

The biochemical and histopathological effects of salusin alpha and salusin beta on cold restricted stress induced gastric injury

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Abstract

Salusin- α and salusin- β are recently discovered bioactive endogenous peptides with both haemodynamic and mitogenic activity. Salusin- α and salusin- β immunoreactivity has been detected in the stomach and intestine. Our study is designed to examine the oxidative stress, cytokines and histological effects of administration of salusin- α and salusin- β on cold-restricted stress (CRS)-induced gastric ulcers. A total of 32 Sprague Dawley, male rats were divided into four groups randomly. Group1: Control; Group2: CRS (Rats were placed individually in the restriction chamber and were subjected to the cold restricted stress at 4°C for 4 h); Group3: CRS+5nmol/kg Salusin- α ; Group4: CRS+5nmol/kg Salusin- β . We determined malondialdehyde (MDA), myeloperoxidase (MPO), superoxide dismutase (SOD), salusin- α and salusin- β levels from stomach tissue, tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) levels from serum. Multiple comparison tests of Kruskal-Wallis and Dunn have been used for the analysis of the data. All the results were presented as mean $\pm$ SD. When compared to the control group; while salusin- α level significantly increases in the group to which CRS has been applied, Salusin- β has shown a slight increase. While MDA, MPO, TNF- α , IL-1 β , and SOD activity in group subjected to the CRS changed, in the groups administered salusin- α and salusin- β , MDA, MPO and TNF- α levels decreased, and SOD activity and IL-1 β levels increased. The mucosal injury and caspase-3 expression increasing with the application of CRS in the histological examinations decreased with the application of salusins. The suppression of salusin- α and salusin- β on caspase-3 expression by means of their effects on oxidative injury and TNF- α levels shows that these two hormones could be an anti-ulcerative agent.

Keywords: Ulcer, Cold-restricted stress, Salusin, Cytokines, Oxidative stress

Introduction

The gastrointestinal tract is particularly sensitive to different stressors. Psychosocial and environmental stressors may play a role in the pathogenesis of gastrointestinal diseases [1]. Stress ulcers are one of the widespread disorders that affect the digestive system due to stress [2]. Stress can induce gastric mucosal injuries and reduce the effectiveness of mucosa which should act as a barrier [3]. In rats, gastric ulcers can be formed by cold-restricted stress (CRS); and CRS is often used as a model for studying the mechanisms of stress in formation of gastric ulcers [4, 5]. In the pathogenesis of gastric lesions induced by CRS, increased lipid peroxidation in gastric tissue [6] and may involve the release of proinflammatory cytokines [7]. It has been demonstrated that CRS causes fast and continuous activation of nuclear factor-kappa B (NF- κ B) in the stomach mucosa. The pharmacological inhibition of NF- κ B may eliminate the inflammation and the stomach damage caused by stress [8].

Salusin- α and salusin- β synthesized from preprosalusin are newly identified bioactive peptides, comprised of 28 and 20 amino acids, respectively [9]. The salusin- α and salusin- β immunoreactivity was detected in the stomach and intestines [10]. In recent studies, it has been reported that salusins is responsible for the regulation of the NF- κ B pathway that stimulates the release of cytokines [11] and prevented the apoptosis [12]

In previous studies, some effects on oxidative stress and cytokines of salusin- α and salusin- β were stated, we investigated the effects on the oxidant/antioxidant parameters, cytokines and apoptosis of salusin- α and salusin- β in stomach injury caused by cold restricted stress in our study.

Materials and Methods

Chemicals

Salusin- α and salusin- β were obtained from Biorbyt (California, USA). The drugs were dissolved in distilled water before use. It was administered to the rats intravenously at 5 nmol/kg doses.

Ethics and Animals

All kinds of treatments and surgical procedures to be conducted on animals were approved by Ataturk

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University, Animal Testing Local Ethics Committee on the 28th of November, 2014 (Protocol No: 28.11.2014/139). The animals to be used in the study were obtained from Ataturk University, Medical Experimental Applications and Research Center (ATADEM). Male rats were subjected to 12 h light/dark cycle in temperature and humidity-controlled environment and they were put in cages and provided continuous access to water and standard food pellets in order to minimize the coprophagia, the rats were deprived of food for 24 h before the procedure, but had free access to drinking water except for the last 1 h prior to the experiment.

Stress-Induced Gastric Ulceration Model

Gastric ulcer was produced in rats by cold restraint stress and the severity of the damage was expressed as ulcer index. The implementation of the CRS, the front and rear legs of the rats were fixed individually with adhesive tape. Rats were placed in a restricted chamber composed of a closed cylinder. The tails were completely taped to the edge of the restriction chamber in order to immobilize the animals. The front side of the chamber was provided with sufficient aeration by large breathing holes. Rats were then placed individually in the restriction chamber and were subjected to the cold restricted stress at 4°C for 4 h [4,5]

Experimental Design

A total of thirty two Sprague dawley (250-280g), male rats were randomly divided into four groups, including eight rats in each group. The first group, control, non-ulcerated group received distilled water intravenously and it was the group of normal, healthy animals without any pretreatment or stress induction. The second group contained CRS-induced ulcer rats. The third group was administered CRS+5 nmol/kg salusin- α ; and the fourth group was administered CRS+5 nmol/kg salusin- β . The salusins were administered right before the experimental model.

At the end of the experiment, the animals were euthanized under anesthesia. The blood samples and stomach tissues were kept at -80°C until cytokines and serum levels of salusin- α and salusin- β were analyzed. A portion of the gastric sample was maintained in a solution containing 10% formaldehyde until histological studies to be conducted.

Determination of the Superoxide Dismutase Activity

The measurements were made as described by Sun et al. [13]. The superoxide dismutase (SOD) evaluation is based on producing superoxide radicals generated by xanthine and xanthine oxidase reacting with nitro-blue tetrazolium (NBT) to form formazan dye. SOD activity was measured at 550 nm by the degree of inhibition of this reaction. 50% inhibition of NBT reduction rate was defined as 1 SOD unit. SOD activity was expressed as unit/mg protein.

Determination of Malondialdehyde Levels

Malondialdehyde (MDA) levels in gastric mucosa determined as an index of lipid peroxidation was measured

according to the method of Ohkawa et al [14]. Gastric mucosa is weighed and homogenized in 10 ml of KCl (10%). The homogenate was boiled at 100 ° C for 1 h by adding 8.1% sodium dodecyl sulfate, 20% acetic acid, and 0.8% TBA. After cooling the reactants 2.5 ml of n-butanol was added, the mix was shaken for 1 min and centrifuged for 10 mins. The absorbance was measured at 532 nm and the results are expressed as nmol/mg protein.

Determination of the Myeloperoxidase Activity

The *Myeloperoxidase* (MPO) activity was measured in accordance with the Bradley technique [15]. The tissues were homogenized in phosphate buffer of 50 mM pH 6,0 containing 0,5 % HTAB (hexadecyl three methyl ammonium bromide). The homogenate was centrifuged for 30 mins at 4 °C at 3500 rpm. The supernatants were used in MPO activity measurement. 5 mins kinetic readings were conducted at 460 nm wave length in ELISA microplate reader. The results are submitted as unit/mg protein.

Measuring tumor necrosis factor- α and interleukin-1 β serum levels and salusin- α and salusin- β from gastric tissue with ELISA

In order to examine the ethanol effect on plasma cytokines and salusins levels in stomach tissue, the ELISA (ELISA, BioTEK powerwave XS Winooski, U.K) was conducted. The measurements were carried out by using interleukin-1 β (IL-1 β) (Elabscience, Beijing, China), tumor necrosis factor- α (TNF- α) (Ebioscience, Sandiego, USA), salusin- α and salusin- β (Sunredbio, Baoshan, Shangai) commercial ELISA kits in accordance with the manufacturer's instructions given.

Histopathological analyses (Hematoxylin and eosin staining)

The stomach samples were fixed in 10% formaldehyde for 48 h, dehydrated in an increasing concentration alcohol series and made pellucid with xylene series. The stomach tissues embedded in paraffin wax and sectioned to a thickness of 5 μ m using a microtome (Leica RM2235, Leica Instruments, Nussloch, Germany) for immunohistochemical and histopathological examinations. In histopathological examination, prepared samples were stained with hematoxylin-eosin and were investigated by light microscopy.

Immunohistochemical analysis (IHC staining)

Deparaffinized in xylene the tissues were rehydrated in ethanol and pursued by water and phosphate-buffered saline. Followed sections after washing in distilled water were immersed in 3% hydrogen peroxide for 15 min. Subsequently applied PBS, the sections were sopped in equilibration buffer at room temperature for 20 min. The tissue sections were then incubated with anti- caspase 3 solution for 1 h at room temperature. The prepared section samples were incubated with phosphate-buffered saline containing normal goat serum but without a primary antibody. The sections were counterstained with Mayer's

hematoxylin. Under a light microscope (Olympus BH-40) the sections were then examined and took picture [(16).

Statistical analysis

All values were reported as mean $\pm$ standard error, including 8 animals per group. The data were analyzed using Kruskal-Wallis test, Dunn's multiple comparison tests. The statistical studies were performed by using SPSS 20 program.

Results

Effect of Salusin- α and Salusin- β on SOD and MPO Activities and MDA Levels in the Stomach Tissue of Rats Exposed to CRS

The level of MDA, an index of lipid peroxidation, was significantly increased ($P<0,05$) in 4 h after exposed to CRS in comparison to the control group. MDA level was significantly decreased by pretreatment with salusin- α ($P<0,005$) and salusin- β ($P<0,05$) (table 1). SOD activities of CRS and salusins groups weren't showed a significant change (table 1). Myeloperoxidase level was used as an indicator of neutrophil infiltration in gastric mucosa. There were higher MPO activity in CRS group than in control group ($P<0.001$), but MPO activities were markedly weakened ($P<0.05$) in the treatment groups with salusins (table 1).

Table 1. Effect of Salusin- α and Salusin- β on SOD and MPO activities and MDA levels in the stomach tissue of rats exposed to

Parameters/Groups	Control	CRS	CRS+Salusin- α	CRS+Salusin- β
MDA ($\mu\text{mol/mg protein}$)	0.124 $\pm$ 0.009	0.235 $\pm$ 0.046*	0.065 $\pm$ 0.009 ⁺	0.121 $\pm$ 0.058 ⁺⁺
SOD (U/mg protein)	219.30 $\pm$ 7.46	205.95 $\pm$ 4.54	210.60 $\pm$ 7.10	206.30 $\pm$ 4.84
MPO (U/mg protein)	71.66 $\pm$ 4.80	144.69 $\pm$ 14.05**	93.88 $\pm$ 14.10 ⁺⁺	71.86 $\pm$ 19.77 ⁺⁺

CRS * $P<0,05$, ** $P<0.001$, versus control group. ⁺ $P<0,005$, ⁺⁺ $P<0,05$, versus CRS group.

Determination of Salusin- α and Salusin- β Levels in CRS Induced Gastric Injuries

The levels of salusins in gastric tissue are determined. Salusin- α level in CRS group was significantly higher than control group. There was no significant change in the level of salusin- β (figure 1).

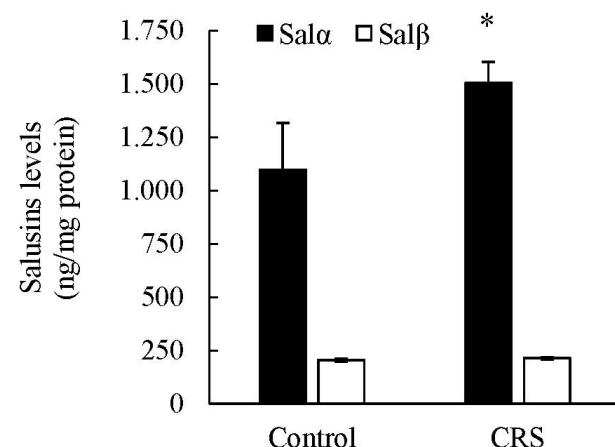


Figure 1. The effects of CRS implementation on salusin- α and salusin- β levels in the gastric tissue. Compared to the control group CRS group is * $P<0,05$.

The Effect of Salusin- α and Salusin- β on Serum TNF- α and IL-1 β Levels in CRS Induced Gastric Injury

The levels of TNF- α and IL-1 β cytokines in ulcerative rats exposed to CRS are presented. Exposed to CRS significantly elevated the levels of TNF- α and IL-1 β compared with control rats ($P<0.005$ and $P<0.05$, respectively). In contrast, the elevation of TNF- α was decreased in rats that received salusins as pretreatment

($P<0.05$). The IL-1 β levels were significantly increased after salusin- α and salusin- β treatment ($P<0.05$) (Figure 2, a and b).

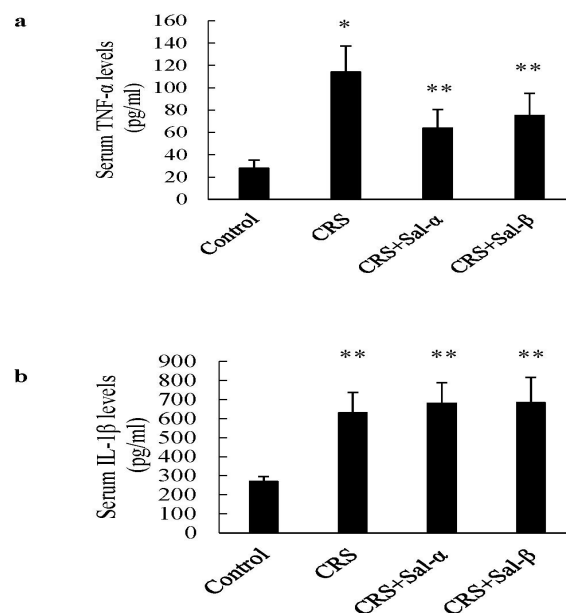


Figure 2. (a) The effect of 5 nmol/kg salusin- α and salusin- β on TNF- α levels in the serum samples. Compared to the control group the CRS group is * $P<0.005$, CRS+sal- α and CRS+sal- β compared to CRS group are ** $P<0.05$. (b) The effect of 5 nmol/kg salusin- α and salusin- β on IL-1 β levels in the serum samples. Compared to the control group the CRS, CRS+sal- α and CRS+sal- β groups are ** $P<0.05$.

Histopathological examination

The histology of the control group's submucosal and gastric gland structures was normal. For the CRS group

such formations draw attention like congestion of veins in lamina propria, the dilation and inflammation in the gastric glands. There was degeneration in the surface epithelium.

All these changes were significantly less expressed in rats pretreated with both salusin- α and salusin- β (figure 3).

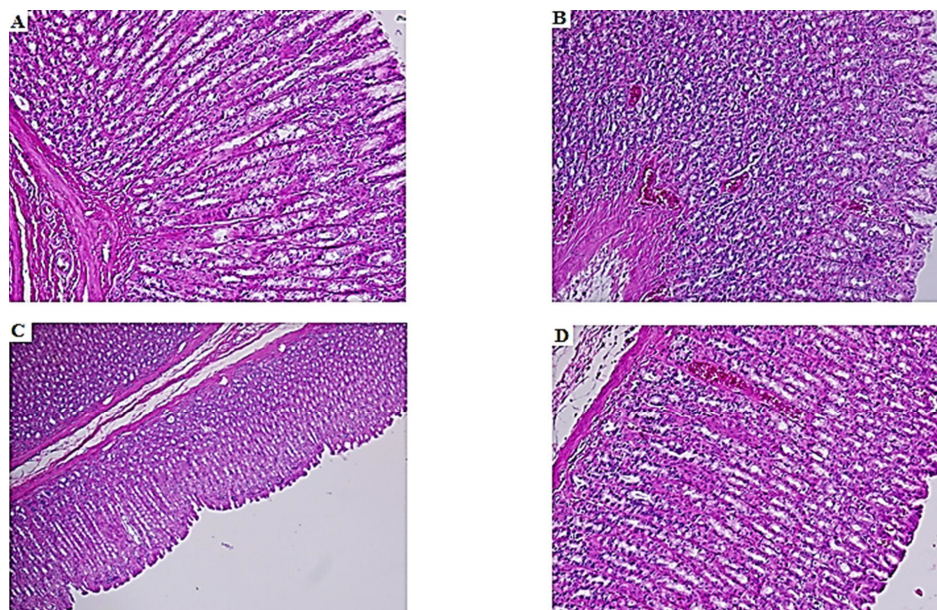


Figure 3. Effects of salusins on histological findings of CRS-induced gastric damage in rats after haematoxylin and eosin staining: The histology of the control group's (A) submucosal and gastric gland structures was normal. For the CRS group (B) such formations draw attention like congestion of veins in lamina propria, the dilation and inflammation in the gastric glands. There was degeneration in the surface epithelium. For the sal- α group (C), the dilation in the abdominal cavity and the gastric glands and the partial erosion that is observed in the surface epithelium and the vessel congestion are bounded in tight spaces compared to the CRS group, but a little more pronounced than the sal- β group. Considering the sal- β group (D), the surface epithelial in the gastric tissue was normal, but there was congestion in the veins, however, decreased significantly compared to the CRS group.

The Evaluation of Caspase-3 Expression in CRS Induced Gastric Mucosal Injury

CRS increased the caspase-3 expression compared to control group. Salusins reduced caspase-3 expression in rats subjected to CRS (figure 4).

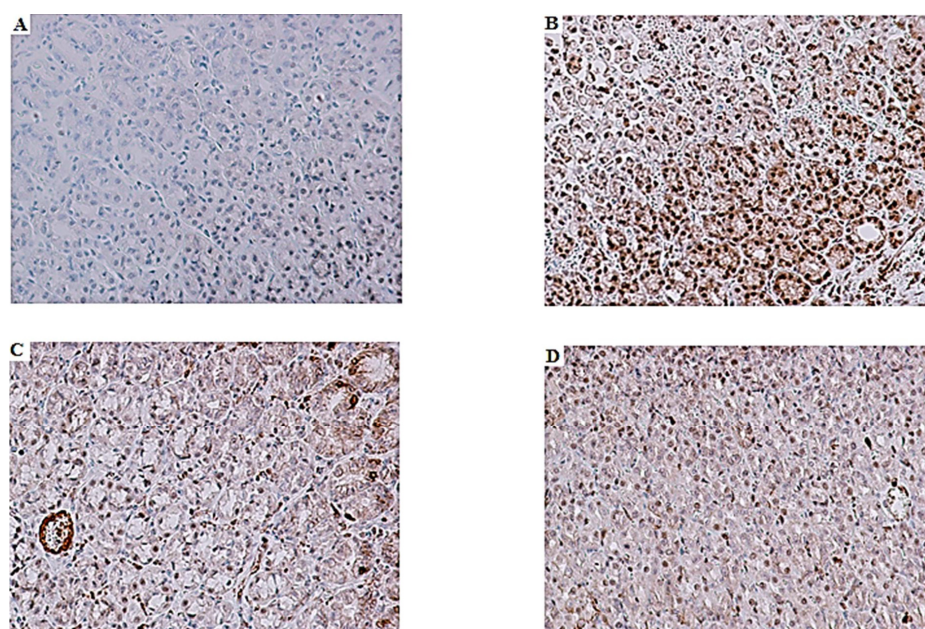


Figure 4. Salusins downregulate the protein expression of caspase-3 in rats with CRS-induced gastric injury. Images submitted for immunohistochemical detection of Caspase-3 expression in stomach tissues. In the control group (A) no expression was observed, but the CRS group (B) was showed an extensive expression. Caspase-3 expression was found to be reduced for the sal- α administered group (C). Caspase-3 expression was found to be reduced for the sal- β administered group (D).

Discussion

Stress may affect different physiologic functions of the gastrointestinal tract including gastric secretion, gut motility, mucosal permeability and barrier function, visceral sensitivity and mucosal blood flow [17]. CRS is reported to cause oxidative stress in animals [18]. The previous studies also showed that the CRS stimulates serious gastric mucosal injury for 4 h long [4,5]. In this study, we demonstrated administration of salusins CRS-associated oxidative damage, inflammatory responses and mucosal structural damage as well as cell apoptosis. To our knowledge, this was the first study to investigate the protective effect of salusins on CRS-induced gastric ulcer.

Our findings showed that salusins (especially salusin- α) increased in CRS-induced gastric injury. Levels of salusin- α and salusin- β increasing gastric damage induced by CRS may play a role in the stomach damage. In the present study, salusins reduced MPO activity, MDA and TNF- α level and increased SOD activity and IL-1 β level in the gastric tissue. The gastric injury can cause ROS induced oxidative gastric stress [19]. Sweeping the ROS is considered to be one of the mechanisms involved in the healing ulcer [20]. As the lipid peroxidation plays a role in cellular injury mechanism, the involved first line of defense against ROS-mediated lipid peroxidation [13]. Thus, in order to address the role of oxidative stress in our model, we assessed several oxidant-antioxidant parameters in gastric tissues of rats. The experimental results showed that CRS markedly increased MDA, an index of lipid peroxidation, accompanied by a decrease of SOD activity is endogenous antioxidant. In the previous studies, for CRS induced gastric injury, the increase in the MDA levels, [21] and an decrease in SOD activity [22] were reported and these results are consistent with our data. After the CRS administration, the treatment with salusins caused a significant decrease in MDA levels, there were partial increases in SOD activity.

Suspension of neutrophil infiltration, was regarded as an important anti-inflammatory mechanism by effective anti-ulcer agents that prevent gastric injury [20]. The increased MPO activity and the invasion of the neutrophils to the gastric tissues cause gastric mucosal injury [23]. Compatible with the previous studies, in CRS group MPO, TNF- α [24] and IL-1 β [25] levels increased significantly in our study and MPO and TNF- α level decreased in salusins administered groups, but IL-1 β level increased. IL-1 β production is stimulated by salusin- β [26]. It has been demonstrated that Salusin- β may up-regulate the number of the active NF- κ B positive cells. It has been reported that salusin- α might decrease the plasma level of the TNF- α without any effects on NF- κ B [27]. The differences in TNF- α and IL-1 β levels might stem from the differences in salusin- α ve salusin- β levels, which change in ulcerative damage. These results are consistent with previous studies [26]. Several genes causing cellular death by apoptosis of

inflammatory signals with oxidative stress are reported to increase the expression [20]. It is believed that the endoplasmic reticulum stress (ERS) that leads to epithelia dysfunction contributes to the inflammation in gastrointestinal system. The pharmacological manipulation of the ERS pathway has been related to the chronic inflammation, it especially makes us consider that this might be a new treatment paradigm for gastrointestinal diseases and for other different diseases [28]. The increased apoptotic death of gastric epithelial cells is shown partially in CRS-induced gastric mucosal injury [29]. The protection of gastric mucosal integrity depends on the balance between proliferation and apoptosis of epithelial cells. The apoptosis has recently been proposed as a casual factor for CRS induced gastric ulcers. Caspase-3 plays a role in apoptotic cell death in stress ulcers [30]. Our study has revealed that the application of salusins suppressed caspaz-3. Previous studies revealed that salusin- α and salusin- β applied at different doses inhibited the ERS, which increased in ischemia reperfusion damage, and decreased the myocardial infarct area [12].

Conclusions

The results of our study indicate that the salusins significantly reduces especially lipid peroxidation and neutrophil infiltration and caspase3 expression injuries either directly or by activating various antioxidant systems.

However, you need to investigate the effects of different doses of Salusins on different models of stomach ulcer for more effective results.

Disclosure

The authors have declared that there is no conflict of interest exists.

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