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Role of mitogen-activated protein kinases in the iNOS production and cytokine secretion by *Salmonella enterica* serovar Typhimurium porins

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Abstract

The expression of inducible nitric oxide synthase (iNOS) is a critical factor in both physiological and pathological functions. The present study examined the role of mitogen-activated protein kinases (MAPKs) in the regulation of iNOS and proinflammatory cytokine production in RAW 264.7 cells in response to *Salmonella enterica* serovar Typhimurium porins. By use of Western blotting for iNOS detection and enzyme-linked immunosorbent assay (ELISA) for quantization of cytokine secretion, selective pharmacological inhibitors of MAPK pathways were tested for dissecting the molecular mechanisms underlying the mediation of these signaling in porins-stimulated murine macrophages. *S. enterica* serovar Typhimurium porins activated iNOS expression, NO production and interleukin (IL)-6, IL-8 and tumor necrosis factor-α (TNF-α) release. Treatment of cells with SB203580 and SP600125 (inhibitors of p38 and JNK, respectively) significantly affected porin-stimulated iNOS and NO production. Concomitant decrease in the proinflammatory cytokine secretion was detected. These data confirm the importance of the MAPKs cascade in macrophage activation by bacterial product opening up new strategies for therapy of septic shock.

Keywords: Porins; MAPKs; Nitric oxide; iNOS; Cytokine

1. Introduction

Nitric oxide (NO)[1] is a small, relatively stable gas that acts as a signaling molecule in various physiological and pathophysiological processes, including neurotransmission, modulation of vascular tone, and both acute and chronic inflammation [1]. During bacterial infection, NO is generated principally by monocytes, macrophages and other cells of the immune system by inducible NO synthase (iNOS) and serves as a cytostatic/cytotoxic agent against several intracellular bacteria [2]. Murine macrophages provide the best studied example of regulation of NO production [3]. NO in murine macrophages can be induced by various stimuli, which include bacterial products or inflammatory cytokines such as lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) or interferon-γ (IFN-γ) [4,5].

Despite the beneficial roles of NO in host defence against viral replication and other factors [3], overproduc-

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[1] *Abbreviations used:* iNOS, inducible nitric oxide synthase; NO, nitric oxide; MAPKs, mitogen-activated protein kinases; ERK, extracellular-signal-regulated kinase; NF-kB, nuclear factor kB; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; LPS, lipopolysaccharide; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; PVDF, poly(vinylidene difluoride); HRP, horseradish peroxidase-linked; ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate buffer saline; PB, polymyxin B; LAL, limulus amebocyte lysate.

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tion of NO and its metabolites can be harmful to the host and has been implicated in the pathogenesis of conditions such as bacterial sepsis and chronic inflammation [6,7]. While iNOS expression is regulated by transcriptional, and post-translational mechanisms, the specific signal transduction systems that mediate the induction of iNOS production in different cell types and the mechanisms through which they regulate iNOS expression are still incompletely understood.

Several authors have reported that activation of MAPKs is significant in the regulation of NO production via controlling the activation of nuclear factor(NF)-κB [8]. In particular, LPS signaling pathways that regulate iNOS have been intensively studied in various cell types. LPS-induced NO production is mediated by a series of signal transduction pathways, such as NF-κB and MAPK including ERK1/2, p38 and SAPK/JNK [9].

In a previous study we have also demonstrated that gram-negative porins are able to modulate inflammatory and immunological responses by affecting the release of NO and the expression of iNOS gene in activated murine macrophages [10,11].

However, the signal transduction pathways that trigger the porin-induced iNOS expression, subsequent NO production and cytokine synthesis in macrophages (or any cell) have not yet been identified. We hypothesized that MAPK cascades are key component of the signaling process in macrophages stimulated with *S. enterica* serovar Typhimurium porins. In this paper, we have addressed this question by studying the effect of selective inhibitors of these MAPK cascades to examine the potential contribution of each pathway in the mediation of porin-induced macrophage accumulation of iNOS protein, NO production and/or secretion of proinflammatory cytokine.

2. Materials and methods

2.1. Materials

Dulbecco's MEM, L-glutamine, penicillin and streptomycin were purchased from Gibco Life Technologies (Grand Island, NY). Fetal calf serum (FCS) containing <0.01 endotoxin U/ml was obtained from HyClone Laboratories (Logan, UT). The poly(vinylidene difluoride) (PVDF) membranes, the conjugated anti-mouse IgG-horseradish peroxidase-linked (HRP), the Enhanced Chemi-Luminescence (ECL) Western-blotting detection kit were purchased from Amersham (Amersham Pharmacia Biotech, UK). The murine monoclonal antibody to macrophage iNOS was obtained from Transduction Laboratories (Lexington, KY). Specific antibodies for the analysis of the total kinases ERK, p38 and JNK were purchased from Santa Cruz (Santa Cruz Biotechnology, Inc.) while for the analysis of the phosphorylated forms were purchased from Transduction Laboratories. Nutrient broth was obtained from Difco (Detroit, MI). Polymyxin-B (PB) and all of the analytical grade reagents were purchased from Sigma (Milano, Italy). Cytokine enzyme-linked immunosorbent assay kit and a cytotoxicity detection kit were obtained from R&D Systems (Minneapolis, MN, USA). *Limulus* amoebocyte lysate (LAL) assay kit was purchased from pbi international (pbi international, Milan Italy). The p38 inhibitor SB203580 (4-(4-fluorophenyl)-5-(4-pyridyl)1H-imidazole), JNK inhibitor SP600125 (Anthra[1,9-cd]pyrazol-6(2H)-one1,9-pyrazoloanthrone) and iNOS inhibitor L-NMMA (N^G-Monomethyl-L-Arginine) were purchased from Calbiochem (La Jolla, CA); the MEK1, 2/ERK inhibitor PD098059 (2′-amino-3′-methoxyflavone) was purchased from New England Biolabs (Beverly, MA). Each inhibitor was reconstituted in dimethyl sulfoxide at stock concentrations of 10 mM, and added into cultures to provide indicated concentrations. Inhibitor concentration used were based on those used in various reports in the literature [12–15] and from preliminary experiments that were effective in preventing porin or LPS-induced NO production in these cells. These inhibitors caused an appreciable decrease in cell viability at higher concentrations and therefore we employed only at the concentrations indicated.

2.2. Cells and cell culture

RAW 264.7 cells, a murine macrophage cell line, were obtained from American Type Culture Collection (Rockville, MD) and were cultured at 37 °C in a moist atmosphere of 5% CO_2 in Dulbecco's MEM supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin. In some experiments, cells (3×10^6 cells/ml) were exposed to SB203580 (10 μM) or PD098059 (30 μM) or SP600125 (10 μM) or L-NMMA (10 μM) 1 h prior to stimulation with porin or LPS and then incubated for additional 6–18 h before collection of supernatants and/or cell lysates.

2.3. Bacterial strain

The bacterial strain used was: *Salmonella enterica* serovar Typhimurium strain SH5014 grown in Nutrient broth for 18–24 h at 37 °C under agitation. The cells were harvested at the end of the exponential growth phase, and outer membranes were prepared from cell envelopes following protocols described by Nurminen et al. [16].

2.4. Preparation of porins and LPS

Salmonella enterica serovar Typhimurium was used to extract and purify porins. Porins were isolated from the lysozyme–EDTA envelopes as described by Nurminen [17]. Briefly, 1 g of envelopes was suspended in 2% Triton X-100 in 0.01 Tris–HCl (pH 7.5, containing 10 mM EDTA); after the addition of trypsin (10 mg/g of envelopes), the pellet was dissolved in sodium dodecyl sulfate buffer (SDS buffer, 4% [wt/vol] in 0.1 M sodium phosphate [pH 7.2]), and applied to an Ultragel ACA 34 column

equilibrated with 0.25% SDS buffer. The fraction containing proteins, identified by absorption at 280 nm (A_{280}), was extensively dialyzed and checked by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) according to Laemmli [18]. The protein content of the porin preparation was determined by the method of Lowry et al. [19]. LPS contamination, revealed on SDS–PAGE gels stained with silver nitrate as described by Tsai and Frasch [20], was determined by the LAL assay [21].

In addition, PB was incubated with porins to neutralize the biological activity of possible traces of LPS. The porins were incubated with PB at room temperature for 1 h in a ratio of 1:100 [22]. LPS, porins and PB mixture were used in pyrogen-free phosphate-buffered saline (PBS).

LPS-R was isolated from *S. enterica* serovar Typhimurium with phenol/chloroform/ether as described by Galanos et al. [23]. Briefly, liquid phenol (90 g of dry phenol plus 11 ml of water–chloroform–petroleum ether in a volume ratio of 2:5:8) was added to 1 g of dried bacteria. After 2 min homogenisation, bacteria were centrifuged and extracted twice. The supernatants was filtered through filter paper and treated as described by Galanos et al. [23].

2.5. Determination of NO concentration

RAW 264.7 cells were plated at 2×10^5 cells/well in 96-well plates. NO production in the culture supernatant was evaluated by measuring nitrite, its stable degradation product, using a colorimetric assay as described previously [10]. The DMEM first was changed to phenol red-free medium, and then the cells were stimulated with porins (5 μg/ml) or LPS (1 μg/ml) in the absence or presence of different inhibitors for 18 h. The isolated supernatant was centrifuged and mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, 5% phosphoric acid) and incubated at room temperature for 10 min. The optical density at 550 nm (OD_{550}) was measured using a microplate reader, and the nitrite concentration was calculated by comparison with the OD_{550} produced using standard solutions of sodium nitrite in the culture medium.

2.6. Preparation of cell extracts and Western blot analysis of iNOS and MAPK

Cell lysates were immunoprecipitated and used for Western blotting following standard procedures as previously reported [12]. Protein concentrations were determined for each sample by use of a standard Bio-Rad/Bradford protein assay. Briefly, lysate sample containing 50 μg of protein were separated on SDS 15% PAGE gels as described by Laemmli [18], transferred to PVDF membrane (sheets 0.45 μm), and blocked with 5% milk in Tris-buffered saline. PVDF membranes were reacted at room temperature for 2 h with a murine monoclonal antibody to iNOS at a dilution of 1:800 or with anti-phosphorylated kinase antibodies diluted 1:2000. The membranes were stripped and re-probed with antibodies against β-actin or non-phosphorylated MAPKs to demonstrate that equivalent amounts of analyzed kinases were present in each sample. Blots were then incubated with anti-mouse IgG-HRP secondary antibody at a dilution of 1:3000 for 2 h, and proteins were detected by ECL.

2.7. Measurement of cytokine concentrations

All assays were carried out using RAW 264.7 cells (3×10^6 cells/ml) stimulated with different concentrations of stimuli for 18 h at 37 °C in 5% CO_2. After incubation, samples were centrifuged at 1800 rpm at 4 °C for 10 min and the supernatants were collected and stored at −70 °C. IL-6, IL-8 and TNF-α protein concentration was measured with enzyme-linked immunosorbent assay (ELISA) as described in the manufacturer's instructions. Standard and sample dilutions were performed at least four times for each individual cell-stimulation assay.

2.8. Lactate dehydrogenase (LDH) assay

LDH assay was carried out according to manufacturer's instructions by using a cytotoxicity detection kit. LDH is a stable cytoplasmic enzyme present in all cells and is rapidly released into cell culture supernatant when the plasma membrane is damaged. LDH activity was determined by a coupled enzymatic reaction whereby the tetrazolium salt was reduced to formazan. An increase in the number of dead or damaged cells resulted in an increase in LDH activity in the culture supernatant. The amount of LDH show that treated and untreated cells are healthy.

2.8.1. Reproducibility

Gels were scanned for densitometry analysis by Sigma Gel software and the results shown are an average of triplicate experiments. The results were expressed as mean values ± standard errors of three independent observations. Statistically significant values were compared using Student's *t*-test and $p \leqslant 0.01$ were considered statistically significant.

3. Results

3.1. Purity of porin preparation

The purification protocols, and methods used to discount the likely contamination by LPS in porin preparation, have been extensively addressed in previous works [24,25]. The purity of the porin preparation and the LPS pattern from *S. enterica* serovar Typhimurium SH5014 were checked by SDS–PAGE (Fig. 1).

By using the LAL test, the LPS contamination in the porin preparation was estimated to be <0.005% (wt/wt) compared with a standard *S. enterica* serovar Typhimurium LPS solution. These traces of LPS did not show any biological activity under our experimental conditions (data

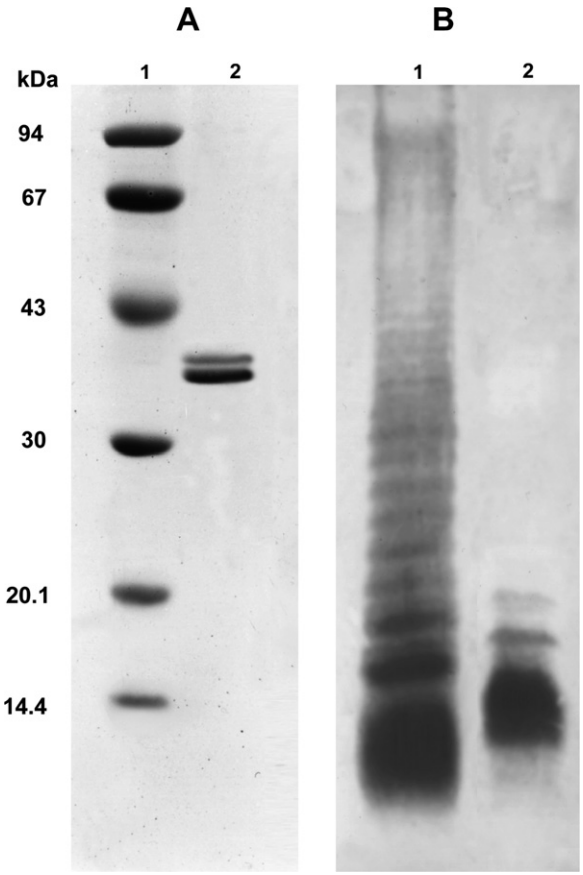

Fig. 1. SDS–PAGE of *Salmonella enterica* serovar Typhimurium porins and LPS. (A) Lane 1, molecular weight standards (Amersham Pharmacia Biotech, Italy) (phosphorylase b, 94,000 Da; albumin, 67,000 Da; ovalbumin, 43,000 Da; carbonic anhydrase, 30,000 Da; trypsin inhibitor, 20,100 Da; α-lactalbumin, 14,400 Da; lane 2, *S. enterica* serovar Typhimurium porins (10 μg). (B) Lane 1, *S. enterica* serovar Typhimurium LPS standard (Sigma–Aldrich S.r.l., Italy); lane 2, *S. enterica* serovar Typhimurium LPS (10 μg).

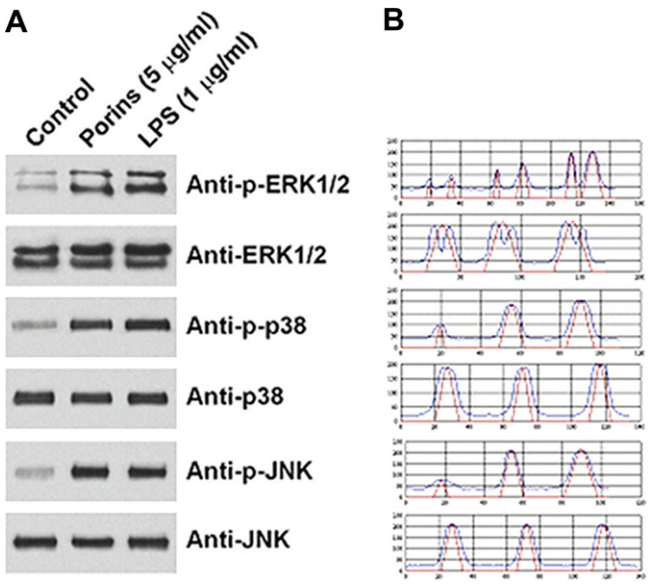

Fig. 2. Phosphorylation level of MAPKs in *S. enterica* serovar Typhimurium porins or LPS-induced RAW 264.7 macrophages. Cells were stimulated with porins or LPS for 20 min. The phosphorylation of MAPKs was detected by Western blot analysis using antibodies against the corresponding phosphorylated or non-phosphorylated forms of these enzymes (A). Band intensity of Western blot signals was quantized densitometrically by Sigma Gel Software (B). In the *x* and *y* axis were indicated the coordinates (arbitrary units) of integration of peak analysis algorithm.

not shown). The biological activities shown by porins in our experiments are not attributable to eventual traces of LPS that could contaminate the porin preparations. The concentration of porins used contains a biologically insufficient percentage of LPS (about 50 pg/μg of porins), which did not induce any enzyme phosphorylation or cytokine release (data not shown). Moreover, when using porins plus PB, in a concentration range that is non-toxic for cells, we did not observe any modification of the tested biological activities (data not shown).

3.2. MAPK pathway implication in iNOS and NO production triggered by porins

Several studies have demonstrated that activation of MAPK is critical in the regulation of NO in LPS-stimulated RAW 264.7 cells [26,27]. As reported in Fig. 2, porins are capable of activating ERK1/2, p38 and JNK pathways in RAW 264.7 cells, although with different intensity.

To explore the signaling pathway mostly involved in porin-mediated iNOS and NO production, we treated RAW 264.7 cells with specific MAPK inhibitors and assayed iNOS and NO generation by Western blot and Griess reaction, respectively. Stimulation of RAW 264.7 cells by porins or LPS can produce iNOS and NO with excellent reproducibility (Figs. 3 and 4). We previously reported that murine macrophages exposed to *S. enterica* serovar Typhimurium porins produced NO in a time and concentration-dependent manner [10]. In these experiments, cells were preincubated with each inhibitor 1 h before exposure to porins (5 μg/ml) or LPS (1 μg/ml) for 18 h.

Consistent with previous results, *S. enterica* serovar Typhimurium porins induced the appearance of iNOS activity as judged by the appearance of nitrite in the medium as well as the iNOS protein itself. The porin-induced synthesis of iNOS protein (Fig. 3) or activity (Fig. 4) was affected by specific inhibitors. As shown in the representative experiment, pretreatment with p38 inhibitor SB203580 and JNK inhibitor SP600125 significantly affected porin-stimulated iNOS (about 63% and 26%) (Fig. 3) and NO (about 69% and 57%) (Fig. 4) production while pretreatment of PD098059 (about 76% for NO and 8% for iNOS) exhibited a little inhibition effect.

The combined treatment resulted in the almost complete reduction of iNOS and NO production (data not shown). Moreover, RAW 264.7 cells were also pretreated with iNOS inhibitor L-NMMA (Fig. 3); nitrite production at 18 h induced by porins or LPS was suppressed by

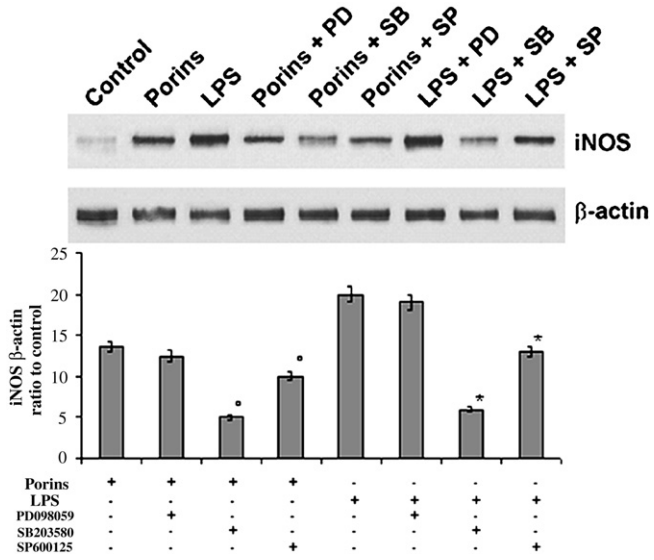

Fig. 3. Effect of different inhibitors on *S. enterica* serovar Typhimurium porins or LPS-induced synthesis of iNOS in RAW 264.7 macrophages. RAW 264.7 cells (3×10^6/ml) were incubated with PD-098059 (PD) (30 μM for 1 h, MEK1 inhibitor) or SB203580 (SB) (10 μM for 1 h, p38 inhibitor) or SP600125 (SP) (10 μM for 1 h, JNK inhibitor) and stimulated with porins (5 μg/ml) or LPS (1 μg/ml) for 18 h. At the end of incubation, Western blot analysis on total cell extracts was performed using anti-iNOS antibody. β-actin protein was used here as an internal control. Western blot signals were quantitated by Sigma Gel Software and reported as the ratio of the value for each stimulation time to the value for a non-stimulated control. The results shown are the average of three independent experiments, and the error bars indicate the standard error of the means. Band intensity was quantified densitometrically. $^{*}p \leqslant 0.01$ indicates statistically significant difference versus porins ($^{\circ}$) or LPS (*) stimulated cells (Student's *t*-test).

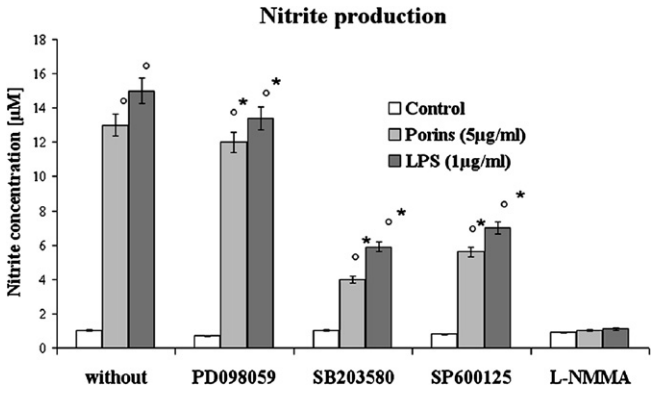

Fig. 4. NO production in *S. enterica* serovar Typhimurium porins or LPS-induced RAW 264.7 cells. Macrophages were incubated for 1 h in the presence or absence of PD-098059 (PD) (30 μM), SB203580 (SB) (10 μM), SP600125 (SP) (10 μM), L-NMMA (10 μM), then stimulated for 18 h in the absence or presence of porins (5 μg/ml) or LPS (1 μg/ml). Nitrite accumulation in the medium was measured by the Griess reaction described in Section 2. The results shown are the average of three independent experiments, and the error bars indicate the standard error of the means. Symbol denoting significance: $p \leqslant 0.01$ versus non-stimulated ($^{\circ}$) or inhibitor pretreated cells ($*$) (Student's *t*-test).

L-NMMA, indicating that iNOS is responsible for the NO production induced by these bacterial surface components.

These data reflected that the MAPKs (JNK and p38) pathway may contribute to the NO generation in response to bacterial porins. The inhibitor concentration used as well as the duration of the treatment did not indicate significant cytotoxicity in these cultures as determined by LDH assay (data not shown).

3.3. MAPK pathway activation is a key event in proinflammatory cytokines production

MAPK are a group of signaling molecules that appear to play important roles in inflammatory processes. Previous results have indicated that the IL-8 response observed in THP-1 cells with *S. enterica* serovar Typhimurium porins requires the independent activation of several pathways, including PTK, MEK/ERK and p38 [13]. Presently, we studied the effects of three well-characterized MAPK inhibitors on the ability of activated macrophages to produce proinflammatory cytokine such as IL-6, IL-8 and TNF-α (Fig. 5). Stimulation with porins increased production of all three cytokine. The pretreatment of RAW 264.7 with the MEK/ERK inhibitor PD098059 strongly decreased cytokine release after LPS stimulation but was less efficient after porin stimulation. As shown in Fig. 4, SB203580 and SP600125 (inhibitors of p38 and JNK, respectively) significantly reduced cytokine production in response to porins. However, pretreatment of cells with SB203580 or SP600125 also attenuated LPS-induced cytokine release. IL-6, IL-8 and TNF-α secretion mediated by porins or LPS were slightly modified by treatment of RAW 264.7 cells with selective iNOS inhibitor, L-NMMA (10 μM for 60 min) since cytokines release would be affected secondarily by other cytokine and NO.

4. Discussion

In the present investigation, our goal was to extend the function of MAPKs to the regulation of iNOS and NO production in porin-activated macrophages.

A greater understanding of the signaling pathways involved in the regulation of iNOS by porins may have potential therapeutic implications in a number of disorders in which gram-negative bacterial components and NO play a crucial role.

To probe the role of each MAPK in the initiation of iNOS and NO expression, we utilize specific pharmacologic antagonists that inhibits the phosphorylation of ERK1/2, p38 and JNK.

Although all chemical inhibitors of enzymatic cascades likely cause some non-specific cellular effects, we used the inhibitors that are highly selective for MAPK cascades. The specificity of these inhibitors has been demonstrated in monocytes and macrophages, including RAW264.7 cells [27–29].

Porin-stimulated iNOS and NO generation into RAW 264.7 macrophages is prevented by SB203580 and SP600125, and at concentrations where they are thought

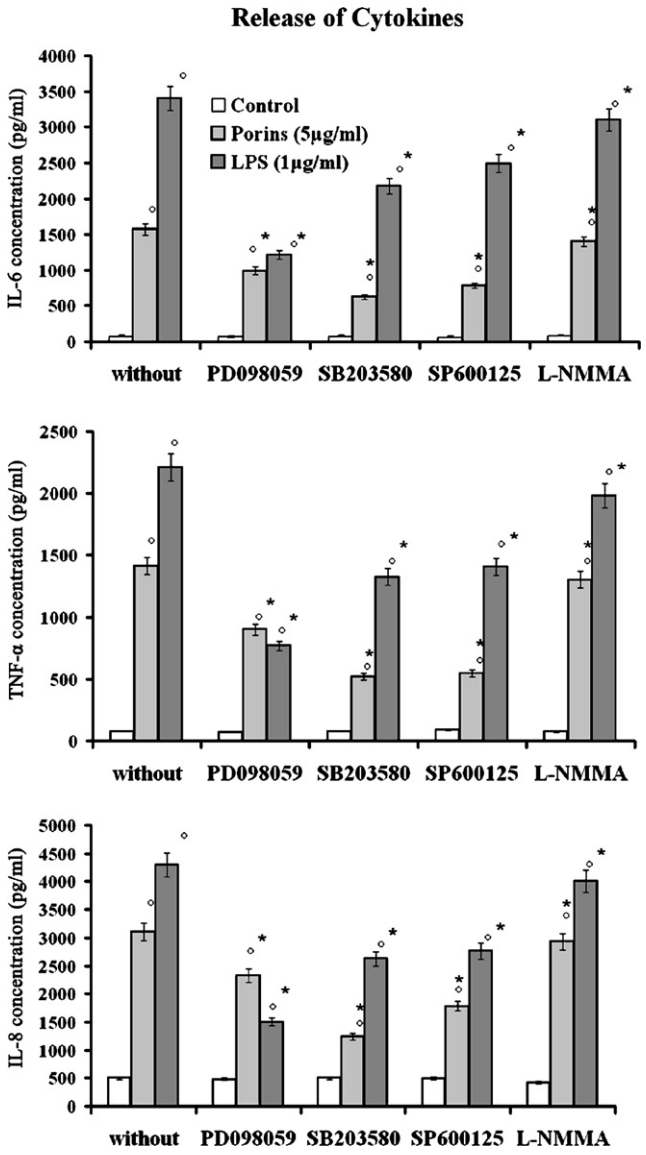

Fig. 5. Cytokines secretion induced by *S. enterica* serovar Typhimurium porin or LPS stimulation after inhibitors pretreatment. IL-6, TNF-α and IL-8 levels in RAW 264.7 cells were measured by capture ELISA. The results are means of three different experiments and error bars indicate the standard errors of the mean. Symbol denoting significance: $p \leqslant 0.01$ versus non-stimulated (°) or inhibitor pretreated cells (*) (Student's *t*-test).

to be specific inhibitors of the activation of p38 and JNK respectively. In our experiments, we used immunoblotting with phosphospecific antibodies that recognize activated forms of the analyzed kinases to confirm that p38 and JNK pathway inhibitor significantly affected the porin-mediated phosphorylation of p38 and JNK kinases but had no effect on ERK phosphorylation. Moreover, the MEK 1,2/ERK inhibitors showed a little inhibition effect while having no effect on p38 and JNK kinase phosphorylation in response to porins. We also demonstrated an inhibitory effect of SB203580, PD98059 and SP600125 on porin-induced proinflammatory cytokine release. Taken together, these results indicate that both p38 and JNK pathways play important roles in the up-regulation of iNOS during macrophage activation by *S. enterica* serovar Typhimurium porins.

It has been previously reported that inhibition of MAPK blocks endotoxin-induced NO production in macrophages [27,30]. Chen and Wang [31] showed that SB203580 but not PD98059 mediated NO synthase by LPS RAW 264.7 stimulation. Others have reported that SB203580 but not PD98059 inhibited gram-positive bacteria component-activated iNOS and NO production in macrophages [28]. These evidences suggested that MAPK pathways are important on macrophage activation by bacterial products but they differentially regulate cell responses.

Recently, the use of specific inhibitors of MAPKs for the treatment of inflammatory disorders due to the inhibitory effect on iNOS and NO production has been suggested [27]. It may be a useful strategy in view of overexpressed NO regulation. Since several observations regarding the cell type and the differential effects of inhibitors on NO production have been shown, the therapeutic use of these compounds should be further evaluated. Nevertheless, the process of iNOS induction remains a promising target for sepsis therapy.

Our data constitute the first direct evidence that a surface component of gram-negative bacteria such as *S. enterica* serovar Typhimurium porins is able to trigger NO release, iNOS and cytokine production through a mechanism involving p38 and JNK activation. These observations suggest that blocking macrophage MAPK pathways could decrease iNOS production leading to reduced NO accumulation in inflammatory conditions. Future studies in the manipulation of these signaling pathways in which porins and NO play critical roles may open new therapeutic strategies of various disease processes such as gram-negative-mediated septic shock.

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