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SOURCES OF PRODUCTION OF REACTIVE OXYGEN AND NITROGEN SPECIES IN TISSUES OF PERIODONTIUM AND SALIVARY GLANDS OF RATS UNDER MODELED SYSTEMIC INFLAMMATION*

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The purpose of the work was to reveal the sources of production of reactive oxygen and nitrogen species in the tissues of periodontium and salivary glands of rats under conditions of experimental systemic inflammation. The study was carried out on 20 white male rats of the Wistar line weighing 180-220 g, divided into 2 groups: the 1st included intact animals, the 2nd was made up of the animals with induced systemic inflammation. Systemic inflammation was induced by intraperitoneal administration of lipopolysaccharide Salmonella typhi (Pyrogenalum) in a dose that stimulated rise in temperature by 1.5 °C according to the scheme: during the first week, 4 minimum pyrogenic doses (MPD) of 0.4 μg/kg of rat mass were administered 3 times a week. During the following seven weeks of the experiment, rats were given 4 MPD / kg of body weight once a week. The research was guided by the principles of biomedical ethics. The animals were decapitated with ethereal anesthesia. Soft tissues of periodontium and submandibular salivary glands were the objects of the study. It has been found out that systemic inflammation is accompanied by an increase in the production of superoxide anion radical in periodontium tissues and salivary glands by mitochondrial and NADPH-dependent electron transport chains of microsomes and NO synthase as well as by leukocyte NADPH oxidase. We have revealed interfering with the mechanism of autoregulation of the physiological NO concentration in the tissues of periodontium and submandibular salivary glands that leads to simultaneous increase in NO formation through the NO-synthase and nitrate / nitrite reductase mechanisms, resulting in the development of oxidative-nitrosative stress with rising peroxynitrite concentration. We have also found out NOS uncoupling in the tissues of periodontium and salivary glands that is an additional source of superoxide anion radical as a leading factor in oxidative-nitrosative stress.

Keywords: reactive oxygen and nitrogen, periodontium, salivary glands, systemic inflammation

Метою роботи було з'ясування джерел продукції активних форм оксигену та нітрогену в тканинах пародонта та слинних залоз щурів за умов експериментального системного запалення. Дослідження були проведені на 20 білих щурах-самцях лінії Вістар масою 180-220 г, розподілених на 2 групи: 1-ша – інтактні тварини, 2-га – після відтворення системної запальної відповіді. Системне запалення відтворювали шляхом внутрішньоочеревинного введення ліпополісахариду Salmonella typhi («Пірогенал») у дозі, яка сприяла у щурів підвищенню температури на 1,5^{0}С, за схемою: протягом першого тижня вводили по 4 мінімальні пірогенні дози (MPD), що складає 0,4 мкг, на 1 кг маси щура 3 рази на тиждень. Протягом наступних семи тижнів експерименту щурам вводили по 4 MPD/кг маси 1 раз на тиждень При проведенні дослідження керувалися принципами біомедичної етики. Тварин декапітували під ефірним наркозом. Об'єктами дослідження були м'які тканини пародонта та тканини піднижньощелепних слинних залоз. Виявлено, що відтворення системного запалення супроводжується збільшенням продукції супероксидного аніон-радикала у тканинах пародонта та слинних залоз мітохондріальним і NADPH–залежними електронно-транспортними ланцюгами мікросом і NO-синтази, а також NADPH-оксидазою лейкоцитів. Виявлено порушення механізму авторегуляції фізіологічної концентрації NO у тканинах пародонта та піднижньощелепних слинних залоз, що призводить до одночасного збільшення утворення NO через NO-синтазний та нітрат- / нітрит-редуктазний механізм, наслідком чого є розвиток окисно-нітрозативного стресу зі збільшенням концентрації пероксинітриту. Виявлено порушення спряження NOS у тканинах пародонта та слинних залоз, що є додатковим джерелом супероксидного аніон-радикала як провідного чинника окисно-нітрозативного стресу.

Ключові слова: активні форми кисню і азоту,пародонт, слинні залози,системне запалення

Introduction

The development of inflammatory diseases of periodontium and salivary glands (SG) is associated with the action of both local and general factors. The local ones include biological (by-products of microorganisms and metabolic wastes), mechanical, physical and chemical damaging factors. Quite often a number of literary reports overestimate the role of local factors in the development of the pathology of the organs mentioned above [12]. These damaging impacts occur under the conditions of trophism and tissue resistance deterioration that arise in the presence of systemic disorders in the body: neurogenic, cardiovascular, endocrine, and metabolic (obesity, metabolic syndrome) disorders. Thus, structural failure and dysfunction of periodontium and SG can result from a series of somatic diseases, the development of which includes systemic inflammation (SI) as a link of pathogenesis [1, 15].

Hence, the development of severe inflammatory diseases of periodontium and SG is determined not only with the direct damage to periodontal tissues by a pathogenic agent, but can also result from the dysregulatory

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effect caused by other altered integrative systems, in particular those associated with triggering SI. It is supposed that the main link in the pathogenesis of this process is the permanent activation of certain transcription factors (in particular, the nuclear factor κB - NF-κB [6, 14]. The consequence is the expression of genes of inflammatory cytokines, inducible NO synthase (NOS), metalloproteinases, cellular adhesion molecules, cyclooxygenase-2, etc., capable of inducing oxidative-nitrosative stress [17]. On the one hand, the latter is associated with an increase in hyperproduction of superoxide anion radical ($^{\bullet}O_2^-$) by various sources, such as mitochondria [4], cytochrome P-450-associated NADPH-dependent microsomal system [5], NOS by itself, which contains NADPH-dependent electron transport chain, under the conditions of so-called uncoupling in the functioning of enzyme under the deficiency of its substrate (L-arginine) or certain cofactors (tetrahydrobiopterin) [10], as well as NADPH-oxidase of phagocytes associated with the function of cytochrome b_{558} [3]. On the other hand, oxidative-nitrogen stress develops under the excess formation of the nitric oxide (NO) by NOS and nitrite reductase, and the generation of reactive nitrogen species (for example, peroxynitrite) [8, 11].

However, mechanisms of the development of metabolic disorders in the periodontium and SG tissues under the conditions of SI are still insufficiently studied. The solution of this issue will contribute to the improvement of approaches and measures aimed at the prevention and treatment of inflammatory and dystrophic diseases of these organs against the progression of SI.

The purpose of the work was to reveal the sources of production of reactive oxygen and nitrogen species in the tissues of periodontium and salivary glands of rats under conditions of experimental SI.

Materials and methods

The study was carried out on 20 white male rats of the Wistar line weighing 180-220 g, divided into 2 groups: the 1st included intact animals, the 2nd was made up of the animals with induced SI. SI was induced by intraperitoneal administration of lipopolysaccharide (LPS) *Salmonella typhi* (pyrogenalum, "Medgamal", Russia) in a dose that stimulated rise in temperature by 1.5°C according to the scheme (modified by [13]): during the first week, 4 minimum pyrogenic doses (MPD) of 0.4 μg/kg of rat mass were administered 3 times a week. During the following seven weeks of the experiment, rats were given 4 MPD / kg of body weight once a week.

The research was guided by the principles of biomedical ethics. The animals were decapitated with ethereal anesthesia. Soft tissues of periodontium and submandibular SG were the objects of the study.

The formation of $^{\bullet}O_2^-$ was evaluated by a test with nitro blue tetrazolium with using spectrophotometry ("Ulab") of the tissue homogenate with the following inductors: NADH was used for the evaluation of $^{\bullet}O_2^-$ production by the mitochondrial electron transport chain, NADPH was used to evaluate $^{\bullet}O_2^-$ production by endoplasmic reticulum and NOS, and LPS (pyrogenalum) was used to assess $^{\bullet}O_2^-$ production by leukocyte NADPH oxidase [9].

The activity of NOS was determined by the difference in the concentration of nitrite ions before and after the incubation of homogenate into the medium containing arginine (NO-synthase substrate) and NADPH. The concentration of nitrite ions was assessed by the formation of diazo-compounds in the reaction with sulfanilic acid, and then we carried out the reaction with α-naphthylethylenediamine, resulting in the production of red color derivatives (azo dyes) [2]. The activity of nitrate reductase (NAR), nitrite reductase (NIR) and concentration of peroxynitrite ions ($NOOH^-$) in the homogenate were evaluated spectrophotometrically [2].

The NOS coupling index was calculated as the ratio between the NOS activity and the $^{\bullet}O_2^-$ generation rate by the NADPH-dependent electron transport chain.

The findings obtained were statistically processed. To verify the normality distribution, the calculation of the Shapiro-Wilk criterion was applied. If they corresponded to the normal distribution, then the Student's t-test was used to compare independent samples. When the results ranges were not subject to normal distribution, statistical processing was performed using a nonparametric method, the Mann-Whitney test. Statistical calculations were performed using the "StatisticSoft 6.0" program.

Results and discussion

SI simulation led to significant changes in the $\bullet O_2^-$production in the tissues of periodontium and SG (Table 1). Thus, the non-stimulated $^{\bullet}O_2^-$ generation increased by 40.8% ($p<0.01$) and 37.9% ($p<0.01$) respectively. The $^{\bullet}O_2^-$ production by NADPH-dependent electron transport chains (microsomal and NOS) increased by 38.3% ($p<0.01$) and 41.7% ($p<0.01$), while by the respiratory chain of mitochondria increased by 40.5% ($p<0.01$) and 37.6% ($p<0.01$).

Table 1

Effect of inductors on the production of superoxide anion radical in tissues of periodontium and SG under modeled SI, nmol / g • s (M + m, n = 20)

Groups of the animals studied	Periodontium				Salivary glands			
	Non-stimulated production	NADPH	NADH	LPS	Non-stimulated production	NADPH	NADH	LPS
Intact animals	1.20±0.09	12.47±0.87	15.41±1.08	1.58±0.12	1.40±0.08	14.65±0.72	18.69±1.02	1.92±0.13
Animals with SI	1.69±0.07 *	17.25±0.66 *	21.65±1.01*	2.10±0.09 *	1.93±0.11 *	20.76±1.01 *	25.72±1.10 *	3.28±0.32 *

*Note: Here and throughout: * – P<0,05 compared with values of intact rats.*

Generation of $^{\bullet}O_2^-$ by leukocyte NADPH oxidase also increased by 32.9% ($p<0.01$) and 70.8% ($p<0.01$) respectively.

According to the literature, the largest amount of $^{\bullet}O_2^-$ is produced by the mitochondrial electron transport chain as a result of the one-electron reduction of oxygen at the level of the following complexes: NADH – ubiquinone oxidoreductase, ubiquinonol – cytochrome c oxidoreductase and cytochrome b-c_1 [4]. This process is accelerated by activating NF-κB [17] and formation of NOOH- in the tissues [7, 16].

At the same time, a powerful level of $^{\bullet}O_2^-$ production under the conditions of SI is provided by NADPH-dependent electron transport chains: in the reactions of microsomal oxidation (with cytochrome P-450) [5] and by NOS itself, which is capable of switching from NO to $^{\bullet}O_2^-$ under unfavorable conditions [10]. When the bacterial LPS are introduced into the mammalian body, the $\bullet O_2^-$ generation by leukocyte NADPH oxidase naturally increases [3]. In addition to LPS, proinflammatory cytokines the synthesis of which depends on the activation of NF-κB [6, 17] are also known as effective stimulants of $^{\bullet}O_2^-$ synthesis.

Under SI conditions, the NOS activity in the periodontium and SG tissues (Table 2) increased by 2.5 times ($p<0.001$) and by 1.9 times ($p<0.001$), respectively. This can be explained by to the ability of LPS to provide NF-κB-dependent activation of inducible NOS [17].

Table 2

Markers of the formation of reactive nitrogen species in the tissues of periodontium and SG under modeled SI (M + m, n = 20)

Groups of the animals studied	Periodontium				Salivary glands			
	NOS, μmol ($N^{\bullet}O_2^-$)/ min • g of protein	NAR, μmol / min • g of protein	NIR, μmol/ min • g of protein	$NOOH^-$, μmol/g	NOS, μmol ($N\bullet O_2^-$) / min • g of protein	NAR, μmol/ min • g of protein	NIR, μmol/ min • g of protein	$NOOH^-$, μmol/g
Intact animals	4.20±0.22	11.98±0.88	3.43±0.25	0.83±0.04	7.27±0.52	32.23±2.42	7.20±0.66	0.99±0.07
Animals with SI	10.32±0.50	16.33±0.74 *	4.61±0.39 *	1.08±0.05 *	13.56±0.86	43.23±3.64 *	9.96±0.82 *	2.56±0.43

At the same time, the activity of NAR grew by 36.3% ($p<0.01$) and 34.1% ($p<0.05$), while the activity of nitrite NIR increased by 34.4% ($p<0.05$) and 38.3% ($p<0.05$), respectively. These enzymatic pathways of the formation of nitrite ions and NO become activated in the tissues, mainly under hypoxia. Activation of these enzymes against increased NO formation by NOS indicates the disturbances in the mechanism of autoregulation of the physiological NO concentration in tissues [8]. An increase in the formation of reactive nitrogen species has been found out to a logical consequence of these processes: $NOOH^-$ content increased in the periodontium and SG tissues by 30.1% ($p<0.01$) and 2.6 times ($p<0.01$), respectively.

The SI modeling was accompanied by a decrease in the NOS coupling index. This index characterizes the presence of substrates (L-arginine, O_2) and co-factor for the NO formation tetrahydrobiopterin, but not for $^{\bullet}O_2^-$, in the oxidative metabolism of L-arginine. That is, under SI, there are conditions favorable for generating $^{\bullet}O_2^-$ by NOS.

Moreover, the unpaired NOS by itself not only becomes a powerful generator of $^{\bullet}O_2^-$, but simultaneously activates other mentioned sources of its formation, thus forming a kind of closed circle of mutual reinforcement of oxidative stress and NOS uncoupling.

Conclusions

1. Simulating of systemic inflammation is accompanied by an increase in the production of superoxide anion radical in periodontal tissues and submandibular salivary glands of rats by mitochondrial and NADPH-dependent microsomal electron transport chains and NO synthase, as well as leukocyte NADPH oxidase.

2. Modeling of systemic inflammation causes impairments in the mechanism of autoregulation of the physiological NO concentration in the tissues of the periodontium and submandibular salivary glands that is manifested by the simultaneous increase in the NO formation through NO-synthase and nitrate / nitrite reductase mechanism. This results in the development of oxidative-nitrosative stress with increasing peroxynitrite concentration.

3. Under the conditions of experimental systemic inflammation, NOS uncoupling has been revealed in periodontal tissues and submandibular salivary glands of rats that is regarded as an additional source of superoxide anion radical as the main factor of oxidative-nitrosative stress.

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