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Preconditioning with the low dose of lipopolysaccharide attenuates apoptosis in the heart during septic shock in mice

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Abstract:
Despite advances in medical treatment, septic shock is associated with very high mortality. Myocardial injury is a common condition in septic shock. The pretreatment with a low dose of lipopolysaccharide (LPS) was proven to diminish injury in the animal heart. The purpose of the study was to investigate whether pretreatment with a low dose of bacterial LPS (preconditioning) may also inhibit apoptosis occurring in the mouse heart during septic shock. Three groups of male C57BL/6J mice were injected intraperitoneally with various doses of LPS (*E.coli* 055:B5): 0.5 mg/kg, 10 mg/kg, and 0.5 mg/kg 24 h prior to 10 mg/kg (the preconditioned group). Control group received solvent. Mice were sacrificed 24 h after the last injection. Apoptosis was evaluated on formalin-fixed slides using TUNEL-fluorescein method. Phosphorylation of STAT3 and expression of Bcl-2 was assessed by Western blotting. High dose of LPS (10 mg/kg) increased the incidence of apoptosis in the heart, whereas pretreatment with the preconditioning dose (0.5 mg/kg) of LPS significantly attenuated this effect. Western blot analysis revealed that a single treatment with low dose of LPS resulted in increased phosphorylation of Stat3. The expression of the Bcl-2 in the preconditioned animals was significantly higher than in animals treated with the high LPS dose alone.
Preconditioning with the low dose of LPS attenuates the activation of apoptosis in the course of septic shock. This effect may rely on activation of Stat3, hence, preservation of higher levels of antiapoptotic proteins, like Bcl-2.

Key words:
lipopolysaccharide, preconditioning, septic shock, heart, apoptosis, mice

Abbreviations: ANOVA – analysis of variance, IL-6 – interleukin 6, iNOS – inducible nitric oxide synthase, LPS – lipopolysaccharide, LV – left ventricle, PAGE – polyacrylamide gel electrophoresis, PBS – phosphate buffered saline, ROS – reactive oxygen species, RIPA – radioimmunoprecipitation assay, SDS – sodium dodecyl sulfate, STAT – signal transducer activator of transcription, TNF-α – tumor necrosis factor α, TUNEL – terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

Introduction

Septic shock complicates often the state of patients with infection. It is one of the most common causes of admission to the intensive care units. Despite progress in diagnosis and treatment, the prognosis in this condition remains relatively poor with in-hospital mortality reaching 50%. Septic shock develops often as a fulminant inflammatory response caused by the lipopolysaccharide (LPS) from Gram-negative bacterial wall [1]. This reaction produces significant decrease in blood pressure, impairment of microvascular circulation and hence tissue ischemia. The typical course of septic shock entails gradual deterioration of function of various organs and systems ending often in multiple organ failure.

From the beginning of the septic shock, the circulatory failure is one of the hallmarks of this illness. Initially this is a high output failure, caused predominantly by increased oxygen demand due to hypermetabolism, diminished peripheral resistance and impaired microvascular flow. Later, however, function of the left ventricle (LV) deteriorates and cardiac failure occurs. In this phase mortality rapidly increases. There is support for the notion that LV function impairment is caused by increased concentration of inflammatory mediators and increased activity of inducible nitric oxide synthase (iNOS) [17, 22, 23]. Other papers suggested an increase in apoptosis in the animal heart exposed to the high doses of bacterial endotoxin and held apoptosis responsible for the LV failure [12, 16]. Enhanced apoptosis occurs during septic shock in various organs, as generalized inflammation was shown to be associated with increased concentrations of proapoptotic factors, like tumor necrosis factor (TNF)-α, Fas Ligand (FasL) and CD40 [5, 11, 17, 23]. All these substances are involved in triggering apoptosis in the heart.

There is support for precondtitioning as an approach to contain apoptosis, because short periods of ischemia may protect myocardium against ischemia-induced apoptosis [21]. It has been shown that a low dose of LPS administered prior to the injury may diminish apoptosis caused by ischemia and reperfusion in the heart and brain in mice [18].

In this study, we addressed the question whether a small dose of LPS may diminish apoptosis in the heart caused by a high dose of bacterial endotoxin in mice.

Materials and Methods

Animals

Twelve-week-old male C57BL/6J mice were used in the study. They were kept at constant temperature of 22 ± 1°C under 12:12 dark-light cycle with constant access to chow and water. The animals received intraperitoneally lipopolysaccharide from bacterial wall (LPS 055: B5 Sigma) or 0.9% sterile saline. One group of mice was given a high dose of LPS – 10 mg/kg and were considered to be the septic shock group. The next one received a preconditioning dose (0.5 mg/kg) of LPS 24 h prior to the high dose (10 mg/kg) and was considered as preconditioned group. The third group was injected with the small dose of LPS only (0.5 mg/kg) [14]. The last group received vehicle (sterile 0.9% saline) in the same volume as all previous groups (20 ml/kg) and these mice were referred to as controls.

All animals were sacrificed 24 h after the last injection. Six mice of each group were sacrificed by cervical dislocation, hearts were rapidly excised, washed in phosphate-buffered saline (PBS) and snap frozen in liquid nitrogen. Tissue awaited further analysis at –80°C.

Other mice were anesthetized with the ketamine-xylazine mixture in sterile 0.9% saline (120 mg/kg and 3 mg/kg, respectively) and sacrificed by potassium chloride infusion. Hearts were fixed *in situ* as described previously [6], by perfusion with phosphate buffered isotonic formalin under normal physiologic pressures (about 80 mmHg in aorta and 6 mmHg in LV).

The experimental procedures were carried out according to the European Council Directive of November 24, 1986 (6/609/EEC) and were approved by the Local Ethics Committee in Białystok.

TUNEL-FITC staining

The formalin-fixed hearts from 7 to 9 mice of each group were cut into transverse rings and were embedded in paraffin. Paraffin blocks were cut into 6 μm thick slices. Subsequently, they were subjected to the fluorescein terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) (ApopTag, Chemicon) [8]. Counterstaining of cell nuclei was performed using DAPI (Chemicon). All slides were analyzed and photographed in fluorescence microscope (Olympus). The ratio between TUNEL positive nuclei and all DAPI-stained nuclei was considered to be the measure of apoptosis.

Western blotting

Protein extracts were prepared from 6 hearts of each group as described previously [3, 8]. Frozen cardiac tissue was homogenized on ice using mechanical disperser (IKA T10) in radioimmunoprecipitation assay (RIPA) buffer containing phosphatase and proteinase inhibitors. Then, extracts were centrifuged at 4°C 12,000 × g for 20 min. Supernatant was collected and protein content was measured using Bradford reagent (Sigma). Samples were frozen at –80°C and kept frozen until further analysis. Proteins extracts were subjected to sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) and blotting onto the nitrocellulose membranes (BioRad). Equal loading was confirmed using Ponceau Red staining (Sigma). Primary antibodies recognizing mouse phosphorylated signal transducer activator of transcription 3 (STAT3) were purchased from Santa Cruz Biotechnology (sc-7993-R) while mouse Bcl-2 was from Sigma-Aldrich (B3170). Secondary antibodies were conjugated with horseradish peroxidase. Blots were visualized using enhanced chemiluminescence reaction (ImmunStar BioRad), and exposed to X-ray film (X-Omat Blue Kodak). Results were scanned and quantified using Scion Image software (Scion Corporation). The results of particular experiments were related to the expression of proteins in the control (or low dose alone) group, which were set as 100%.

Statistical analysis

Results were presented as the mean ± standard error of the mean. One-way ANOVA and Fisher test were used for statistical analysis. The group where normal distribution of the variable could not be presumed was compared using the non-parametric Mann Whitney test. A p value lower than 0.05 was considered statistically significant.

Results

TUNEL staining

One-way ANOVA of the degree of apoptosis in mouse hearts, assessed with TUNEL-fluorescein staining, yielded $F(3, 26) = 7.2268$, $p = 0.001$. *Post-hoc* comparison made with Fisher test revealed that high dose of LPS significantly enhanced apoptosis in comparison to the vehicle treated controls ($p = 0.0002$). Pretreatment of animals with the low dose of LPS significantly diminished the percentage of apoptotic nuclei in comparison with the group injected with 10 mg/kg alone (Fisher test $p = 0.009$) (Fig. 1).

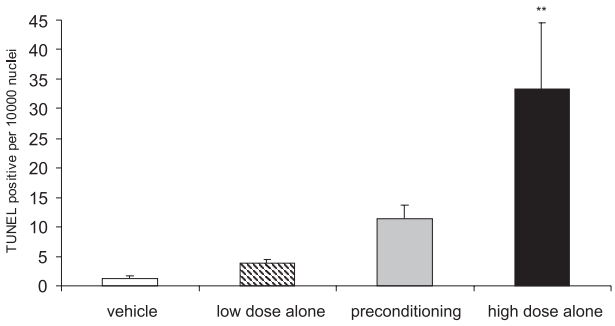

Fig. 1. The ratio of TUNEL-positive nuclei to all DAPI positive nuclei in the heart cross sections from animals treated with the vehicle or with LPS: 0.5 mg/kg (low dose alone); 0.5 mg/kg 24 h prior to 10 mg/kg (preconditioning) and 10 mg/kg (high dose alone). ** $p < 0.01$ *vs.* all other groups. (ANOVA and Fisher test)

Western blotting

STAT3 phosphorylation was not observed in any control animal, whereas already a small dose of LPS caused noticeable phosphorylation of this protein (Fig. 2 A). As distribution of the data was not normal (all values were equal 0), this group was compared to the others, using the non-parametric test (Mann-Whitney test). The STAT3 phosphorylation in preconditioned mice as well as in mice treated with the low

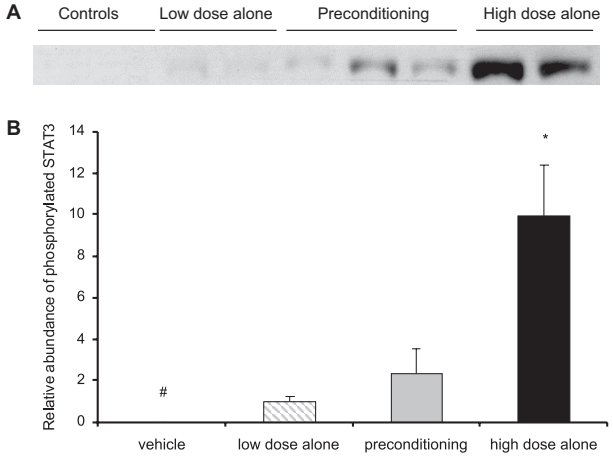

Fig. 2. The phosphorylation of STAT3 protein, measured in Western blotting, in the hearts of mice injected with the vehicle or with LPS: 0.5 mg/kg (low dose alone); 0.5 mg/kg 24 h prior to 10 mg/kg (preconditioning) and 10 mg/kg (high dose alone). (**A**) Representative Western blot presenting phosphorylation of the STAT3 protein. No phosphorylation of STAT3 was detected in the control animals, whereas, all animals injected with LPS presented detectable levels of phospho-STAT3 protein. (**B**) Quantification of Western blots of protein extracts from hearts of mice treated with vehicle or with LPS. * $p < 0.005$ *vs.* groups: low dose alone and preconditioning. ANOVA and Fisher test, # $p < 0.005$ *vs.* all other groups. Mann-Whitney test

dose of LPS alone, was significantly lower in comparison to the animals treated with the high dose of LPS alone [ANOVA $F(2, 14) = 8.2128$, $p = 0.004$; Fisher test $p = 0.005$ and $p = 0.003$ respectively] (Fig. 2 B).

Bcl-2 expression was significantly diminished after the LPS challenge in comparison to the vehicle-treated animals. [ANOVA $F(3, 17) = 5.4320$, $p = 0.008$]

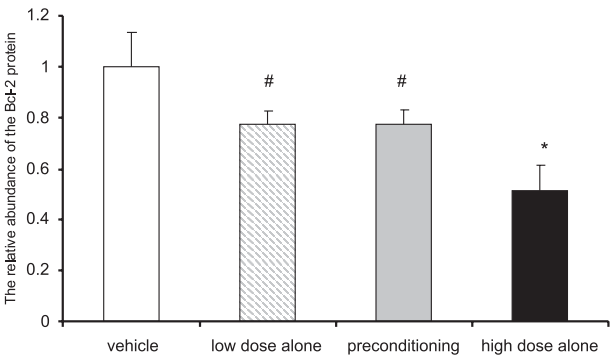

Fig. 3. The expression of Bcl-2 protein in the hearts of mice injected with the vehicle or with LPS: 0.5 mg/kg (low dose alone); 0.5 mg/kg 24 h prior to 10 mg/kg (preconditioning) and 10 mg/kg (high dose alone). # $p = 0.05$ *vs.* the vehicle-treated group. Fisher *post-hoc* test; * $p < 0.05$ *vs.* all other groups. Fisher *post-hoc* test

There were no significant differences, however, between animals treated with the low dose of LPS alone and the preconditioning group. Moreover, a significantly higher Bcl-2 protein abundance was observed in the preconditioning group in comparison to the mice treated with the high dose of LPS alone ($p = 0.03$ Fisher test) (Fig. 3).

Discussion

The results of the present study indicate that the low dose of bacterial LPS decreases apoptosis of cardiac cells during septic shock. Based on the Western blotting results, we hypothesize that this effect may be mediated by the activation of the STAT3. This, in turn, diminished the reduction of the abundance of anti-apoptotic protein Bcl-2.

It has been previously shown that septic shock is associated with severe cardiac dysfunction and activation of apoptosis [7, 9, 16]. It is believed that this effect is mediated by secretion of proapoptotic cytokines, like TNF-α and extensive activation of iNOS [1, 17]. The precise pathways of this phenomenon, however, are still unclear as experimental models yielded equivocal results [10]. Taking into account the high incidence of septic shock and unusually high, for modern medicine, mortality [1], there is an urgent need for new therapeutic modalities. One of the novel approaches involves preconditioning [21]. Among substances, which may exert preconditioning effect, there is a bacterial endotoxin.

Previous studies have reported a protective effect of a low dose of LPS on the ischemia-induced apoptosis in the heart and in the brain in animals [18, 24]. Similarly to the short periods of ischemia, the low dose of LPS resulted in the late preconditioning with the smaller extent of injury and attenuated apoptosis [24]. This effect is considered to be mediated by the activation of iNOS and inhibition of nuclear factor (NF)-κB [21]. In this study, we describe a similar effect in the model of septic shock. It has been previously reported that preconditioning with a low dose of LPS ameliorates the deterioration of LV contractility in septic shock [14]. Our study suggests that this effect, at least in part, may be explained by the diminished injury due to the lower number of apoptotic cardiac cells. This notion may be supported by the fact

that caspase inhibitors decreasing cardiomyocyte apoptosis prevent cardiac dysfunction in the animal model of sepsis [15].

There is a debate which factors predominantly contribute to the cardiac dysfunction during sepsis. Suliman et al. [22] have reported that the main cardiac injury during endotoxemia is caused by reactive oxygen species (ROS). In our study, we did not measure the production of ROS, nevertheless, STAT3 has been shown to play a crucial role in protection against ROS in the heart [8]. It seems to be possible that upregulation of the elements of antioxidant defense system by STAT3 may attenuate the oxidative stress and explain the protective effect of the low dose of LPS. On the other hand, ROS are known to induce phosphorylation of STAT3 [20]. There is an apparently surprising finding that STAT3 phosphorylation is the highest in the group treated with the high dose of LPS alone. We hypothesize that such high activation is secondary to the extensive injury, production of ROS and high concentration of circulating hormones activating STAT3. Pretreatment with the low dose of LPS may result in weaker activation of leukocytes [18], attenuation of oxidative stress [14], hence, in the decreased activation of STAT3.

In our study, we have noticed a decreased abundance of antiapoptotic protein Bcl-2 in the group injected with the high dose of endotoxin. This effect of suppressing expression or/and increasing cleavage of antiapoptotic proteins results in markedly enhanced apoptosis of cardiac cells. Similar effect in the septic shock was previously reported by MacDonald et al. [13]. We, however, were able to show that pretreatment with the low dose of LPS resulted in maintaining higher expression of Bcl-2. This phenomenon, at least in part, may explain decreasing incidence of apoptosis by preconditioning with the low dose of LPS. This finding is in agreement with the previous studies investigating LPS-mediated preconditioning in other experimental settings [12].

In the present study, we only described the findings observed during preconditioning. We have not modified any transduction pathway possibly participating in antiapototic effect of low-dose LPS. There are two potentially interesting factors, whose involvement should be thoroughly investigated in further studies. One of them is the activation of iNOS, because its extensive activity is thought to contribute to vasodilatation, decline in blood pressure and deterioration of the heart contractility [23]. In this context particularly interesting seems to be the role of native NOS inhibitors like asymmetric dimethylarginine (ADMA) in septic shock [2]. On the other hand, iNOS is known to play a key role in the preconditioning exerted by either ischemia or a low dose of LPS [4, 24]. Since previous studies failed to show decisively whether the low dose of LPS enhances expression of iNOS [24], this question still remains to be solved.

Another pivotal factor mediating the ischemic preconditioning is interleukin 6 (IL-6) [4]. Large amounts of IL-6 secreted to the circulation in the model of endotoxemia decrease the concentration of other proinflammatory cytokines, like TNF-α [19]. Locally, in the heart during septic shock, protective effects of the increased IL-6 expression were observed [19]. Moreover, IL-6 enhanced activity of iNOS and conferred ischemic preconditioning by increasing expression of this protein [4]. Whether IL-6 has similar role in antiapoptotic effects of LPS preconditioning as in ischemic preconditioning still remains to be determined.

Based on the described findings, we conclude that the low dose of bacterial endotoxin applied 24 h prior to the high dose of LPS, activates the cytoprotective pathways, maintains the concentration of antiapoptotic proteins and attenuates the apoptosis in the heart.

This paper presents only an initial step in search for novel mechanisms that may help to attenuate cardiac dysfunction in septic shock and thus increase survival in patients suffering from severe endotoxemia. Hopefully, in the future new therapeutic modalities promoting cell survival will enable to achieve this goal.

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