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Influence of lipopolysaccharide and the general adaptation syndrome on the development of oxidative-nitrosative stress in the lacrimal glands of rats

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The lacrimal glands play a key role in the visual organ functioning due to the production of tear fluid. From a pathogenetic point of view, it is interesting to study the combined effect of the general adaptation syndrome and systemic inflammatory response syndrome (SIRS) on lacrimal glands metabolism. The purpose of this study is to assess changes in the L-arginine-dependent part of nitric oxide cycle, nitric oxide metabolites concentration, and pro- and antioxidant balance in the rat lacrimal glands during modelling of chronic stress and SIRS. The experiments were performed on 18 mature male rats weighing 190-240 g. The animals were divided into 3 groups: I – control, II – water avoidance stress (WAS) group, III – WAS rats injected with lipopolysaccharide (WAS+LPS) group. The NO cycle parameters and markers of oxidative stress were determined in the rat lacrimal glands homogenate. The superoxide anion production and malondialdehyde concentration in the lacrimal glands of WAS+LPS rats increased by 2.48 and 1.86 times, respectively, compared to the control group and by 1.35 and 1.11 times compared to WAS group. The catalase activity in WAS+LPS rats decreased by 1.68 times and superoxide dismutase activity increased by 1.34 times compared to the control group; if compared to WAS group, catalase activity increased by 1.26 times, and superoxide dismutase activity elevated by 6.52 times. The activity of inducible NO-synthase in WAS+LPS rats decreased by 1.29 times compared to the control and increased by 1.23 times compared to WAS group. The concentration of peroxynitrites, nitrites, and nitrosothiols in WAS+LPS rats increased by 2.6, 3.02, and 3.68 times, respectively, compared to the control group and by 1.43, 1.41, and 2 .91 times compared to WAS group. Thus, administration of bacterial LPS to rats under the conditions of stress modeling enhances antioxidant protection and increases nitric oxide production from iNOS; at the same time, such stimulation increases damage to protein and lipid structures.

Keywords: oxidative stress; lacrimal glands; lipopolysaccharide; nitric oxide; general adaptation syndrome.

INTRODUCTION

The lacrimal glands play a key role in the visual organ functioning due to the production of tear fluid in sufficient quantity and with a defined qualitative composition. Damage to these glands by systemic processes, such as Sjögren syndrome, leads to impaired tear secretion and the dry eye syndrome development [1]. In addition to the visual organ moisturizing, tear fluid performs a number of other important functions, namely: promotes wound healing, prevents inflammation, has antiradical and antimicrobial protection [2].

Tear fluid production depends on NO-ergic regulation. As shown in the studies of Szarka et al. [3], the stimulating effect of α-adrenergic agonists on the production of tear fluid is mediated by nitric oxide. The nitrate-nitrite reductase pathway for the nitric oxide formation is important for normal functioning of the visual organ in general and the lacrimal glands in particular [4].

The general adaptation syndrome can increase nitric oxide production by increasing the inducible isoform of NO synthase (iNOS) expression [5]. Long-term modeling of the general adaptation syndrome leads to the development of oxidative damage to various organs and tissues [6]. However, in the scientific

literature, there is a limited amount of data on this syndrome effects on the production of nitric oxide and indicators of oxidative stress in the lacrimal glands.

Bacterial lipopolysaccharide during infectious processes can cause systemic changes in the body. These changes are characterized by increased expression of pro-inflammatory cytokines and iNOS, as well as the development of oxidative-nitrosative damage to various organs [7, 8]. The effect of bacterial lipopolysaccharide, which triggers and maintains the systemic inflammatory response syndrome (SIRS), on the lacrimal glands remain poorly understood.

Combined action of bacterial lipopolysaccharide and general adaptation syndrome on the body, despite involvement of similar mechanisms in development of tissue damage, shows some controversy in effects. For instance, modelling of predictable chronic mild stress facilitates the recovery from bacterial lipopolysaccharide-induced depressive- or anxiety-like behavior and suppresses bacterial lipopolysaccharide-induced proinflammatory cytokine expression, microglia activation, and oxidative stress in hippocampus [9]. On the other hand, single intranasal administration of bacterial lipopolysaccharide one day prior to stress exposure prevented stress-induced increase in levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and IL-1β mRNA in the hippocampus and prefrontal cortex [10].

Scientific literature provides limited amount of data on combined influence of the general adaptation syndrome and SIRS on lacrimal glands functioning. Therefore, it is interesting from the pathogenetic point of view to study the effect of bacterial lipopolysaccharide on lacrimal glands metabolism in water avoidance stress (WAS) rats.

The aim of our study was to assess changes in the functioning of L-arginine-dependent part of nitric oxide cycle, concentration of nitric oxide metabolites, and pro- and antioxidant balance in the rat lacrimal glands during modelling of chronic stress and SIRS.

METHODS

The experiments were performed on 18 mature male rats weighing 190-240 g. The animals were divided into 3 groups of 6 rats. I group - intact animals, which were injected with 0.1 ml of a 0.9% aqueous solution of Sodium Chloride (Control group). II group - animals on which we modeled a stress syndrome by keeping them above water for 1 hour daily for 30 days. In addition, they were administered 0.1 ml of 0.9% sodium chloride aqueous solution (WAS group). III group of animals was injected intraperitoneally with 0.4 μg/kg of bacterial lipopolysaccharide (LPS) of *S. typhi* (pyrogenal) in the first week 3 times, then once a week throughout the experiment [11] simultaneously with simulation of WAS as in group II (WAS+LPS group).

The animals were kept in ordinary vivarium conditions. They were sacrificed on the 30th day of the experiment by blood sampling from the right ventricle of the heart under thiopental anesthesia.

The research was conducted in accordance with the standards of the Council of Europe Convention on Bioethics "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (1997), general ethical principles of experiments on animals approved by the First National Congress on Bioethics of Ukraine (September 2001) and other international agreements and national legislation in this area. The animals were kept in a vivarium accredited in accordance with the "Standard rules of order, equipment and maintenance of experimental biological clinics (vivarium)".

An aliquot of 0.1 ml of blood after sedimentation of red blood cells by 15 min centrifugation (3000g) was taken for analysis of cortisol concentration. To determine the concentration of cortisol, 2 ml of ammonium tetramethylhydroxide pentahydrate solution (100 mg of ammonium tetramethylhydroxide pentahydrate was dissolved in 5 ml of distilled

water, then 5 ml of the resulting solution was mixed with 45 ml of methyl alcohol) and 2 ml of nitroblue tetrazolium chloride solution (100 mg of nitroblue tetrazolium chloride in 50 ml of methyl alcohol). As a result, a red-colored dye was formed with a maximum light absorption at a wavelength of 510 nm [12].

We studied the activity of inducible NO-synthase (iNOS), constitutive isoforms of NO-synthase (cNOS), activity of arginase, concentrations of nitrites (NO_2^-) and peroxynitrites ($ONOO^-$) of alkali and alkaline earth metals [13, 14]. To determine SOD activity, we used adrenaline auto-oxidation reaction in an alkaline environment with superoxide generation [15]. Catalase activity estimation was based on the determination of colored products formed by the reaction of hydrogen peroxide with ammonium molybdate [16]. The concentrations of malondialdehyde (MDA) [17], nitrosothiols (S-NO) [18], and sulfide anion [19] were also evaluated. The method for estimation of superoxide anion radical (SAR) production was based on Nitroblue tetrazolium (NBT) reduction by superoxide with the formation of diformazan [20]. Oxidatively modified proteins (OMP) determination was based on spectrophotometric estimation of carbonyl groups of proteins, which were formed in reaction of reactive oxygen species with amino acids residues [21].

Statistical processing of the results of biochemical studies was carried out using the pairwise non-parametric Mann-Whitney test. All statistical calculations were performed with Microsoft office Excel software and its extension Real Statistics 2019. The difference was considered statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

We found that the blood concentration of cortisol in WAS and WAS+LPS rats increased by 2.63 and 4.7 times compared to the control, respectively. Thus, a combined influence of the general adaptation syndrome and SIRS increased cortisol level by 1.78 times compared to animals with chronic stress (Table).

The iNOS activity in WAS rats decreased by 1.58 times compared to the control animals. In WAS+LPS group it decreased by 1.29 times compared to the control and increased by 1.23 times compared to WAS group. This result shows that simulating the general adaptation syndrome for 30 days leads to a decrease in the inducible NO-synthase activity in the lacrimal glands. This somewhat contradicts the data of Gurel et al. [5], who claim that under the conditions of modeling chronic stress, the activity of inducible NO-synthase in the gastric mucosa increases. The possible explanation is that these researches were performed on different tissues: we studied the glandular epithelium, while Gurel et al. studied the prismatic epithelium of the stomach. Also, the duration of exposure in our experiment was significantly longer, this could lead to exhaustion stage of the general adaptation syndrome. Considering the fact that the expression of iNOS is controlled by the transcription factor NF-κB, the decrease in its activity can be explained by an excessive increase in the blood concentration of cortisol in rats under the conditions of our experiment, which has a strong inhibitory effect on the activation of NF-κB [22, 23]. Increased iNOS activity compared to WAS group may also be associated with NF-κB-independent activation of iNOS expression under the influence of bacterial lipopolysaccharide, namely due to the transcription factor AP-1 activation [24].

The cNOS activity in WAS rats increased by 1.93 times compared to the control, while in WAS+LPS animals it increased by 1.71 times compared to the control and decreased by 1.13 times compared to WAS group (Table). An increased cortisol concentration can also reduce the activity of the endothelial isoform of NO synthase (eNOS) and cause endothelial dysfunction [25]. According to our results, the activity of constitutive isoforms of NO-synthase (eNOS and the neuronal isoform of NO-synthase) significantly increases after

30 days of the general adaptation syndrome simulation. These changes can be explained by the stimulating effect of H_2S on the eNOS activity, the concentration of which, according to the results of our study, significantly increases in the lacrimal glands of rats [26].

The arginase activity decreased in WAS rats by 1.54 times compared to the control, while in WAS+LPS group it decreased by 2.98 times compared to the control and by 1.93 times compared to WAS group. A decrease in arginase activity may be associated with the inhibitory effect of excessive nitric oxide, which comes from the nitrate-nitrite reductase system of the visual organ. Also, chronic stress increased concentration of nitrosothiols, $ONOO^-$ and NO_2 in lacrimal glands of rats by 1.26, 1.82 and 2.13 times, respectively, while in WAS+LPS group these concentrations increased by 3.68 (2.91), 2.6 (1.43) and 3.02 (1.41) times compared to the control and WAS group, respectively (Table). An increase in the formation of peroxynitrites, nitrites and nitrosothiols may be associated with activation of the nitrate-nitrite reductase system of the visual organ [4]. Thus, such a nitrite reductase as xanthine oxidoreductase, which can simultaneously produce the superoxide anion radical, can be a source of nitric oxide necessary for the formation of peroxynitrites. It should also be noted that the activity of xanthine oxidoreductase under the conditions of our experiment can be stimulated by an increased concentration of sulfide anion [27]. Administration of bacterial lipopolysaccharide to rats did not significantly change the tendency for increase in the concentration of nitrites,

Cortisol concentration in the blood and biochemical changes in the lacrimal glands of rats under conditions of administration of bacterial lipopolysaccharide on the background of general adaptation syndrome (M ± m)

Parameters	Control group	WAS group	WAS+LPS group
Cortisol concentration, nmol/l	17.36±0.17	45.69±1.93*	81.53±9.69*^
Inducible NO-synthase, μmol/min per g of protein	0.84±0.02	0.53±0.02*	0.65±0.03*^
Constitutive NO-synthase, μmol/min per g of protein	0.0474±0.0002	0.0913±0.0003*	0.0811±0.0004*^
Arginase activity, μmol/min per g of protein	1.28±0.02	0.83±0.009*	0.43±0.01*^
S-NO, μmol/g	0.53±0.003	0.67±0.004*	1.95±0.06*^
$ONOO^-$, μmol/g	2.45±0.06	4.45±0.06*	6.38±0.06*^
NO_2^- concentration, nmol/g	6.99±0.11	14.9±0.11*	21.08±0.39*
Superoxide anion radical production, nmol/s per g	0.31±0.004	0.57±0.01*	0.77±0.008*^
Catalase activity, μkat/g	0.57±0.002	0.27±0.001*	0.34±0.003*^
Superoxide dismutase activity, c.u.	1.51±0.08	0.31±0.02*	2.02±0.11*^
Malondialdehyde concentration, μmol/g	9.14±0.06	15.36±0.04*	17.02±0.09*^
OMP, c.u.	0.063±0.001	0.129±0.003*	0.144±0.0007*^
Sulfide anion concentration, μmol/g	8.83±0.16	21.13±0.5*	25.94±0.06*^

*indicates statistically significant difference from control group ($P < 0.05$); ^ indicates statistically significant difference from WAS group ($P < 0.05$)

peroxynitrites, and nitrosothiols, but lead to even further elevation of their concentrations. These changes clearly show synergetic effect of SIRS and chronic stress on accumulation of nitric oxide metabolites.

Analyzing anti-radical protection and reactive oxygen species (ROS) production under conditions of combined exposure to chronic stress and administration of lipopolysaccharide, we found that the production of SAR during WAS increased by 1.84 times, catalase and SOD activities decreased by 2.11 and 4.87 times, respectively, compared to the control group. SAR production in WAS+LPS group increased by 2.48 times, SOD activity increased by 1.34 times and catalase decreased by 1.68 times compared to the control group. Introduction of LPS during WAS increased SAR production and by 1.35 times, increased catalase and SOD activities by 1.26 and 6.52 times, respectively, compared to the WAS group (Table). A decrease in the activity of antioxidant enzymes may be associated with the inhibitory effect of cortisol on the activity of NF-κB, which controls transcription of studied antioxidant enzymes. A decrease in the activity of antioxidant enzymes against the background of an increase in the production of the superoxide anion radical and peroxynitrite leads to increased damage to lipid and protein structures of the lacrimal glands under the general adaptation syndrome conditions.

The decrease in the activity of studied antioxidant enzymes (SOD and catalase) in WAS group is consistent with the literature data. Ilderbayev et al. [28] also established a decrease in antioxidant protection in stressed animals during immobilization stress modeling. The SOD activity decrease in WAS group may be connected to inhibition by increased concentration of cortisol of TNF-α/COX-2 axis in non-canonical NF-κB activation pathway, which leads to decreased expression of SOD-2 [29]. Addition of LPS to WAS-modeling leads to activation of TLR-4-dependent or canonical NF-κB activation pathway and as a result to an increased SOD-1 expression [30]. Canonical NF-κB activation pathway is less dependent on COX-2, so increased cortisol concentration observed in WAS+LPS group has limited influence. Therefore, the increase in SOD activity observed in WAS+LPS group compared to WAS group can be explained by additional activation of canonical NF-κB activation pathway by bacterial lipopolysaccharide used in our study.

The increased activity of antioxidant enzymes in WAS+LPS rats, compared to WAS group, may also be associated with activation of Nrf-2 transcription factor, which, unlike NF-κB, does not reduce the degree of its activation under the influence of cortisol [31]. Activation of Nrf-2 in WAS+LPS group can be a result of increased production of nitric oxide from L-arginine-dependent pathway of NO production. This is evidenced by an increased iNOS activity in WAS+LPS rats compared to WAS group [32].

MDA concentration and OMP content increased by 1.68 and 2.05 times in WAS rats, respectively, compared to the control. In WAS+LPS group this parameters were 1.86 (1.11) and 2.29 (1.12), respectively compared to the control and WAS group. Therefore, we can state that LPS introduction during WAS modeling potentiates lipid peroxidation and protein damage in lacrimal glands. Also, we found increasing in the concentration of sulfide anion by 2.39 times under the conditions of general adaptation syndrome compared to the control and by 2.94 (1.23) times under the combined effect compared to the control and stress syndrome group, respectively. The causes and mechanisms of this increasing require further research.

The authors see the perspectives of further researches in studying of activities of nitrate and nitrite reductases and xanthine oxidoreductase complex, since results of our study suggest that they can play a crucial role in changes in concentration of nitric oxide metabolites.

CONCLUSIONS

1. General adaptation syndrome modelling leads to a decrease in nitric oxide production from inducible NO-synthase, while simultaneously the activity of constitutive (endothelial and neuronal) isoforms and concentration of components of the nitrate-nitrite reductase pathway of nitric oxide production increase.

2. General adaptation syndrome reduces antioxidant protection and increases production of reactive oxygen and nitrogen species, which leads to destruction of protein and lipid structures in lacrimal glands.

3. Administration of bacterial lipopolysaccharide to rats under the conditions of general adaptation syndrome modeling enhances antioxidant protection and increases the production of nitric oxide from inducible NO-synthase; at the same time, this stimulation increases damage to protein and lipid structures due to an increased production of superoxide anion radical and peroxynitrite.

The authors of this study confirm that the research and publication of the results were not associated with any conflicts regarding commercial or financial relations, relations with organizations and/or individuals who may have been related to the study, and interrelations of co-authors of the article.

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ВПЛИВ ЛІПОПОЛІСАХАРИДУ ТА ЗАГАЛЬНОГО АДАПТАЦІЙНОГО СИНДРОМУ НА РОЗВИТОК ОКСИДАТИВНО-НІТРОЗАТИВНОГО СТРЕСУ В СЛІЗНИХ ЗАЛОЗАХ ЩУРІВ

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Слізні залози відіграють ключову роль у функціонуванні зорового органу за рахунок продукції слізної рідини. З патогенетичної точки зору цікавим є вивчення поєднаного впливу загального адаптаційного синдрому та синдрому системної запальної відповіді (ССЗВ) на функціонування та метаболізм слізних залоз. Мета дослідження - оцінити зміни у функціонуванні L-аргінінзалежної частини циклу оксиду азоту та концентрацію його метаболітів, про- та антиоксидантного балансу в слізних залозах щурів під час моделювання хронічного стресу та ССЗВ. Експеримент виконаний на 18 статевозрілих щурах-самцях масою 190–240 г. Тварин розділили на 3 групи: I – контрольна, II – щури, яким моделювали загальний адаптаційний синдром, III – щури, яким вводили ліпополісахарид і моделювали загальний адаптаційний синдром. В гомогенаті слізних залоз щурів визначали показники циклу оксиду азота та маркери оксидативного стресу. Поєднання загального адаптаційного синдрому і введення ліпополісахариду збільшує продукцію супероксид-аніона в 2,48 раза і концентрацію малонового діальдегіду в 1,86 раза, знижує активність каталази в 1,68 раза та індуцибельної NO-синтази в 1,29 раза, активність супероксиддисмутази зростає в 1,34 раза, концентрація пероксинітритів, нітритів та нітрозотіолів в слізних залозах підвищується відповідно в 2,6, 3,02 та 3,68 раза порівняно із контрольною групою. При порівнянні із групою загального адаптаційного синдрому продукція супероксид-аніона знижується в 1,35 раза, а концентрація малонового діальдегіду в 1,11 раза, активність каталази зростає в 1,26, індуцибельної NO-синтази в 1,23 раза і супероксиддисмутази в 6,52 раза, концентрація пероксинітритів, нітритів та нітрозотіолів в слізних залозах підвищується відповідно в 1,43, 1,41 та 2,91 раза. Таким чином, введення щурам бактеріального ліпополісахариду за умов моделювання загального адаптаційного синдрому посилює антиоксидантний захист та збільшує продукцію оксиду азоту від індуцибельної NO-синтази, разом з тим така стимуляція посилює ушкодження білкових та ліпідних структур.

Ключові слова: оксидативний стрес; слізні залози; ліпополісахарид; оксид азоту; загальний адаптаційний синдром.

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