SP-D-Dependent Regulation of NO Metabolism in Lipopolysaccharide-Stimulated Peritoneal Macrophages

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This work was designed to study the role of surfactant protein D in the regulation of NO synthesis by "non-alveolar" microphages. We evaluated whether the effects of surfactant protein D depend on the phenotype of macrophages. In the absence of surfactant protein D, the LPS-induced iNOS response was shown to decrease in macrophages of native and proinflammatory phenotypes by 30%, and in macrophages of the antiinflammatory phenotype (by 63%). Under the influence of lipopolysaccharide in high doses (500 ng/ml), NO_2^- production by mouse macrophages without surfactant protein D was reduced in native cells (by 25%), but increased in proinflammatory (by 40%) and antiinflammatory phenotypes (by 12% compared to mouse macrophages with surfactant protein D). Our results suggest that surfactant protein D is involved in the immune response in the whole organism, but not only in the lungs. The effect of surfactant protein D depends on the phenotype of macrophages.

Key Words: *surfactant protein D; macrophages; lipopolysaccharides; nitric oxide*

Surfactant protein D (SP-D) is a multimeric Ca^{2+}-binding protein, which belongs to a family of collagen-like lectins. SP-D is mainly produced by type 2 cells and nonciliated bronchiolar cells in the lungs [6]. The major function of SP-D is modulation of the immune protection during lung inflammation [8]. However, little is known about the mechanism for action of SP-2. Previous studies showed that SP-D stimulates chemotaxis of neutrophils [7] and plays a role in the engulfment of microbes by alveolar macrophages and neutrophils [9]. SP-D content increases in inflammation and infection. SP-D gene-knockout SP-D(-/-) mice are characterized by the increased number and size of alveolar macrophages, changes in the profile of surfactant phospholipids, increase in metalloproteinase activity, and oxidative and nitrosative stress [13]. Moreover, SP-D(-/-) mice exhibit an increased basal incidence of lung inflammation and emphysema [4] and high susceptibility to bacterial [10] and viral infections [11]. Alveolar macrophages of SP-D(-/-) mice produce a greater amount of superoxide radicals and hydrogen peroxide as compared to macrophages from normal animals [10]. Due to the direct antioxidant effect, SP-D protects the lungs and alveolar macrophages from oxidative damage [5]. These data illustrate an important role of SP-D in antiinflammatory processes.

Previous experiments showed that the expression of inducible NO synthase (iNOS) is elevated in alveolar macrophages of SP-D(-/-) mice. These features contribute to NO overproduction and nitro-

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sative stress [2]. It was hypothesized that iNOS serves as an important factor of inflammation. Moreover, SP-D can regulate the production and metabolism of NO [1]. This hypothesis was confirmed by the results of experiments with blockade of iNOS [3]. Studying the effect of SP-D on NO production is required to understand the regulatory role of SP-D in inflammation and immune response in the lungs. Alveolar macrophages probably serve as the main cells in this process. SP-D is bound to alveolar macrophages in a receptor-dependent manner [9]. SP-D production in the lungs is greater compared to other organs and tissues. Therefore, the lungs have attracted considerable attention. Strong evidence exists that SP-D plays a role in the regulation of alveolar macrophages. It should be emphasized that SP-D production occurs in the heart, stomach, and intestine [12]. It remains unclear whether SP-D regulates NO synthesis in alveolar macrophages or also affects these cells in other organs and tissues. In other words, whether SP-D serves as a local factor of pulmonary immunity or plays a role in the immune response of the whole organism.

Macrophages plays the major role in the induction of inflammation. Native macrophages acquire a proinflammatory or antiinflammatory phenotype during the immune response. The transformation of macrophage phenotype was described and studied *in vitro* [15]. A proinflammatory phenotype os characterized by increased production of proinflammatory cytokines including interleukin-1 (IL-1), IL-12, and tumor necrosis factor-α (TNF-α). Antiinflammatory macrophages produce antiinflammatory cytokines (*e.g.*, IL-10). It is unknown whether the regulatory effect of SP-D depends on the phenotype of secretory activity of macrophages.

These two questions should be answered to understand the role of SP-D in the regulation of NO synthesis. We compared the dynamics of LPS-induced iNOS expression and NO production in various phenotypes of peritoneal macrophages from SP-D(-/-) mice and wild-type animals.

MATERIALS AND METHODS

SP-D(-/-) mice were bred from C57Bl/6 mice as described by Botas [4]. C57Bl/6 mice of comparable age served as the control (8-10 weeks). The animals were maintained in a special vivarium, which avoided the exposure to pathogenic microorganisms. Our study was performed in accordance with the protocol of the Animal Care Committee (University of Pennsylvania, USA). Peritoneal macrophages were isolated from peritoneal lavage of mice, which received an intraperitoneal injection of thioglycollate broth (4%, 2 ml) 4 days before the start of experiments. The peritoneal fluid was centrifuged at 400*g* and 4°C for 10 min. The pellet was resuspended in 1 ml phosphate buffered saline with Ca^{2+} and Mg^{2+}. The cells were counted using a Z1 Particle Counter (Beckman-Coulter). To study the shape and size of cells, the cell suspension was centrifuged in a Thermo Shandon Cytospin-3 centrifuge at 750 rpm and room temperature for 3 min, stained with a standard dye Diff Kwik (Thermo Shandon), and examined under a microscope.

Peritoneal macrophages from control animals and SP-D(-/-) mice were cultured in RPMI-1640 medium with 10% serum, 100 U/ml penicillin, and 100 mg/ml streptomycin in 48-well plates (5×10^5 cells per well). Proinflammatory and antiinflammatory phenotypes of macrophages were obtained as descried elsewhere [15]. The primary culture of native peritoneal macrophages was divided into three pools. LPS in a dose of 0.5 ng/ml was added to pool 1 to obtain a proinflammatory phenotype. LPS in a dose of 5.0 ng/ml was added to pool 2 to obtain an antiinflammatory phenotype. LPS was not added to pool 3 (native phenotype, control). After addition of LPS, macrophages were maintained in an incubator at 5% CO_2 and 37°C for 6 h. The medium and cells were sampled for a further analysis (0 h). LPS in a dose 500 ng/ml was added to all wells to activate macrophages. Incubation was performed for 24 and 72 h under similar conditions. The medium and cells were analyzed. Experiments were performed with *Escherichia coli* O111:B4 LPS (List Biologic Laboratories, Campbell).

Peritoneal macrophages of the proinflammatory phenotype produced proinflammatory cytokines TNF-α and IL-12 (4695 and 20.2 pmol/ml, respectively) and antiinflammatory cytokines IL-10 and IL-13 (99.4 and 60.1 pmol/ml, respectively) in response to LPS stimulation (500 ng/ml). Peritoneal macrophages of the antiinflammatory phenotype produced proinflammatory cytokines TNF-α and IL-12 (3117 and 1.8 pmol/ml, respectively) and antiinflammatory cytokines IL-10 and IL-13 (509.7 and 103.9 pmol/ml, respectively) in response to LPS stimulation (500 ng/ml). Hence, this treatment [15] was followed by alternative transformation of the phenotype of secretory activity of peritoneal macrophages into proinflammatory and antiinflammatory phenotypes.

Quantitative analysis of iNOS expression was performed by the method of reverse transcription (RT) and polymerase chain reaction (PCR) and TaqManR Gene Expression Assays (Applied Biosystems). The measurements were performed on an

ABI 7500 FAST Real Time PCR device. Total RNA was isolated from macrophages by the standard method with thiazole. The RT reaction of total RNA was conducted with RETROscriptR First Strand Synthesis Kit for RT-PRC (Applied Biosystems) and set of primers. The data were normalized by primer 18S. NO production was evaluated from the concentration of nitric (end metabolite of NO). The measurements were performed on an Ionics/Sievers Nitric Oxide Analyzer 280 (Ionics Instruments, Boulder) as described elsewhere [2].

The results were analyzed by Student's *t* test. Inter-group differences were significant at $p<0.05$.

RESULTS

SP-D gene knockout in mice is followed by a significant increase in the number and size of alveolar macrophages [13]. As differentiated from alveolar macrophages, the morphology of peritoneal macrophages remained unchanged in the absence of SP-D (Fig. 1). Basal iNOS expression and NO production were not observed in peritoneal macrophages from SP-D(-/-) mice (time 0, 0 ng/ml LPS; Figs. 2 and 3). However, alveolar macrophages from SP-D(-/-) mice are characterized by greater activation of NO synthesis as compared to the control (SP-D(+/+) mice) [2]. The absence of morphological changes in peritoneal macrophages from SP-D(-/-) mice are probably related to the absence of iNOS expression and NO production in cells.

The LPS-induced transformation of secretory activity of peritoneal macrophages into the proinflammatory phenotype (0.5 ng/ml LPS, 6 h) was followed by an increase in iNOS transcription (iNOS response) from 0 to 2.3±0.3 iNOS/18S. The concentration of $NO_2^{\bullet-}$ remained practically unchanged under these conditions (Figs. 2 and 3). The LPS-induced transformation into an antiinflammatory phenotype (5 ng/ml LPS, 6 h) was followed by a significant increase in iNOS transcription (from 0 to 52.7±5.2 iNOS/18S) and $NO_2^{\bullet-}$ concentration (from 0.1 to 1.3 mM). iNOS transcription and $NO_2^{\bullet-}$ concentration in native macrophages remained practically unchanged after 6-h culturing (0 ng/ml LPS; Figs. 2 and 3). A correlation was found between the increase in iNOS expression and NO production after treatment with LPS in concentrations of 0-5.0 ng/ml.

Activation of macrophages with 500 ng/ml LPS was followed by the iNOS response in all phenotypes of macrophages (Fig. 2). However, the dynamics of this response differed in various phenotypes. iNOS transcription in native macrophages increased 24 h after treatment with LPS in a dose of 500 ng/ml (from 0 to 125.1±8.5 iNOS/18S), but decreased twofold by the 72nd hour. Therefore, the LPS-induced iNOS response in native cells was described by a dome-shaped curve. iNOS transcription in proinflammatory and antiinflammatory phenotypes increased 24 h after treatment with LPS (similarly to native cells; Fig. 2). During this period, various phenotypes of macrophages did not differ in the transcription of iNOS. As differentiated from native macrophages, iNOS transcription in proinflammatory and antiinflammatory phenotypes did not decrease 72 h after treatment with LPS in a dose of 500 ng/ml. The LPS-induced iNOS respon-

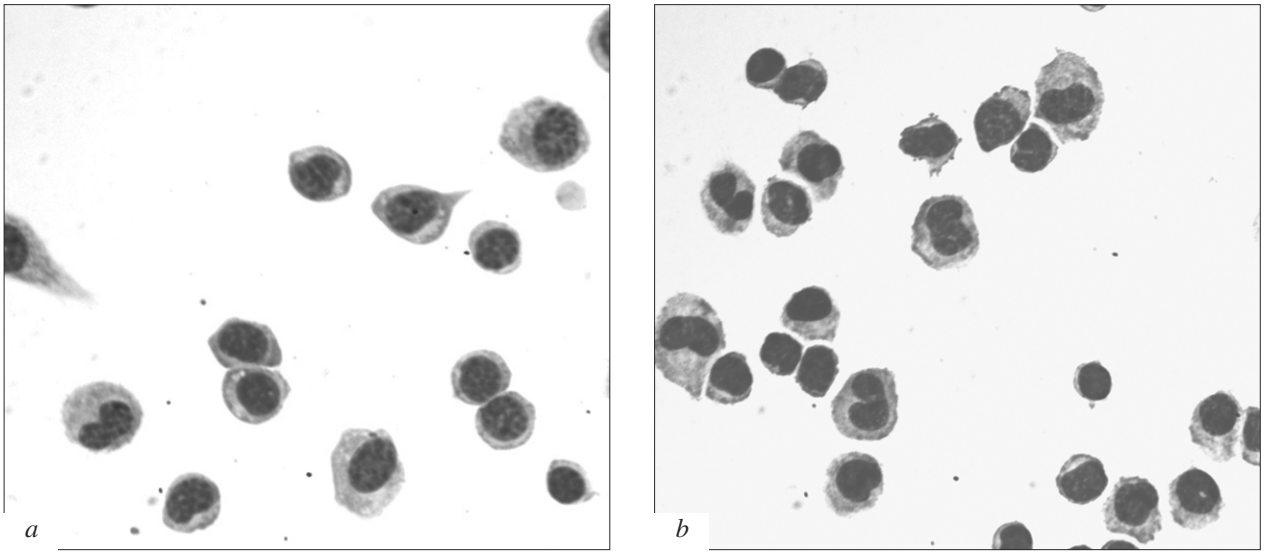

Fig. 1. Peritoneal macrophages from control mice (*a*) and SP-D(-/-) mice (*b*). Representative image for each group, which consisted of 5 specimens.

se in proinflammatory and antiinflammatory phenotypes increased progressively and reached a plateau after 24 h. By the 72nd hour, iNOS transcription in both phenotypes was much higher than in native cells (Fig. 2).

Therefore, the iNOS response of proinflammatory and antiinflammatory macrophages from control mice to LPS stimulation was more prolonged than that of native cells.

Under the influence of LPS in high doses, NO production depended strongly on the phenotype of macrophages. After 24-h stimulation with LPS, $NO_2^{\bullet-}$ concentration in the culture medium of native, proinflammatory, and antiinflammatory macrophages was 20.90±1.04, 11.10±0.48, and 26.40± 0.99 mM, respectively. Significant differences in the concentration of $NO_2^{\bullet-}$ in various phenotypes persisted after 72 h (Fig. 3). It should be emphasized that phenotypic differences in the LPS-induced production of NO did not correspond to differences in the expression of iNOS in macrophages of various phenotypes (Figs. 2 and 3).

The following results were obtained in experiments with peritoneal macrophages from SP-D(-/-) mice. The absence of SP-D had no effect on the "LPS dose—iNOS transcription" curve and "LPS dose—$NO_2^{\bullet-}$ production" curve at low concentrations of LPS (0-5.0 ng/ml; Figs. 2 and 3). However, the response of peritoneal macrophages from SP-D(-/-) mice to high concentrations of LPS (500 ng/ml) differed significantly from that of cells from control animals. First, the LPS-induced iNOS response of native macrophages from SP-D(-/-) mice was described by a dome-shaped curve (similarly to cells from control animals). However, iNOS transcription in macrophages from SP-D(-/-) mice was much lower than in the control. After stimulation with LPS for 24 and 72 h, iNOS transcription in native macrophages from SP-D(-/-) mice was lower than in cells from control animals (by 1.63 and 1.43 times, respectively; Fig. 2). Second, the LPS-induced iNOS response of proinflammatory macrophages from SP-D(-/-) mice increased progressively, but did not reach a plateau after 24-72 h (as distinct from cells from control animals). At the same time, iNOS transcription in proinflammatory macrophages from SP-D(-/-) mice was much lower than in cells from control animals after 24 and 72 h (Fig. 2). And third, high concentrations of LPS did not induce further increase in iNOS transcription in antiinflammatory macrophages from SP-D(-/-) mice (as distinct from cells from control animals; Fig. 2). By the 72nd hour of LPS stimulation, iNOS transcription in antiinflammatory macrophages was twofold lower than in proinflammatory macrophages from SP-D(-/-) mice. These data indicate that the iNOS response of peritoneal macrophages from SP-D(-/-) mice depends strongly on the phenotype of cells. The absence of SP-D gene is accompanied by a decrease in iNOS gene transcription in peritoneal macrophages after treatment with high doses of LPS, but has no effect on gene transcription upon stimulation with LPS in low doses.

After 24 h stimulation with LPS, differences in $NO_2^{\bullet-}$ concentration in native, antiinflammatory, and proinflammatory macrophages from SP-D(-/-) mice were similar to those in cells from control animals (Fig. 3). The differences were observed after 72 h of stimulation with LPS in a dose of 500

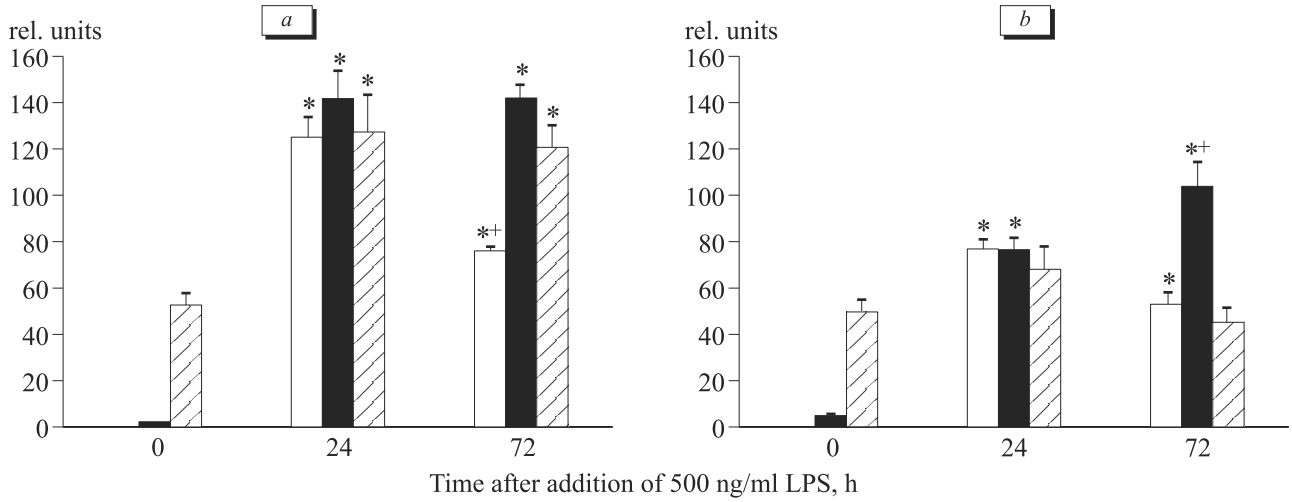

Fig. 2. LPS-induced expression of iNOS. Here and in Fig. 3: each group consists of 3 specimens. (*a*) Control mice; (*b*) SP-D(-/-) mice. Light bars, 0 ng/ml; dark bars, 0.5 ng/ml; shaded bars, 5 ng/ml. $p<0.05$: *compared to 0 h; +compared to 24 h.

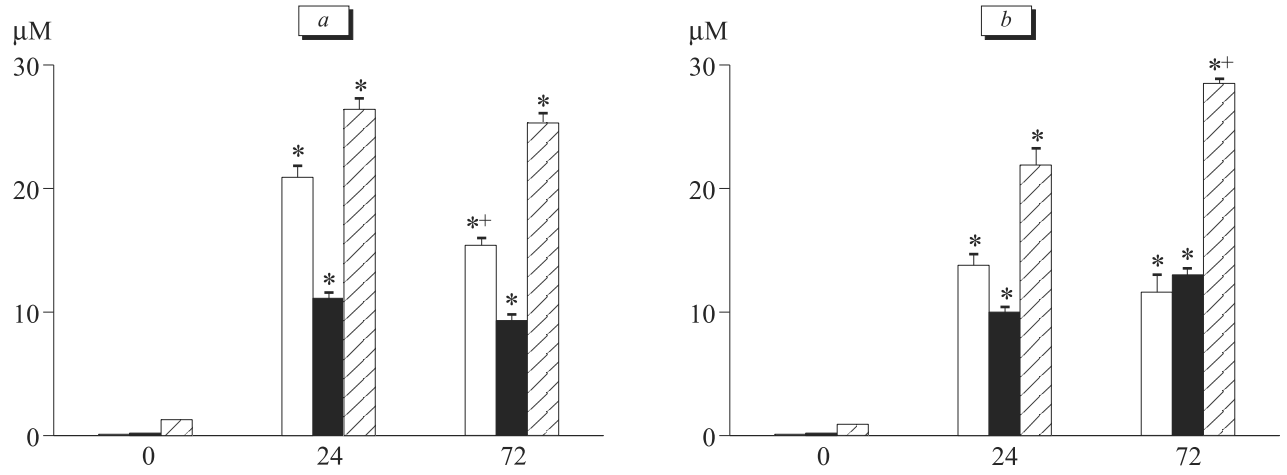

Fig. 3. LPS-induced production of nitrites.

ng/ml. $NO_2^{\bullet-}$ production was reduced in native macrophages, but increased in proinflammatory cells from SP-D(-/-) mice. Hence, significant differences in NO production by native and proinflammatory macrophages were not observed in SP-D(-/-) mice (as distinct from SP-D(+/+) mice).

The absence of the SP-D gene had an adverse effect on the transformation of the native phenotype into the proinflammatory phenotype, but did not modulate the transformation of the native phenotype into the antiinflammatory phenotype (as confirmed by NO production).

Little is known about the SP-D-dependent regulation of macrophage response. Particular attention was paid to the effect of SP-D on iNOS and NO production.

As differentiated from alveolar macrophages of SP-D(-/-) mice, the basal expression of iNOS and production of NO are not elevated in peritoneal macrophages of these animals [2]. Previous studies showed that basal activity of NF-κB is high in alveolar macrophages from SP-D(-/-) mice [14]. NF-κB serves as a transcription factor for iNOS and various cytokines. These data suggest that SP-D decreases basal activity of iNOS in alveolar macrophages and reduces the severity of lung inflammation due to inhibition of NF-κB. It remains unclear why this effect of SP-D is not observed in peritoneal macrophages. We conclude that SP-D has an organ-specific role in the regulation of basal iNOS expression and NO production.

Peritoneal macrophages from SP-D(-/-) mice differed from macrophages of wild-type animals by lower activation of the iNOS response under the influence of LPS in high doses. SP-D is probably involved in the regulation of NO production and immune response not only in the lungs, but also in the whole organism. Knockout of the SP-D gene had no effect on iNOS expression and NO production by peritoneal macrophages after treatment with LPS at low doses. In the absence of SP-D, addition of LPS at high doses was followed by a decrease in the iNOS response, suppression of NO production in native macrophages, and increase in NO production by the proinflammatory phenotype of macrophages (72-h stimulation).

We showed that the LPS-induced iNOS response is reduced in all phenotypes of peritoneal macrophages from SP-D(-/-) mice. However, the type and dynamics of the inhibitory effect depended strongly on the phenotype of macrophages. For example, the iNOS response of native and proinflammatory macrophages decreased only by 30% in the absence of SP-D (72-h period). However, the iNOS response in macrophages of the antiinflammatory phenotype was reduced by 63% under these conditions. The effect of SP-D on NO production also depended on the phenotype of macrophages. After 72-h stimulation with LPS, $NO_2^{\bullet-}$ concentration was reduced in native macrophages (by 25%), but increased in cells of proinflammatory (by 40%) and antiinflammatory phenotypes (only by 12%). Therefore, SP-D has a phenotype-dependent effect on the iNOS response and NO production. Further investigations should be performed to evaluate the mechanisms mediating the dependence of SP-D-induced changes on the phenotype of macrophages.

Our study provides additional information on the role of SP-D in the regulation of macrophage function. SP-D is probably involved in the immune response not only in the lungs, but also in the whole organism. Regulatory activity of SP-D depends on the phenotype of macrophages.

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