 cells was used to determine the phagocytic activity [16].

The chemotactic activity was determined according to the method previously described [15]. In brief, zymosan activated serum (ZAS, 10%) was prepared by the method of Goldstein *et al*. [17] by incubation of rat serum with zymosan particles at a concentration of 1 mg/ml for 45 min at 37 ºC. The zymosan particles were removed by centrifugation (1,000 rpm for 10 min) and the supernatant was collected and stored at -20 ºC in small aliquots until used. For the measurement of chemotactic activity, ZAS was diluted at 1:10 in the incubation medium.

The chemotaxis assay was performed in Boyden chamber using a nitrocellulose filter 8 μm pore size (Shleicher & Schuell AE 9 Membrane Filter). RPMI-1640 (0.5 ml) containing 2 x 10^6 cells/ml was added to the upper compartment of the Boyden chamber. ZAS (0.5 ml) was injected to the lower compartment of the chamber and incubated for 90 min at 37 ºC. After the incubation period, the membrane filter was removed and stained with hematoxyline. The distances (μm) between macrophages which migrated to the lower face of the filter were determined using light microscope (x1000; Olympus B201). The data are expressed as the average of three randomly chosen fields.

Determination of plasma orexin-A levels

Plasma orexin-A levels were determined in the blood samples that were obtained from anesthetized animals via the abdominal aorta. Blood sample was collected in a Lavender vacutainer tube which contains EDTA and was transferred from the lavender vacutainer tube to centrifuge tube containing aprotinin (0.6 TIU/ml of blood), centrifuged at 2,500 rpm for 15 min at 4 °C, and then the plasma was separated and stored at -80 °C until used. Orexin-A ELISA kit was purchased from Phoenix Pharmaceuticals (Burlingame, USA). Plasma orexin-A was extracted using C18 Sep-Columns (Strata C18-E, Phoenix Pharmaceuticals, USA). Aliquots were further evaporated to dryness using Speedvac (Savant Instruments, Holbrook, NY). The dry residue was dissolved in assay buffer and used for ELISA according to the manufacturer's instructions [18]. The results are expressed as pg/ml.

Determination of the expression of iNOS and COX-2 of the exudate cells

The cells isolated from the pouch fluid were also used to determine the expression of iNOS and COX-2 by western blot analysis. Cell lysates were prepared by treating the cells with a lysis buffer (10 mM Tris at pH 7.4, 100 mM NaCl, 1 mM EDTA, 1mM ethylene glycol tetraacetic acid (EGTA), 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 1% Triton-X, 10% glycerol, 0.1% SDS and 0.5% deoxycholate)

supplemented with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA, P2714). The mixture was incubated on ice for 30 min and sonicated for 30 seconds (Bandelin Electronic, Berlin, Germany, UW2070). The samples were centrifuged at 15,000 rpm at 4 °C and supernatants were solubilized in Laemmli sample buffer (Sigma-Aldrich, St. Louis, MO, USA, S3401).

Prior to electrophoresis, samples were boiled for 5 min at 95 °C. Amounts of protein were determined using commercial kit (Pierce, Rockford, IL, USA, R3236) according to Bradford *et al.* [19] and 80 µg of total protein was loaded on SDS-polyacrylamide gel along with a molecular weight marker (Fermentas, SM1811) and run at 80 volts on 10% gradient gel, followed by transfer onto nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA, 162-0112) at 4 °C and subsequently hybridized with the primary antibodies iNOS (Sigma Aldrich, N7782, dilution; 1:100), COX-2 (Cell Signal, 4842, dilution; 1:500), β-actin (Cell Signal, 4967, dilution; 1:500). After incubation with the primary antibodies at 4 °C overnight, membranes were washed with Tris-buffered saline (TBS)-Tween-20 buffer for 1 h. Membranes were incubated with horse radish peroxidase (HRP)-conjugated secondary antibody (dilution; 1:2,000) (Chemicon, Temecula, CA, USA, AP132P) dissolved in TBS-Tween-20 buffer containing 5% non-fat milk (Bio-Rad Laboratories, Hercules, CA, USA, 170-6404) at room temperature for 1 h. Blots were visualized using chemiluminecent detection system kit according to the manufacturer's instructions (Chemicon, Temecula, CA, USA, 2600). Membranes were exposed to Hyperfilm (Amersham Biosciences, Buckinghamshire, UK, RPN3103K) which was subsequently analyzed using image J, 1.37v software.

Statistical analysis

Data are presented as the mean ± S.E. Statistical analyses were performed with SPSS version 13.0 software (SPSS, Chicago, IL, USA) by using Kruskal Wallis and Mann-Whitney U test. Differences between groups were considered significant at a level of $P < 0.05$.

RESULTS

Plasma orexin-A level

As shown in figure 1, the significant increase in plasma orexin-A levels was observed 6 h after carrageenan administration. The high orexin-A level which was observed 6 h after the administration of carrageenan in the carrageenan group were not similar to the carrageenan+SB-334867 group. The data presented in figure 1 demonstrate that OX1R antagonist administration partially prevented the increase in plasma orexin-A level due to inflammation.

The pouch fluid volume and number of infiltrating cell

To assess the role of endogenous orexin-A in inflammatory processes, we investigated the inflammation index in carrageenan-induced air pouch inflammation model in rats. Pouch fluid volume and number of infiltrating cells were evaluated at

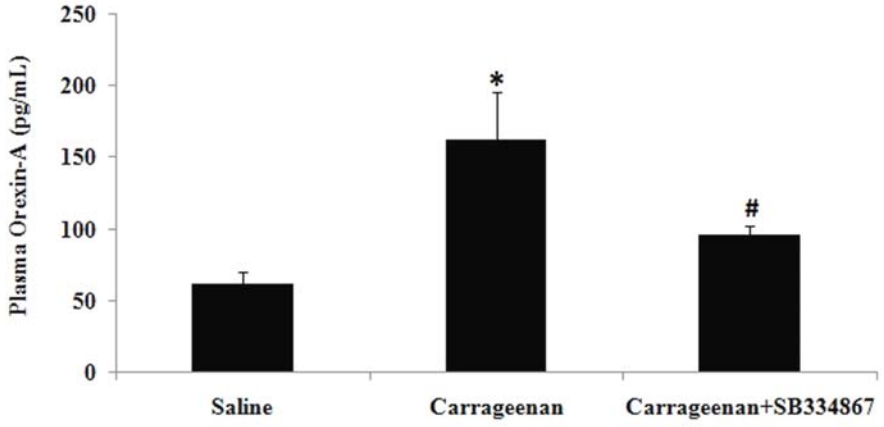

Figure 1. Plasma orexin-A levels. The results are the mean ± S.E. of six experiments performed in duplicate. *$p < 0.05$ compared to the saline group, $^{\#}p < 0.05$ compared to the carrageenan group.

6 h after induction of inflammation by intra-pouch injection of carrageenan. To evaluate the effect of endogeneus orexin-A on air pouch inflammation, animals were treated with SB-334867 intraperitoneally prior to the inflammation. As indicated in figure 2, SB-334867 decreased the total number of cells present in exudates and also the exudate volume observed 6 h after the injection of carrageenan.

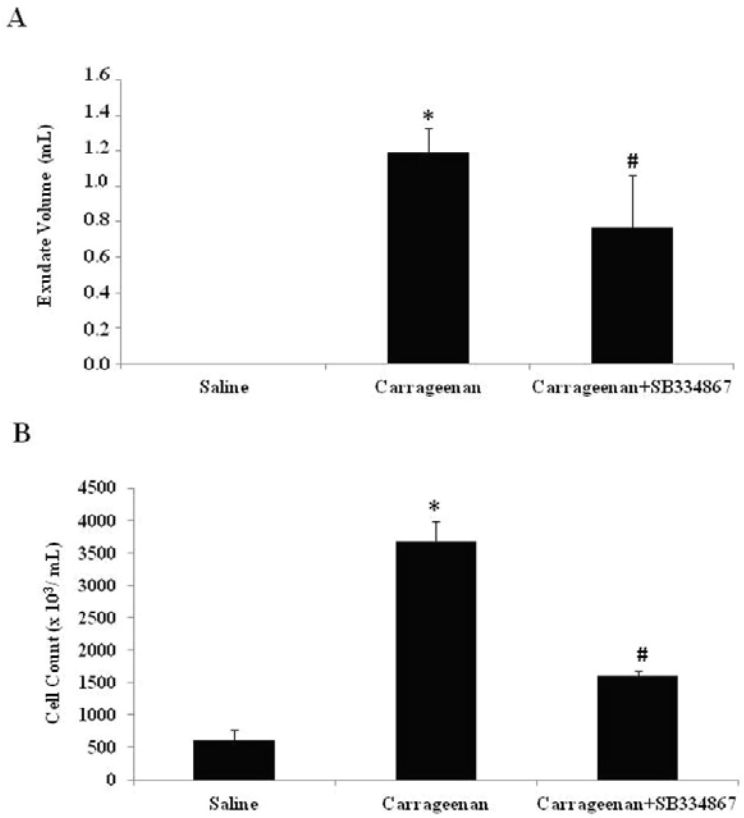

Figure 2. Inflammation index. The exudate volume (A) and the cell count of exudates (B) from the carrageenan-injected rat air pouches. The results are the mean ± S.E. of eight experiments per group. *$p < 0.05$ compared to the saline group, $^{\#}p < 0.05$ compared to the carrageenan group.

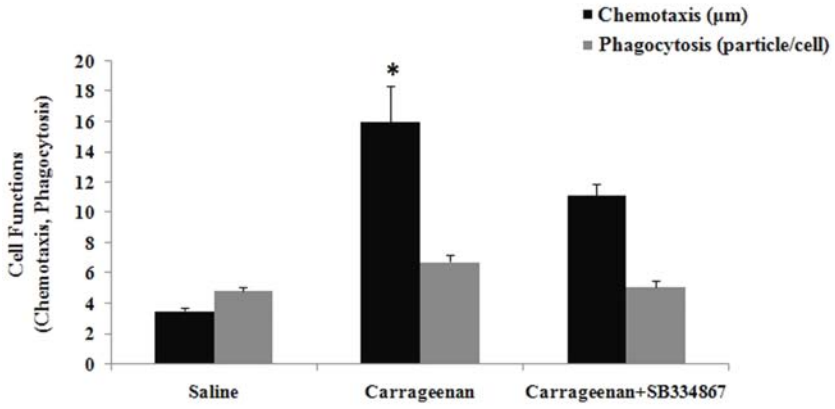

Figure 3. Chemotaxis and phagocytosis of the exudate cells from the air pouches of the carrageenan-injected rats. The results are the mean ± S.E. of eight experiments per group. *$p < 0.05$ compared to the saline group.

The activities of chemotaxis and phagocytosis of the exudate cells

The activities of chemotaxis and phagocytosis of the cells obtained from the pouch exudates were evaluated. The functions of the exudate cells were stimulated by carrageenan injection, but the increase in phagocytic activity was not statistically significant. SB-334867 had no effect on the chemotactic and phagocytic activities of exudates cells (Figure 3).

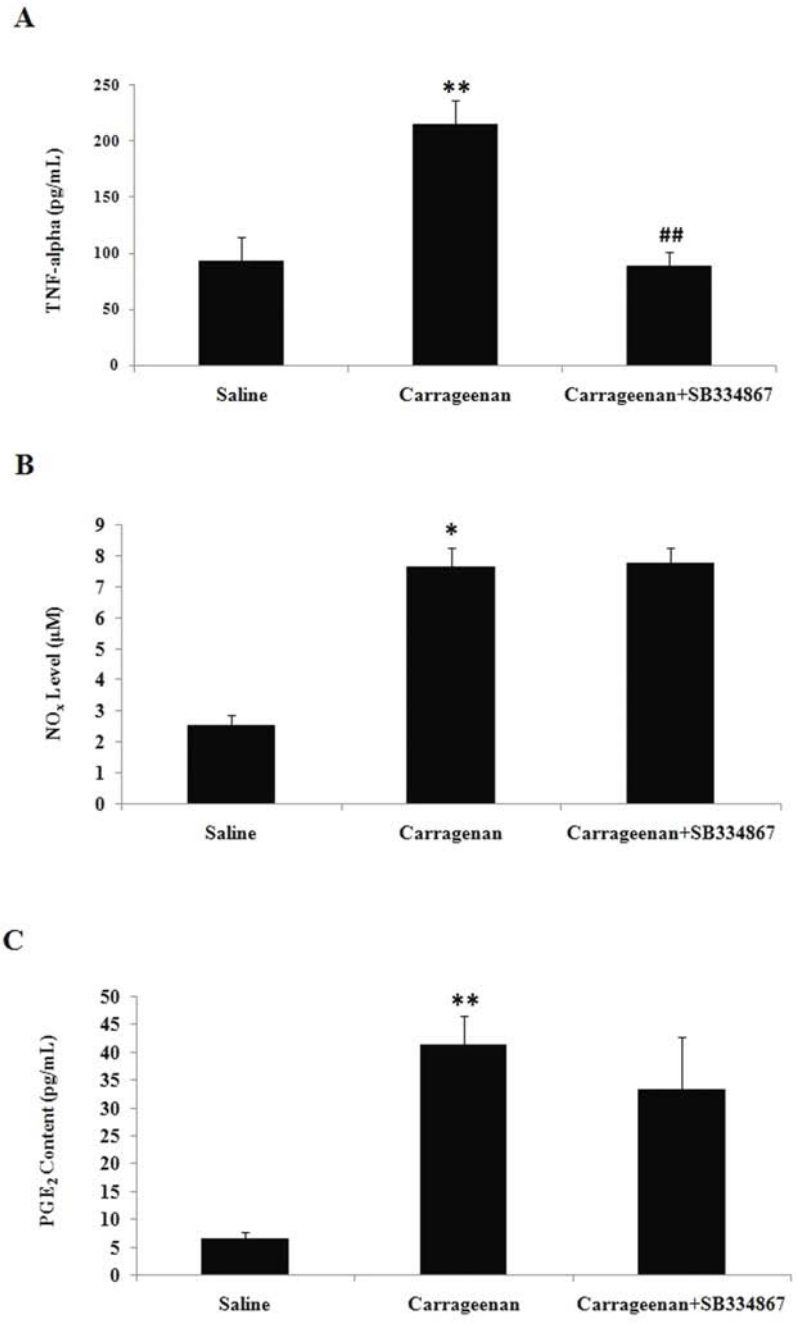

Figure 4. TNF-α (Figure 4A), NO_x (Figure 4B) and PGE_2 (Figure 4C) levels of exudates from the carrageenan-injected rat air pouches. The results are the mean ± S.E. of eight experiments per group. *$p < 0.05$, **$p < 0.01$ compared to the saline group, $^{\#\#}p < 0.01$ compared to the carrageenan group.

The concentrations of TNF-α, PGE_2 and NOx in the pouch fluid

To investigate the effect of endogenous orexin-A on the release of pro-inflammatory cytokines and mediators, we measured the concentrations of TNF-α, NOx and PGE_2 in the exudate. As seen in figure 4, after 6 h following carrageenan injection into the air pouch, TNF-α, NOx and PGE_2 levels were greatly increased in the rat air pouch exudates. When OX1R receptors were antagonized with SB-334867, a significant decrease in TNF-α concentration was observed. Nevertheless, no significant change of PGE_2 and NOx levels in pouch fluid was observed in the carrageenan+SB-334867 group.

The expression of iNOS and COX-2

Western blot analysis of the extracts of the inflammatory cells were performed to assess possible effect of orexin-A on the expressions of iNOS or COX-2. Figure 5 and 6 depicts that carrageenan increased iNOS as well as COX-2 protein expression in the exudate cells. In the group treated with OX1R receptor antagonist SB-334867, the expressions of iNOS and COX-2 in exudate cells were similar to the carrageenan group.

DISCUSSION

To evaluate the role of endogenous orexin-A in the inflammatory response, we used the well-established rat air pouch inflammation model which is a convenient animal model to study acute inflammation [2]. The air pouch model of inflammation, described previously [20], consists of injection of air into the subcutaneous tissue of interscapular region of rodents. Air injection causes the proliferation of cells that cover the surface of the cavity to form a pouch after 6 days [21]. Injection of carrageenan into the air pouch induces granulomatous inflammation characterized by an increased production of biochemical mediators in the fluid exudate, including prostaglandins and nitric oxide as well as a

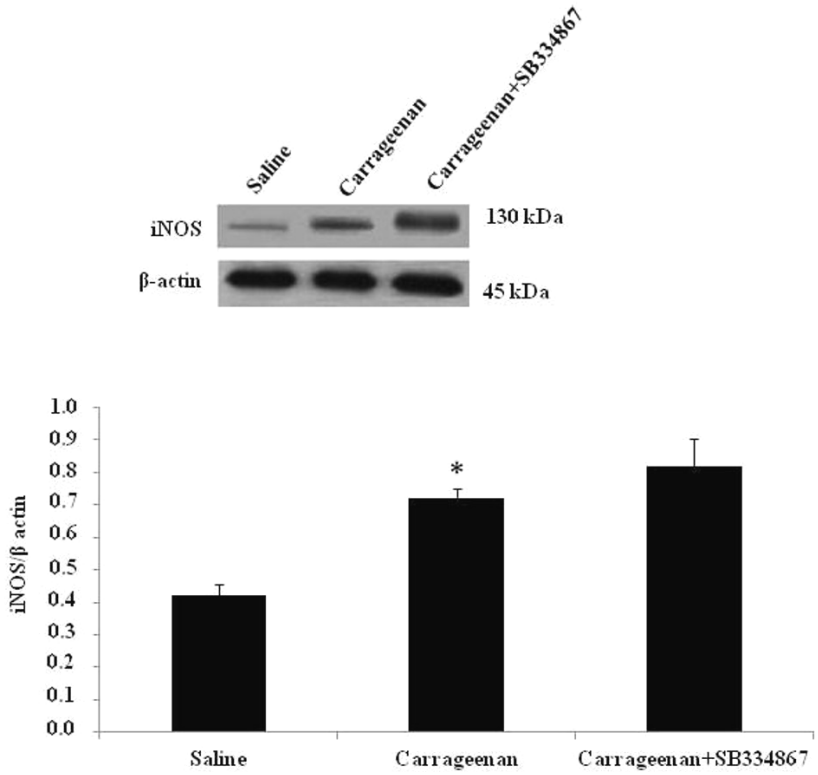

Figure 5. iNOS protein expression in the exudate cells from the carrageenan-injected rat air pouches. The results are the mean ± S.E. of four experiments per group. *$p < 0.05$ compared to the saline group.

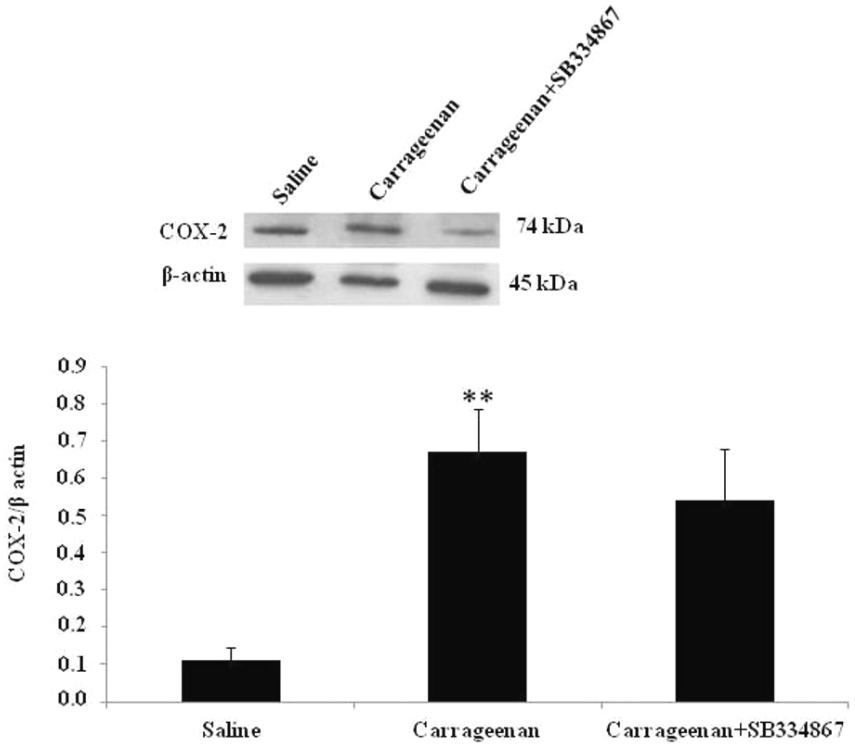

Figure 6. COX-2 protein expression in the exudate cells from the carrageenan-injected rat air pouches. The results are the mean ± S.E. of four experiments per group. **$p < 0.01$ compared to the saline group.

significant influx of polymorphonuclear leukocytes and macrophages [22]. In our study, carrageenan injection successfully created acute inflammation in the peripheral tissue, as indicated by increasing number of inflammatory cells and inflammatory mediators in air pouch exudate. These results are consistent with previous observations showing that carrageenan induced infiltration of immune cells and resultantly increased inflammatory response [10, 23]. This carrageenan-induced acute inflammation model provided us to examine the role of endogenous orexin-A in inflammation process. In the present study, we demonstrated that the administration of the specific OX1R antagonist SB-334867 to rats exposed to carrageenan-induced inflammation significantly decreased the inflammatory index and TNF-α concentration in air pouch exudate. Our findings for the all parameters studied in rats treated with SB-334867 alone were not different from the saline group. These results provide strong evidence for a role of orexin-A in inflammatory reactions.

Hunger is the most efficient stimulant for orexin-A production within the central nervous system and gastrointestinal system [2, 3, 8]. Previous study demonstrated that 36-h starvation increased the level of plasma orexin-A in rats [24]. Furthermore, recent findings suggest a link between the regulation of orexin-A expression and inflammation. It has been shown that orexin-A has a delayed response to intestinal ischemia-reperfusion injury and may function as an inflammatory cytokine in the metabolic disorders caused by acute inflammation [25]. It is known that inflammatory responses are associated with ischemia-reperfusion and the intense inflammation triggered by ischemia-reperfusion augments the ischemic organ damage. In the present study, we demonstrated that carrageenan-induced inflammation led to the increase in plasma orexin-A level. Our findings demonstrated for the first time that the levels of orexin-A in plasma were regulated by inflammation. This finding supports that there could be a relationship between inflammation and orexin-A.

The cellular phase of acute inflammation comprises the adhesion of inflammatory cells to endothelial lining and emigration of the leukocytes. Granulocytes are the first cells to enter into the inflamed tissue, followed by monocytes (macrophages) and lymphocytes. Polymorphonuclear leukocytes, especially neutrophils are the main cellular components recruited to acute inflammatory sites induced by carrageenan [26]. Granulocytes, particularly neutrophils are among cells known as phagocytes and capable of producing pro-inflammatory cytokines including TNF-α [26-28]. In accordance to our results, the inflammatory cells migrated into the carrageenan-injected air pouches. SB-334867 suppressed cell migration to 56% of the level determined in the carrageenan group. In addition to the decreasing effect of OX1R antagonist on the number of cells in inflammatory site, the production of TNF-α which has been increased by carrageenan injection was decreased by OX1R antagonist compared to the carrageenan group. These findings were confirmed by other researchers who showed stimulatory action of TNF-α, which is the major inflammatory mediator produced by inflammatory cells on infiltration of neutrophils and T lymphocytes and the expression of adhesion molecules [26, 29]. TNF-α is an important cytokine which plays a key role in acute and chronic inflammation. The production of a number of inflammatory mediators is linked with TNF-α and thus contributes to the various mediators that control inflammation [29, 30]. Pro-inflammatory cytokine TNF-α induces the synthesis of PGE_2 and NO and its effect is related to NF-κB activation which is a well known critical transcription factor for the expression of COX-2 and iNOS protein [31]. In our study, the increase in TNF-α content of air pouch exudate is accompanied by the increase in NO and PGE_2 concentration. The signaling molecule NO is involved in many physiological and pathophysiological processes including inflammation. The accumulation of NO metabolites in exudates may be dependent on migrating cells, as is demonstrated by the fact that carrageenan injection increased NO production in parallel to cell count in exudates. It is well known that the involvement of NO during the inflammatory response is related to its ability to increase leukocyte migration to the inflammation site [28, 30] and inhibition of NOS reduces leukocyte migration to inflammatory sites [28, 32]. The anti-inflammatory effects of NOS inhibitors in acute and chronic models of inflammation have been shown [33]. Previous observations indicate that iNOS accounts for NO release in the carrageenan-injected rat air pouch [30]. In agree with Salvemini *et al.* [22], we demonstrated the increase in iNOS expression and the production of NO in the cell fraction of exudates after carrageenan administration.

Enhanced NO production by iNOS and increased PGE_2 production by COX-2 are associated with the development of inflammatory diseases. It has been shown that carrageenan promotes the release of NO as well as PGE_2 in the peripheral tissue. In our study, we demonstrated that OX1R antagonist prevented the augmentation in the TNF-α levels due to inflammation and did not change NO and PGE_2 levels. The reason of this finding cannot be explained with the available data. In parallel to this finding, SB-334867 did not alter iNOS and COX-2 protein expressions which are increased depending on the carrageenan injection.

CONCLUSION

In conclusion, we demonstrated that carrageenan-induced inflammation led to the increase in plasma orexin-A level. Orexin-A exhibited inflammatory effects which were pronounced at the level of inflammation index (the exudate volume, and the number of exudate cells) as well as in TNF-α content of exudate in this study. The present study is the first to demonstrate that orexin-A has an inflammatory effect. The present findings suggest that orexin-A could be a mediator in inflammation process. The inflammatory effect of orexin-A is independent of NO and PGE_2 production. Further studies are necessary to better characterize the inflammatory effect of orexin-A.

ACKNOWLEDGMENTS

This study is supported by the Scientific Research Projects Coordination Unit (Project Number: 2008.07.103.008) of Akdeniz University, Turkey.

CONFLICT OF INTEREST STATEMENT

None of the authors or cooperative members has a proprietary, commercial or other financial interest in this study, its procedure or result.

ABBREVIATIONS

OX1R, orexin type 1 receptor; OX2R, orexin type 2 receptor; NO, nitric oxide; PGE_2, prostaglandin E_2; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; PBS, phosphate buffered saline; ELISA, linked immunosorbent assay kit; EDTA, ethylenediaminetetraacetic acid; ZAS, zymosan activated serum; TNF-α, tumor necrosis factor; NF-κB, nuclear factor kappa B; DMSO, dimethyl sulfoxide; EGTA, ethylene glycol tetraacetic acid.

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