Intracellular and Extracellular NO Differentially Modulates the Stress Response and Apoptosis in Macrophages Exposed to the Influence of Biological or Physical Factors

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We studied the role of extracellular and intracellular NO in the regulation of the stress response and apoptosis in macrophages of proinflammatory and antiinflammatory phenotypes under the influence of *S. aureus* and heat shock. Blockade of extracellular nitric oxide synthesis in cells with antiinflammatory phenotype inhibited the stress response induced by *S. aureus* and heat shock. The decrease in extracellular nitric oxide concentration around antiinflammatory macrophages potentiated the stress response induced by *S. aureus*, but had no effect on the stress response induced by heat shock. Hence, intracellular NO mediates the stress response induced by *S. aureus* and heat shock, while extracellular NO inhibits the stress response induced by *S. aureus*, but has no effect on the stress response induced by heat shock. In cells with antiinflammatory phenotype, intracellular NO plays an antiapoptotic role. *S. aureus* and heat shock did not cause apoptosis in macrophages with proinflammatory phenotype, while intracellular NO did not play a role in antiapoptotic activity of the proinflammatory phenotype. Extracellular NO synthesized by macrophages protects these cells from apoptosis induced by *S. aureus* and heat shock.

Key Words: *stress response; apoptosis, macrophages; nitric oxide*

Studying the mechanisms mediating the effect of microbial products on the host immune response at various stages of infection is an urgent problem of modern immunology. Macrophages play an important signal and regulatory role in immune reactions of the organism. Macrophages adequately change secretory activity, thus adapting to microenvironmental conditions and maintaining the immune balance. Exposure of macrophages to bacterial lipopolysaccharide (LPS) in substimulatory concentrations modulates the native cytokine pattern to proinflammatory or antiinflammatory profile during further stimulation of these cells with microbial products, including LPS [10]. It should be emphasized that antiinflammatory phenotype is characterized by more potent production of antiinflammatory cytokines and nitric oxide (NO) than proinflammatory phenotype. The LPS-dependent acquisition of a specific phenotype for the cytokine response of macrophages *in vitro* was called reprogramming.

In the inflammatory focus, macrophages are exposed to adverse factors. Activation of a self-protecting stress response in macrophages prevents toxic effect of cytokines and oxidative and nitrosative stress. Inducible heat shock proteins HSP70

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play a key role in this process [5]. The protective effect of HSP70 is related to chaperone activity [6], disaggregation of denatured protein aggregates [8], activation of the antioxidant defense system [5], transport of damaged proteins to lysosomes [9], prevention of NO overproduction [7], and antiapoptotic influence [4]. However, long-term activation of macrophages and overproduction of proinflammatory mediators can cause a variety of diseases. The excessive inflammatory response in activated macrophages can be limited by triggering apoptosis. The balance between the protective stress response and apoptosis determines survival of activated macrophages.

NO plays an important role in the regulation of LPS-induced stress response and apoptosis in macrophages [2]. It depends on extracellular or intracellular localization of NO. LPS is a component of gram-negative bacterium cell wall, which mediates receptor-dependent induction of the macrophage response. The interaction of macrophages with gram-positive bacteria *S. aureus* is realized via receptors and phagocytosis. It was demonstrated that *S. aureus* stimulates the stress response and apoptosis in macrophages with the antiinflammatory phenotype, while LPS in cells with the proinflammatory phenotype [1].

The concentration of bacterial products modulating activity of macrophages increases at the early stage of infection. Body temperature increases when the infection is not localized by the immune response. Hence, macrophages are exposed to the influence of a physical factor (heat shock). In contrast to LPS, heat shock stimulates the stress response and apoptosis in macrophages with antiinflammatory phenotype [1].

Here we studied the role of NO in the regulation of the stress response and apoptosis induced by the biological (*S. aureus*) and physical (heat shock) factors.

MATERIALS AND METHODS

Experiments were performed on cultured mouse macrophages. Proinflammatory and antiinflammatory phenotypes of macrophages were obtained by the standard method [10]. Primary culture of naive macrophages was divided into 3 pools. LPS was added to pools I (0.5 ng/ml) and II (5 ng/ml) for 6 h for induction of proinflammatory and antiinflammatory phenotypes, respectively. Pool III served as the control. The stress response and apoptosis were induced by heat shock (42°C, 1 h) and *S. aureus* (bacterium/macrophage ratio 200:1). The stress response was evaluated by HSP70 content (Western blot analysis). Apoptosis was assayed by DNA fragmentation (flow cytometry). The intracellular and extracellular effects of NO on the macrophage response were studied using ITU (S-(2-aminoethyl)isothiourea), a selective inhibitor of inducible NO synthase (iNOS), and c-PTIO ((2-4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxil-3-oxide), an NO trap that does not enter the cell [3]. The results were analyzed by Student's *t* test.

RESULTS

Figure 1 illustrates the effects of c-PTIO (NO trap not entering the cell) and ITU (selective iNOS inhibitor) on induction of HSP70 synthesis in macrophages by *S. aureus*. In macrophages with proinflammatory and native phenotypes *S. aureus* did not induce HSP70 synthesis. ITU (50 μM) and c-PTIO (100 μM) did not modulate the ability of *S. aureus* to induce the stress response in cells of these phenotypes. *S. aureus* induced the stress response, while blockade of intracellular NO synthesis completely abolished activation of HSP70 synthesis in antiinflammatory macrophages exhibiting the stress response to *S. aureus*. The decrease in extracellular NO concentration around antiinflammatory macrophages due to binding to c-PTIO potentiated the stress response under the influence of *S. aureus*.

Hence, the role of NO in the regulation of the *S. aureus*-induced stress response in macrophages with antiinflammatory phenotype depends on NO localization. Intracellular NO probably serves as a mediator of the *S. aureus*-induced stress response. However, extracellular NO inhibits the stress response in macrophages.

ITU 4-fold increased the degree of *S. aureus*-induced apoptosis in cells with antiinflammatory phenotype. This compound had no effect on apoptosis in the proinflammatory phenotype. ITU significantly potentiated the proapoptotic effect of *S. aureus* in native macrophages (Table 1). Binding of extracellular NO by c-PTIO was followed by a 2-fold increase in apoptosis in cells with antiinflammatory phenotype, but had no effect on proinflammatory and native phenotypes.

These data allowed us to make two conclusions. First, intracellular NO produced by iNOS in cells with native and antiinflammatory phenotypes plays an antiapoptotic role. However, *S. aureus* does induce apoptosis in cells with proinflammatory phenotype. It can be hypothesized that intracellular NO is not involved in antiapoptotic activity of proinflammatory macrophages. And second, extracellular NO synthesized by macrophages with proinflammatory phenotype protects these cells from

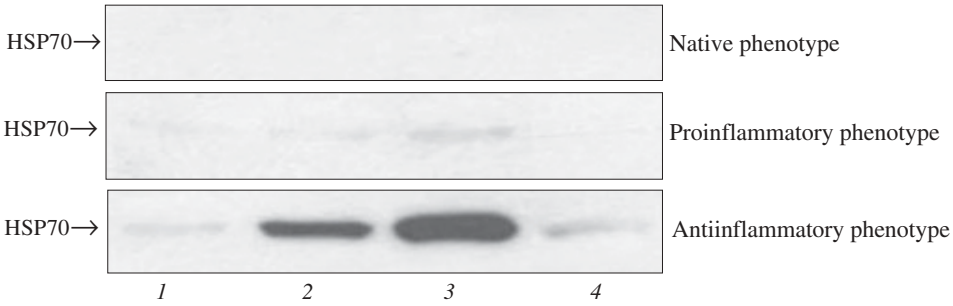

Fig. 1. Effects of c-PTIO and ITU on the ability of *S. aureus* (SA) to activate HSP70 synthesis in macrophages of various phenotypes. Control (*1*), SA (*2*), SA+c-PTIO (*3*), and SA+ITU (*4*).

S. aureus-induced apoptosis. This mechanism probably serves as a component of autocrine protection of antiinflammatory macrophages in the infection focus. At the same time, extracellular NO does not contribute to the increased resistance of macrophages with native and proinflammatory phenotypes to *S. aureus*-induced apoptosis.

Heat shock activated HSP70 synthesis in macrophages with antiinflammatory phenotype, but not in proinflammatory and native phenotypes (Fig. 2). ITU in a concentration of 50 μM decreased the heat shock-induced accumulation of HSP70 in macrophages with antiinflammatory phenotype. c-PTIO (100 μM) did not enter the cell and had no effect on heat shock-induced HSP70 synthesis in antiinflammatory macrophages. These data suggest that heat shock-induced HSP70 synthesis is realized via the intracellular mechanisms regulated by intracellular NO in cells with antiinflammatory phenotype. Extracellular NO is not involved in this process.

Heat shock caused DNA fragmentation in 30% macrophages with antiinflammatory phenotype, but had no effect on DNA integrity in proinflammatory and native phenotypes. The inhibition of iNOS by ITU induced a 2-fold increase in the number of antiinflammatory macrophages, which were characterized by DNA fragmentation during heat shock (Table 2). ITU had no effect on cells of proinflammatory and native phenotypes under control conditions and during heat shock. c-PTIO increased the severity of heat shock-induced damage to DNA in macrophages of various phenotypes. These data indicate that NO regulates the intracellular mechanisms for DNA protection from heat shock only in cells with antiinflammatory phenotype. The extracellular signal pathways regulated by extracellular NO are involved in DNA protection from heat shock in macrophages of various phenotypes.

Our results indicate that NO produces various effects on the stress response and apoptosis in macrophages under the influence of biological and physical factors, which depends on the target for this compound (extra- or intracellular components). SH groups in various proteins and iron-containing proteins are the main molecular targets for NO. NO induces direct modification of proteins via nitrosylation or has an indirect effect on methylation and ribosylation. NO increases activity of cytoplasmic guanylate cyclase and inhibits iNOS and some mitochondrial enzymes. Extracellular NO is probably involved in nitrosylation of SH groups in extracellular domains of membrane receptors and, therefore, has a role in specific signal pathways.

Binding of extracellular NO increases the stress response during LPS-induced receptor-mediated [2] and *S. aureus*-dependent phagocytosis-mediated activation of macrophages. The stress response to heat shock results from denaturation of intracellular proteins. Binding of extracellular NO during heat shock had no effect on the stress response of macrophages. The inhibition of intracellular NO syn-

TABLE 1. Effect of *S. aureus* (SA) on the Number of Macrophages with Different Phenotypes with Fragmented DNA (%) and Effect of c-PTIO and ITU

Phenotype	N, 6 h	N, 72 h	SA, 72 h	SA+c-PTIO, 72 h	SA+ITU, 72 h
Native	<0.5	2±1	9±3	15±4	22±3^{+}
Proinflammatory	<0.5		7±3	10±3	9±2
Antiinflammatory	<0.5		17±1*	30±4^{+}	61±5^{+}

Note. N, normal conditions. $p<0.05$: *compared to normal conditions after 72 h; $^{+}$compared to SA after 72 h.

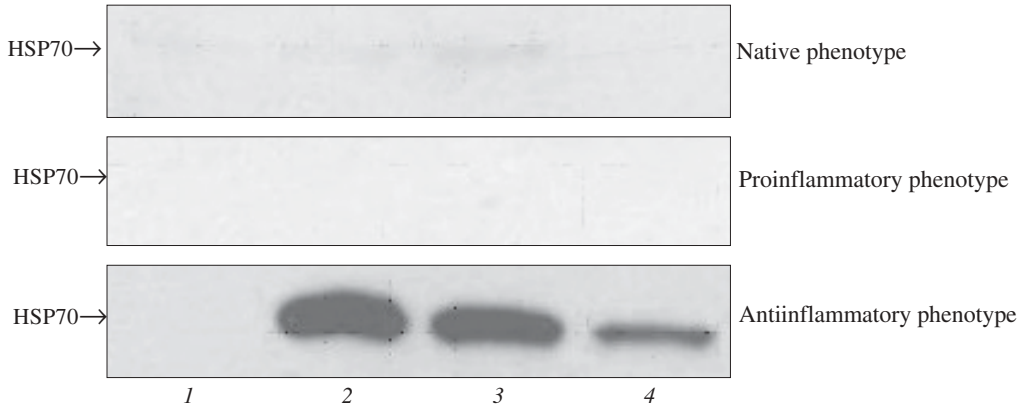

Fig. 2. Effects of c-PTIO and ITU on heat shock-induced activation of HSP70 synthesis in macrophages with antiinflammatory phenotype. Control (*1*), HS (*2*), HS+c-PTIO (*3*), and HS+ITU (*4*).

thesis was followed by blockade of the stress response induced by LPS [1], *S. aureus*, or heat shock. These data show that intracellular NO mediates the stress response in macrophages under the influence of bacterial or physical factors. It does not depend on the mechanism for induction of the stress response (receptor mechanism, phagocytosis, or denaturation of intracellular proteins). Extracellular NO inhibits the stress response to bacterial factors, but has no effect during exposure to physical factors.

LPS caused apoptosis in cells with proinflammatory phenotype [1], while *S. aureus* and heat shock had an effect on antiinflammatory cells. It remains unclear, why the immune system eliminates proinflammatory macrophages, but retains antiinflammatory cells during treatment with LPS in high doses. By contrast, the immune system eliminates antiinflammatory macrophages and retains proinflammatory cells under the influence of *S. aureus* and high temperature. Independently on the nature of apoptosis-inducing agent (biological or physical factor) and phenotypic characteristics of cells, binding of extracellular NO was followed by an increase in apoptosis. These data are consistent with the results of previous experiments [2]. Hence, a biological role of extracellular NO secreted by macrophages is the autocrine protection of these cells from adverse effects of biological and physical factors.

We compared the effects of iNOS blockade on apoptosis in macrophages of various types under conditions of exposure to LPS, *S. aureus*, and heat shock. Intracellular NO has a differential specific role in macrophage apoptosis. The iNOS antagonist significantly decreased LPS-induced apoptosis in cells with proinflammatory phenotype [2], i.e. intracellular NO played a proapoptotic role under these conditions. At the same time, iNOS antagonist significantly increased apoptosis induced by *S. aureus* or heat shock. Hence, intracellular NO has an antiapoptotic role under these conditions.

Studying the effect of heat shock on macrophages after treatment with ITU or c-PTIO showed that the intracellular NO-mediated protection of DNA during heat shock is related to activation of HSP70 synthesis. However, extracellular NO is involved in the protection of DNA in cells of various phenotypes. This effect is not mediated by activation of HSP70 synthesis.

Our results indicate that NO is a multimodal signal, which activates various responses depending on the intracellular or extracellular localization of NO, nature of inducing factor, and immune status of macrophages (phenotype).

TABLE 2. Effect of Heat Shock (1 h) on the Number of Macrophages of Various Phenotypes with Fragmented DNA (%). Influence of c-PTIO and ITU (*M*±*m*)

Phenotype	N, 6 h	N, 72 h	HS+N, 72 h	(HS+cPTIO), +cPTIO, 37°C, 72 h	(HS+ITU), +ITU, 37°C, 72 h
Native	<1	4±1	5±1	12±2^{+}	6±3
Proinflammatory	<1		4±1	13±3^{+}	5±3
Antiinflammatory	<1		31±4*	68±8^{+}	58±7*

Note. N, normal conditions; HS, heat shock. $p<0.05$: *compared to normal conditions after 72 h; $^{+}$compared to HS+N after 72 h.

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