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Induction of tyrosine phosphorylated proteins in THP-1 cells by *Salmonella typhimurium*, *Pasteurella haemolytica* and *Haemophilus influenzae* porins

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Abstract

The effect of porins purified from *Salmonella typhimurium*, *Pasteurella haemolytica* and *Haemophilus influenzae* on induction of tyrosine phosphorylation in THP-1 cells and C3H/HeJ macrophage was investigated. Incubation of porins at concentration of 1.0–5.0 μg ml^{-1} with either THP-1 or macrophage from C3H/HeJ mice resulted in tyrosine phosphorylation of specific host cell proteins. After porin stimulation a pattern of tyrosine phosphorylated proteins appeared in the soluble cytoplasmic fraction, in the membrane fraction and in the insoluble protein fraction. The observed effects were dependent on the porin concentrations; they reached a maximal expression at 3 min and declined at 60 min. Porin and lipopolysaccharide treatments induce a similar phosphorylation pattern in all of the three cellular fractions studied. A difference can be observed in the cytoplasmic fraction bands of 50–60 kDa, which are more evident after treatment with lipopolysaccharide, and in the insoluble fraction band of 80 kDa and the cytoplasmic fraction band of 250 kDa, which are more evident after treatment with porins. The events of tyrosine protein phosphorylation were present in macrophage from lipopolysaccaride-hyporesponsive C3H/HeJ mice stimulated with porins, while they were markedly reduced when the cells were stimulated with lipopolysaccharide. Staurosporine, genistein and cytochalasin D induced in the three cellular fractions a different inhibition pattern of tyrosine protein phosphorylation in porin stimulated cells. Porins extracted from the three bacterial species investigated behave in a similar way as stimuli more or less potent; Hib porin seems to be the most powerful stimulator and, moreover, it specifically induces phosphorylation of a 55 kDa band.

Keywords: Porin; Tyrosine phosphorylation; Signal transduction

1. Introduction

Endotoxin, a term given by Pfeiffer to a complex of proteins, polysaccharides and peptides extracted from Gram-negative bacteria [1], can cause shock and death if released by blebbing or bacterial lysis [2] into the systemic circulation of the host. Techniques previously used in the extraction of lipopolysaccharide (LPS) from the endotoxin had greatly favoured the study of this portion of the complex, ignoring the protein fraction and identifying most of the effect of the endotoxin with those of the LPS. Subsequent extraction techniques for outer membrane proteins (OMPs) then allowed the study of the protein fraction which was globally extracted from the endotoxin. Besides their transport functions, OMPs are major antigens, and there are now several reports of their ability to stimulate a protective role [3–7]. Among OMPs, proteins which behave like porins play an important role in the pathology associated with infections by Gram-negative bacteria. Antiporin antibodies have been demonstrated to be bactericidal and opsonic in a patient with pelvic inflammatory disease, whose self-cure is represented by high levels of antiporin antibodies [7–9].

Porins of *Salmonella typhimurium* have endotoxin-like effects such as lethal action, the ability to elicit a local Shwartzman reaction and pyrogenicity [10]. *S. typhimurium* porins also activate the complement system in vitro and in vivo [11]. Furthermore, the porins have the ability to stimulate pro- and anti-inflammatory cytokine synthesis

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and release [12–14]. Porins stimulate the release of granulocyte–monocyte colony stimulating factor (GM-CSF), soluble intercellular adhesion molecule-1 (ICAM-1) and soluble E-selectin and surface expression of the adhesion molecules (E-selectin and ICAM-1) from human endothelial cells [15]. The mechanisms by which porins activate these cells are poorly characterised. LPS and porins or other OMPs probably activate the cells through similar but not identical mechanisms. Signal transduction pathways known to occur during immune cell activation have been investigated by numerous authors. It has been established that protein tyrosine phosphorylation plays a central role in the bacterium or LPS [16,17] or toxin [18] mediated transduction process [19–21].

In this report, we show that porins, and not possible traces of contaminating molecules (LPS), rapidly increase tyrosine phosphorylation of several proteins in THP-1 cells.

2. Materials and methods

2.1. Cells lines

A clone of the human monocytic cell line, THP-1 cells (202-TIB; ATCC, Rockville, MD, USA), was grown at 37°C in 5% CO_2 in RPMI 1640 (Labtek, Eurobio) with HEPES supplemented with 10% (v/v) heat inactivated fetal calf serum (FCS), glutamine (2 mM), penicillin (100 U ml^{-1}) and streptomycin (100 U ml^{-1}) (Labtek, Eurobio) in 150-cm^2 tissue culture flasks (Corning, New York, NY, USA). Cell viability was determined by the trypan blue (Sigma Chemical Co., St. Louis, MO, USA) exclusion test.

2.2. Bacterial strains

The bacterial strains used were: *S. typhimurium* SH5014 grown in Nutrient broth (Difco, Detroit, MI) for 18–24 h at 37°C under agitation; *Pasteurella haemolytica* ATCC 14003 grown in brain–heart infusion (BHI) broth for 18 h at 37°C while stirring; *Haemophilus influenzae* type b strain ATTC 9795 grown in CY medium for 18–24 h at 37°C in agitation. Cells were harvested at the end of the exponential growth phase, and outer membranes were prepared from cell envelopes following protocols described by Nikaido [22].

2.3. Preparation of porins

Porins were extracted as described by Nurminen [23]. Briefly, 1 g (wet wt.) of cell envelopes was suspended in 2% Triton X-100 in 0.01 M Tris–HCl pH 7.5, containing 10 mM ethylenediaminetetraacetic acid (EDTA); after the addition of trypsin (10 mg/g of envelopes), the pellet was dissolved in sodium dodecyl sulfate (SDS) buffer (4% w/v in 0.1 M sodium phosphate, pH 7.2) and applied to an Ultragel ACA34 column (Pharmacia, Uppsala, Sweden) equilibrated with 0.25% SDS buffer (to obtain the Hib porin, the bacterial envelopes extracted from 13 g (wet wt.) of pellet were not digested with trypsin, since *H. influenzae* porin seems to be degraded by this enzyme). The protein containing fraction, identified by its optical density at 280 nm (OD_{280}), was extensively dialysed and checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis in slabs (SDS–PAGE) according to Laemmli [24]. The protein content of the porin preparation was determined by the method of Lowry et al. [25]. LPS contamination was revealed on SDS–PAGE staining with silver nitrate as described by Tsai and Frasch [26] and quantified by *Limulus*–Amoebocyte–Lysate assay (*Limulus* test) according to Thye Yin et al. [27]. This was compared with a standard LPS solution which was *Limulus*–Amoebocyte–Lysate-positive at 0.1 EU ml^{-1} (QCL-1000 Quantitative Chromogenic LAL, Biowhittaker, Walkersville, MD, USA). In some assays the biological activity of traces of LPS that could be present in the preparation was neutralised by adding polymyxin B for 1 h at room temperature [28,29]. Porins and the mixtures containing polymyxin B were used in pyrogen-free PBS, unless otherwise specified.

2.4. Macrophage preparation from C3H/HeJ mice

Murine macrophages from female C3H/HeJ mice (Harlan UK Ltd. Shaws Farm, Blackthon, Bicester, Oxon, UK) were prepared according to conventional procedures from the peritoneal cavity. Resident macrophages were washed from the peritoneal cavity with RPMI 1640. After centrifugation at $170 \times g$ for 10 min at 4°C, the cell pellet was resuspended in RPMI 1640 medium supplemented with 10% heat inactivated FCS, glutamine (2 mM), penicillin (100 U ml^{-1}) and streptomycin (100 U ml^{-1}) (complete medium) at a concentration of 5×10^6 cells ml^{-1}. Adherent macrophage monolayers were obtained by plating the cells in six-well plastic trays at 5×10^6 cells/well for 2 h at 37°C in 5% CO_2. Non-adherent cells were removed by suction and freshly prepared complete medium was added with the indicated experimental reagents.

2.5. Cellular stimulation and preparation of cell lysates

Suspension of cells (3×10^6 ml^{-1}) in RPMI 1640 medium was stimulated with *S. typhimurium* LPS (Sigma Chemical Co., St. Louis, MO, USA) (1 μg ml^{-1}), and porins of *S. typhimurium* or *P.haemolytica* or Hib at the concentrations of 1 μg ml^{-1} and 5 μg ml^{-1} for 3 min, 20 min, 1 h and 2 h.

After stimulation, cells (5×10^6) were washed in situ two times with 10 ml of ice-cold PBS without Ca^{2+} and Mg^{2+} and permeabilised by adding 250 μl of 0.2% saponin buffer (in 50 mM Tris–HCl (pH 7.5) containing 0.4 mM $NaVO_4$, 1 mM NaF and 0.1 mM phenylmethylsulfonyl fluoride (PMSF)). After 10 min of incubation on ice, the samples

were centrifuged (5 min at 14 000 × *g*, 4°C) and the soluble cytoplasmic protein fraction was removed. The insoluble pellet was washed two times with 1 ml PBS and the membrane proteins were separated from the insoluble components by the addition of an appropriate lysis buffer (50 mM Tris–HCl (pH 7.5) containing 0.4 mM $NaVO_4$, 1 mM NaF, 0.1 mM PMSF, 10 μg ml^{-1} of leupeptin and 1% Triton X-100). The lysate was then centrifuged at 14 000 × *g* for 5 min. After removal of the Triton X-100 soluble supernatant, the pellet (insoluble fraction) was washed two times with cold PBS. The samples were resuspended with Laemmli sample buffer (Bio-Rad Laboratories). All the fractions obtained were kept at −70°C [20,21].

2.6. Immunoprecipitation

A total of 20 μg of agarose conjugated with anti-phosphotyrosine (IgG 2bk, clone 4G10) (Upstate Biotechnology, Inc.) was used to immunoprecipitate THP-1 protein fractions from 5×10^6 cell lysate. The samples were precleared with Sepharose A/G beads (20 μl) for 45 min. The immunoprecipitation steps were all carried out at 4°C overnight with gentle rotation. After incubation the beads were pelleted (6000 × *g*), washed three times with 1% Triton X-100 lysis buffer and boiled in 20 μl Laemmli sample buffer with 5% β-mercaptoethanol for 5 min [20]. The samples were pelleted and the supernatant obtained, containing cell phosphorylated proteins, was resolved by SDS–PAGE as described by Laemmli [24].

2.7. Western immunoblotting

Membrane and cytoskeletal protein samples were loaded onto SDS–10% PAGE gels, whereas cytoplasmic protein samples were loaded onto SDS–8% PAGE and transferred to nitrocellulose membrane (sheets 0.45 μm, Bio-Rad, Hercules, CA, USA) for 1 h and 15 min at 100 V. Blots were blocked overnight in Tris buffered saline (TBS (150 mM NaCl, 20 mM Tris–HCl, pH 7.5)) containing 5% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO, USA). Mouse monoclonal anti-phosphotyrosine (clone 4G10, Upstate Biotechnology, Inc.) diluted 1/2000 in TBS containing 1% BSA was used. For detection, we used anti-mouse IgG alkaline phosphatase conjugated secondary antibodies diluted 1/3000 (GAM-AP 0.5 ml, Bio-Rad, Hercules, CA, USA).

2.8. Inhibitors of signal transduction

In some experiments, prior to the addition of LPS or porins, cells (5×10^6) were pretreated for the indicated times with cytochalasin D (1 μM) (30 min), genistein (250 μM) (15 min) and staurosporine (1 μM) (60 min). The inhibitors were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All stock solutions were prepared by dissolving the drugs in dimethyl sulfoxide (DMSO; Fisher Scientific, Pittsburgh, PA, USA). Stock solutions were stored at −20°C as recommended by the manufacturer.

3. Results

3.1. Purity of porin preparation

The purification and contamination by LPS in the preparations obtained have been amply addressed in previous works [13,30]. All possible traces of LPS were identified by the *Limulus*–Amoebocyte–Lysate test using a standard LPS solution which was positive to the *Limulus* test at 0.1 EU ml^{-1}. The LPS contamination in the porin preparation was estimated to be < 0.005% w/w. These traces of LPS did not show any biological activity under our experimental conditions (data not shown). The purity of the porin preparation from *S. typhimurium* SH5014, checked by SDS–PAGE (Fig. 1), revealed two bands with molecular masses of 34 and 36 kDa. In all the tests performed, the porins plus polymyxin B gave the same results as the porins alone.

3.2. Induction of tyrosine phosphorylated proteins by porins in THP-1 cells

Induction of tyrosine phosphorylation of several minor and some major protein substrates could be detected in THP-1 cells treated with porins extracted from some

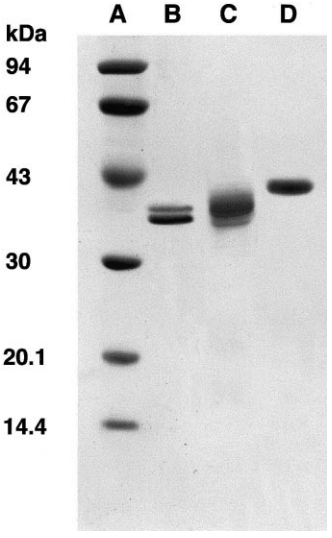

Fig. 1. Protein pattern of the porin (10 μg) prepared from *S. typhimurium* SH5014, *P. haemolytica* ATCC 14003, and *H. influenzae* type b ATCC 9795. The gel was stained with Coomassie blue. Lane A, molecular mass standards (phosphorylase B: 94 000 Da, albumin: 67 000 Da, ovalbumin: 43 000 Da, carbonic anhydrase: 30 000 Da, trypsin inhibitor: 20 100 Da, α-lactalbumin: 14 400 Da). Lane B, *S. typhimurium* porins. Lane C, *H. influenzae* type b porin. Lane D, *P. haemolytica* porin.

Gram-negative bacteria (*S. typhimurium*, *P. haemolytica*, Hib). Examination of the cytoplasmic, membrane and insoluble protein extracts derived from THP-1 cells treated with porins or control cells by Western blotting analysis revealed several tyrosine phosphate containing proteins, as detected using mouse monoclonal anti-phosphotyrosine antibody and alkaline phosphatase conjugated secondary antibody. The molecular masses of these proteins were estimated by comparison in polyacrylamide gels to the mobility of standard proteins. Porins from *S. typhimurium* rapidly increased tyrosine phosphorylation of the cytoplasmic proteins with apparent molecular masses of about 40–45, 50–60, 70, 150 and 250 kDa (Fig. 2). Increased tyrosine phosphorylation reached a maximum after 3 min, declined after 20–60 min and was completely abolished after 2 h.

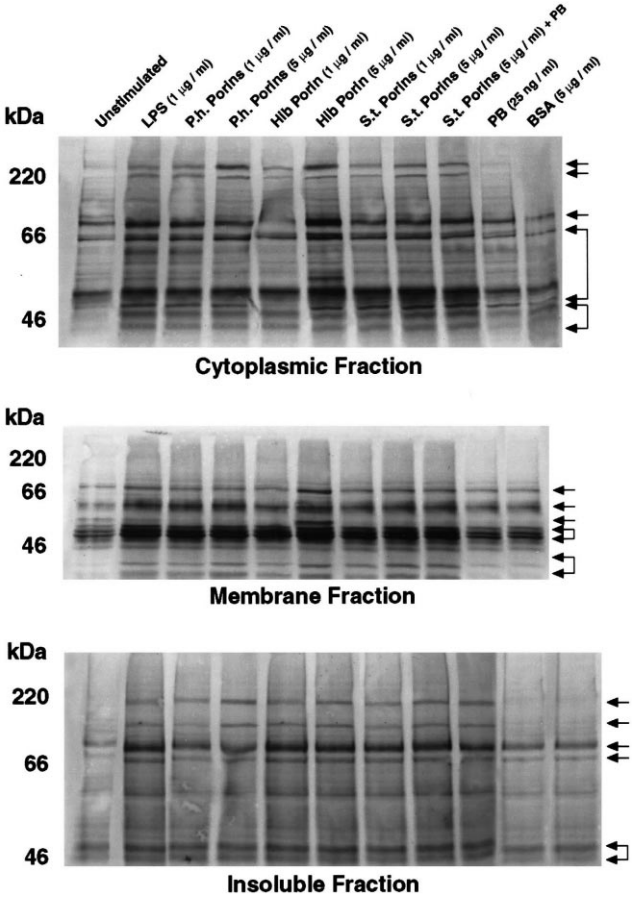

Fig. 2. Western blotting analysis of THP-1 cell (3×10^6 ml^{-1}) tyrosine phosphorylation events induced by porins at 3 min after stimulation. The cell lysates were separated into cytoplasmic, membrane and insoluble fractions, immunoprecipitated and resolved by 8% (cytoplasmic fraction) or 10% (membrane and insoluble fraction) SDS–PAGE. The fractions were normalized for protein content. After being transferred to nitrocellulose, the blots were probed with anti-phosphotyrosine-specific antibodies. The results obtained were confirmed by quantitation of developed Western blots using SIGMA GEL software. Similar results were obtained in three separate experiments. P.h., *P. haemolytica*; Hib, *H. influenzae*; S.t., *S. typhimurium*; PB, polymyxin B; BSA, bovine serum albumin. The cellular tyrosine phosphorylation events altered after porin treatment are indicated by the arrows.

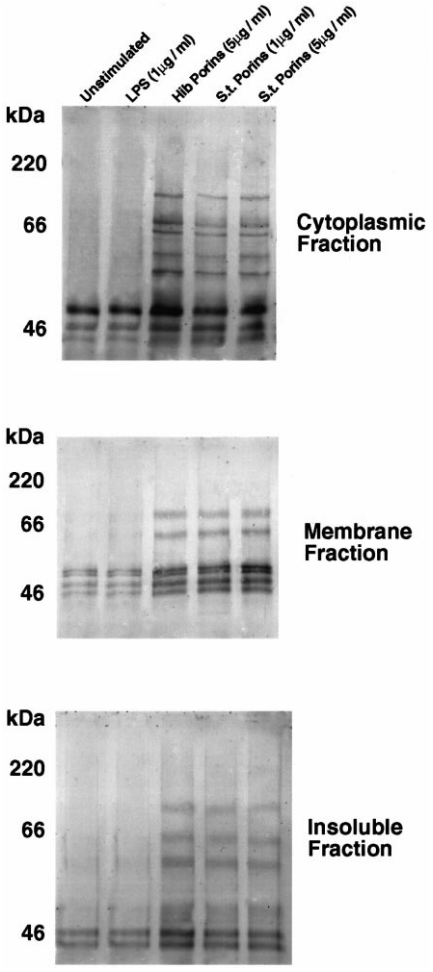

Fig. 3. Western blotting analysis of C3H/HeJ macrophage (5×10^6 cells ml^{-1}) tyrosine phosphorylation events induced by porins. Experimental procedure as described in Fig. 2. Hib, *H. influenzae*; S.t., *S. typhimurium*.

Following stimulation with porins, the tyrosine phosphorylation response was detectable after stimulation with 1 μg ml^{-1} and was maximal after stimulation with 5 μg ml^{-1}. In the membrane fraction particularly evident are bands corresponding to proteins of apparent molecular masses of about 42–44, 48–52, 55, 60 and 70 kDa while the proteins of the insoluble fraction that are more phosphorylated have apparent molecular masses of about 42–46, 70, 80, 160 and 200 kDa.

The same profile of protein tyrosine phosphorylation in the three cellular fractions was observed when porins of *P. haemolytica* or Hib were used as a stimulus. However, the Hib porin revealed to be a more powerful activator of tyrosine phosphorylation, in fact the band of 55 kDa was strongly evident both in the cytoplasmic and membrane fractions.

We found that porin stimulated tyrosine phosphorylation of several proteins in the membrane, cytoplasmic and insoluble fractions appeared to be similar to tyrosine phosphorylation patterns due to LPS stimulation. However,

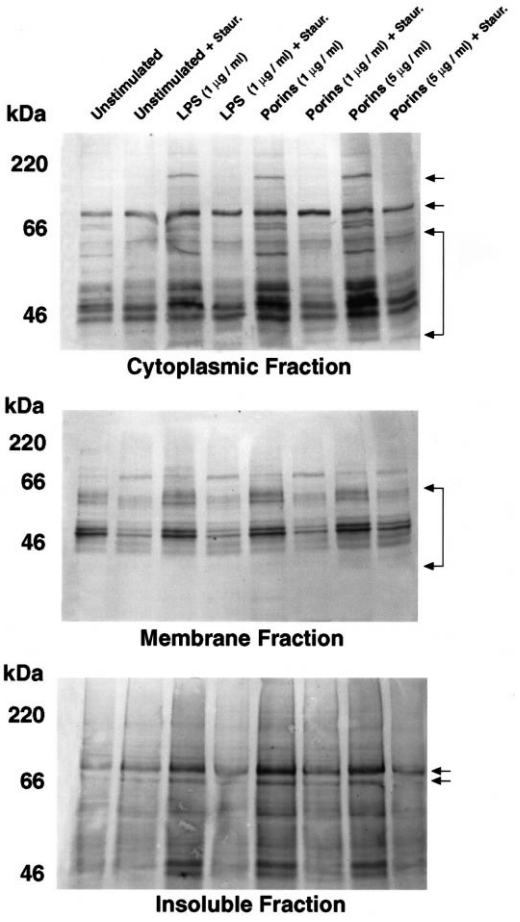

Fig. 4. Western blotting analysis of alteration by staurosporine (Staur.) (1 μM) of THP-1 cell tyrosine phosphorylation events induced after 3-min stimulation by *S. typhimurium* porins. The cells (3×10^6 ml^{-1}) were preincubated at 37°C with staurosporine for 60 min. Similar results were obtained in three separate experiments. The cellular tyrosine phosphorylation events altered after porin treatment are indicated by the arrows.

the patterns of modulated proteins by LPS and porins were different in some respect: porin from Hib in the membrane and the cytoplasmic fraction stimulated the tyrosine phosphorylation of proteins of a molecular mass of about 55 kDa. In the insoluble fraction porins induced a stronger phosphorylation of the 70 and 80 kDa proteins compared to LPS.

Tyrosine phosphorylation of proteins in the different fractions was still observed when using a mixture of porins and polymyxin B. Using LPS at a concentration of 50 pg, which is the amount that could be present in a porin preparation, we were not able to observe any activation (data not shown). An unspecific stimulus, such as bovine albumin, did not elicit any response in tyrosine phosphorylation. Similar results were observed when THP-1 cells were fractionated after using a homogeniser rather than detergents to release the cytoplasmic proteins (data not shown).

Inhibitors of eucaryotic protein synthesis, such as actinomycin D (20 μg ml^{-1}) added 30 min prior to stimulation with porins, had an undetectable effect on the tyrosine phosphorylation events. These results suggest that the increased tyrosine phosphorylation was not due to increased synthesis of constitutively phosphorylated proteins (data not shown).

3.3. Induction of tyrosine phosphorylation proteins by porins from S. typhimurium in macrophage from C3H/HeJ mice

Results obtained when stimulating THP-1 cells with porins were not related to eventual traces of LPS present in the porin preparations. Stimulations performed with *S. typhimurium* porins at a concentration of 5 μg ml^{-1} on LPS-hyporesponsive C3H/HeJ peritoneal macrophages induced a pattern of protein phosphorylation. Consequently, stimulation of C3H/HeJ cells with LPS (1 μg ml^{-1}) did elicit a tyrosine phosphorylation pattern markedly reduced and similar to that of controls. Results are reported in Fig. 3.

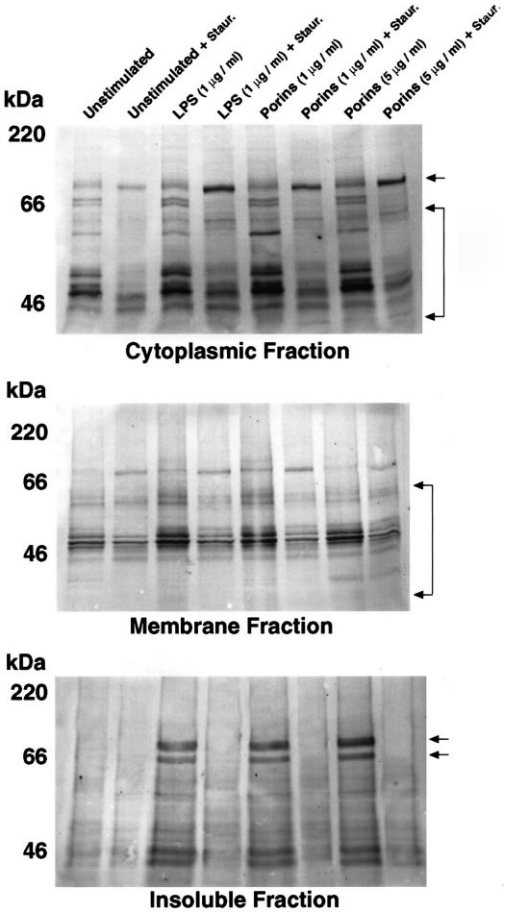

Fig. 5. Western blotting analysis of alteration by staurosporine (Staur.) (1 μM) of THP-1 cell tyrosine phosphorylation events induced after 20-min stimulation by *S. typhimurium* porins. Experimental procedure as described in Fig. 4. The cellular tyrosine phosphorylation events altered after porin treatment are indicated by the arrows.

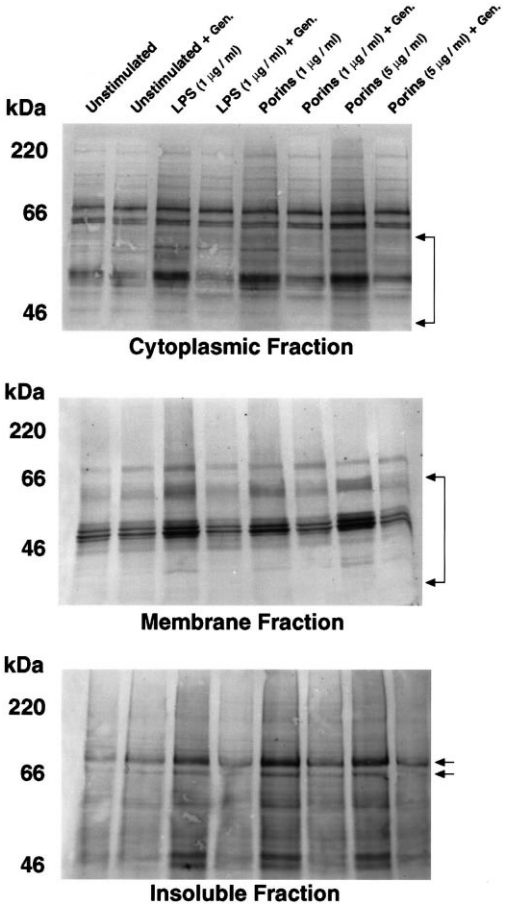

Fig. 6. Western blotting analysis of alteration by genistein (Gen.) (250 μM) of THP-1 cell tyrosine phosphorylation events induced after 3-min stimulation by *S. typhimurium* porins. The cells (3×10^6 ml^{-1}) were pre-incubated at 37°C with genistein for 15 min. Similar results were obtained in three separate experiments. The cellular tyrosine phosphorylation events altered after porin treatment are indicated by the arrows.

3.4. Effect of inhibitors on THP-1 cells in inducing tyrosine phosphorylation events by porins

To investigate which phosphorylation pathway may be activated by porins, we used a general kinase inhibitor (staurosporine), a tyrosine protein kinase inhibitor (genistein) and an inhibitor of actin polymerisation (cytochalasin D). To examine the effects of these inhibitors, THP-1 cell medium was replaced with medium containing inhibitors before porin treatment [21]. We observed that staurosporine inhibited the tyrosine phosphorylation of THP-1 cells stimulated by *S. typhimurium* porins. In the three cellular fractions already after 3 min (Fig. 4) several bands of tyrosine phosphorylation appeared much less marked compared to samples stimulated with LPS or porins, but not treated with staurosporine. In particular, both in the cytoplasmic and in the membrane fractions, bands between 40 and 60 kDa were considerably reduced; a similar reduction in the cytoplasmic fraction was observed in phosphorylation of bands around 150 kDa. After 20 min (Fig. 5) in the presence of staurosporine, the phosphorylation of bands of 40–60 kDa in the cytoplasmic fraction resulted markedly reduced. Moreover, after 20 min, a band of a molecular mass of about 95 kDa appeared much stronger in the cytoplasmic fraction of cells treated with staurosporine compared to controls. This band also appeared in cells stimulated with LPS and treated with staurosporine. Treatment of cells with genistein at 3 and 20 min (Figs. 6 and 7) gave the same pattern of inhibition, but in a less marked way compared with the results obtained using staurosporine. The insoluble fraction showed an evident alteration of phosphorylation events in the presence of staurosporine and genistein; these inhibitors did not present an inhibiting activity on the bands of molecular masses of 70 and 80 kDa of the insoluble fraction, after 3 min of stimulation, but each single band was obviously reduced in intensity after 20 min of stimulation.

Porins induce actin polymerisation [31]; therefore, the inhibiting activity of cytochalasin D in phosphorylation of these proteins was analysed. Phosphorylation of the 70 and 80 kDa bands in the cytoplasmic fraction was considerably evident after 3 min of stimulation and was not inhibited by cytochalasin D (Fig. 8), while after 20 min

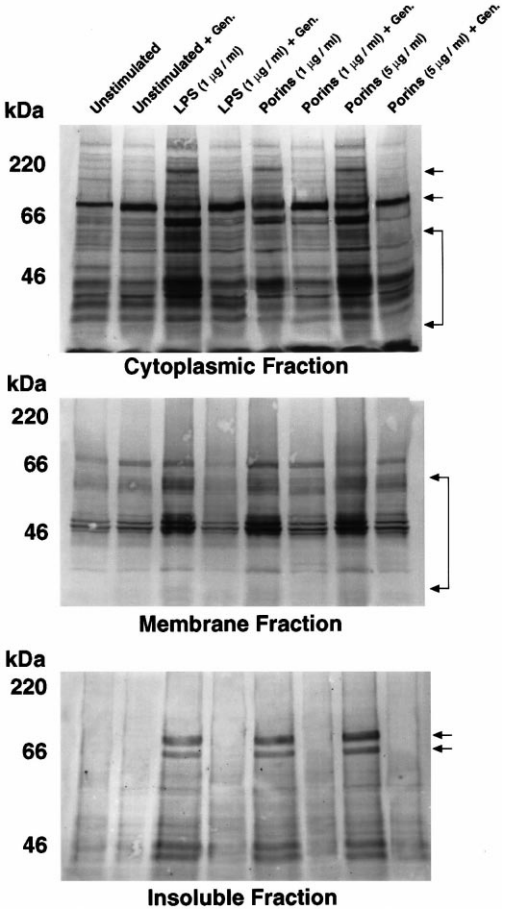

Fig. 7. Western blotting analysis of alteration by genistein (Gen.) (250 μM) of THP-1 cell tyrosine phosphorylation events induced after 20-min stimulation by *S. typhimurium* porins. Experimental procedure as described in Fig. 6. The cellular tyrosine phosphorylation events altered after porin treatment are indicated by the arrows.

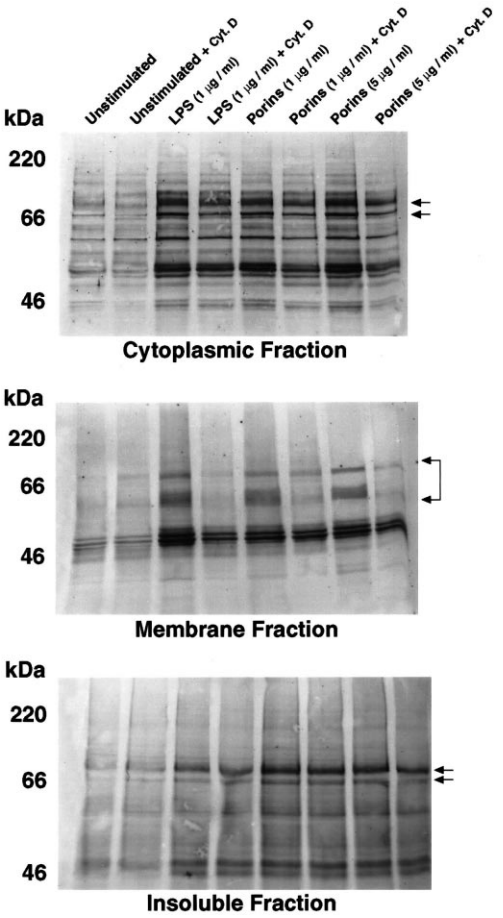

Fig. 8. Western blotting analysis of alteration by cytochalasin D (Cyt. D) (1 μM) of THP-1 cell tyrosine phosphorylation events induced after 3-min stimulation by *S. typhimurium* porins. The cells (3×10^6 ml^{-1}) were preincubated at 37°C with cytochalasin D for 30 min. Similar results were obtained in three separate experiments. The cellular tyrosine phosphorylation events altered after porin treatment are indicated by the arrows.

of stimulation there was a marked decrease in phosphorylation events (Fig. 9). After 3 min of incubation, in the membrane fraction, a reduction of phosphorylation of bands of molecular masses between 60 and 70 kDa was observed, with a further decrease after 20 min. In the insoluble protein fraction, after 3 min of stimulation, we were not able to observe any significant modification of the bands of molecular masses between 70 and 80 kDa, as previously observed when using staurosporine and genistein as inhibitors.

4. Discussion

Invasive *S. typhimurium*, non-invasive *invA* mutant and *S. typhimurium* bacterial culture supernatants induce similar tyrosine phosphorylation of different host cell lines [32]. The mechanisms by which *Salmonella* spp. or their components induce tyrosine phosphorylation remain unclear. This activation is independent from the *invA*-mediated invasion pathway, and it appears that a bacterial product secreted in culture supernatant is also able to induce tyrosine phosphorylation [32]. The most abundant product in a Gram-negative bacterial supernatant is represented by the LPS–protein complex. Therefore, in this study we focused our efforts on these two components.

The molecular mechanisms by which porins activate cells are unknown, as well as porin receptors or other events that occur at the surface of cellular membranes are still unknown. In this study, our major finding is the demonstration that also cell activation by porins is associated with tyrosine phosphorylations of specific host cell proteins. The detection of phosphotyrosine protein bands was more specifically examined by immunoblot analysis of soluble and insoluble protein fractions from porin treated cells. After porin stimulation a pattern of tyrosine phosphorylated proteins appeared in the three fractions. Many phosphorylated protein bands appeared intensely stained in the soluble cytoplasmic fraction. The insoluble fraction yielded fewer bands, but of the same molecular mass as

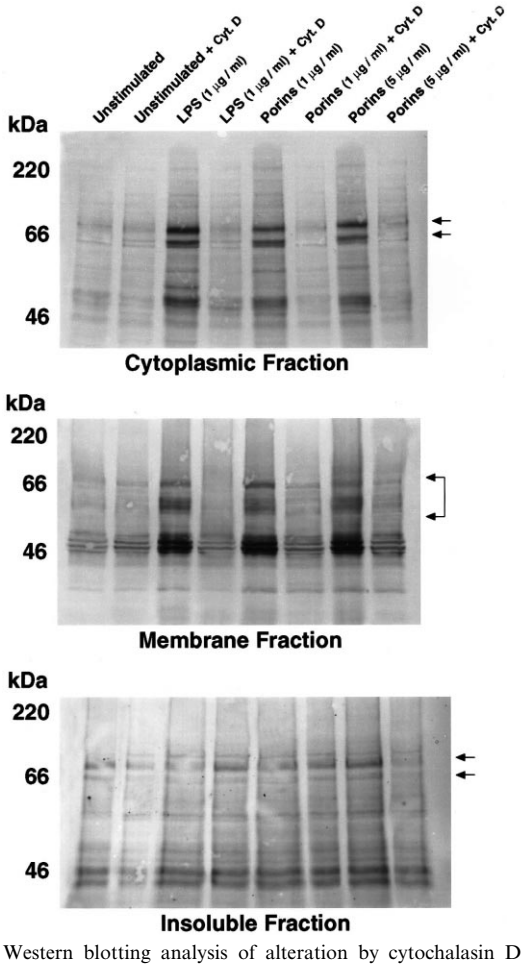

Fig. 9. Western blotting analysis of alteration by cytochalasin D (Cyt. D) (1 μM) of THP-1 cell tyrosine phosphorylation events induced after 20-min stimulation by *S. typhimurium* porins. Experimental procedure as described in Fig. 8. The cellular tyrosine phosphorylation events altered after porin treatment are indicated by the arrows.

proteins that appeared in the cytoplasmic fraction. In the membrane fraction from cells treated with porins or LPS, 48–52 kDa bands were particularly evident.

Porin induced tyrosine phosphorylation was detectable within 3 min, persisted up to 20 min, and after 2 h could not be revealed any more. It showed a dose-dependent response, in fact, porin preparations of 5 μg ml^{-1} induced a much more marked tyrosine phosphorylation than porin preparations of 1 μg ml^{-1}. These phosphorylation events were differentially inhibited by staurosporine, genistein and cytochalasin D. This observation suggests that porin stimulations involve a different protein kinase pathway. Besides, the protein pattern that can be observed after 3 min of incubation with the inhibitor is different from that obtained after 20 min of incubation with the same inhibitor. In the cytoplasmic fraction of cells treated with staurosporine, after 20 min of stimulation the intensity of the phosphorylation signal was strongly reduced, except for the bands of about 95 kDa which exhibited an increase in intensity compared to cells which were treated only with porins or LPS. It has been shown that porins get internalised [33,34]; therefore, it is possible that after internalisation, these molecules induce specific pathways of phosphorylation which are not inhibited by staurosporine. In the insoluble fraction of cells treated with porins, phosphorylation events could be observed only for a few minutes at the beginning of the stimulation (70–80 kDa), and they declined after 20 min; these phosphorylation events were not inhibited or only partially inhibited by incubation with staurosporine, genistein and cytochalasin D. These events, in cells treated with porins, may involve pre-existing proteins which are not phosphorylated through the usual protein kinase cascade. The uptake signals for *S. typhimurium* invasion of epithelial cells are insensitive to genistein and staurosporine [32].

The same pattern of phosphorylation was observed using porins extracted from *P. haemolytica* or *S. typhimurium*. Using the same concentration, the Hib porin was considerably more active than the other two porins; besides, the Hib porin showed an evident band of phosphorylation of about 55 kDa, that is less marked in cells treated with porins from the other species. The phosphorylation pattern present in the three cellular subfractions of cells treated with *S. typhimurium* porins follows the same results that are obtained using 1 μg ml^{-1} of LPS. Treatment with porins at a concentration of 5 μg ml^{-1} (about 0.2 μM) caused an increase in tyrosine phosphorylation that was superior to the one obtained with 1 μg ml^{-1} of LPS (about 0.5 μM).

Besides, the different phosphorylation patterns of the cellular subfractions have been shown to depend on porins and not on eventual traces of LPS that could be present in the preparations. When adding polymyxin B to porin preparations, we could not observe any inhibition of phosphorylation in the studied cellular fractions, while the same amount of polymyxin B was able to completely inhibit phosphorylation when added to LPS. Therefore, the amount of LPS present in our preparation was unable to stimulate our experimental model. Moreover, some additional experiments were done with macrophages from LPS-hyporesponsive C3H/HeJ mice. We found that porin did stimulate tyrosine phosphorylation from the C3H/HeJ macrophages, whereas LPS did not elicit any response different from that of controls.

The current results suggest that tyrosine phosphorylation is required for LPS induced cytokine production, because inhibition of tyrosine kinase correlates with inhibition of LPS induced macrophage activation and cytokine production [16,17,35–38]. This may also be valid in porin stimulated cells, but it has not been demonstrated yet. The porin induced activation, however, was not due to secondary stimulation of cells by porin induced cytokines, because activation of signal transduction molecules induced by porins reached its maximum at a time that was not sufficient for porins to induce transcription of cytokine genes. Taken together, our results indicate that porins and LPS activate the cells through similar but probably not identical mechanisms. Also the peptidoglycan has been shown to induce rapid tyrosine phosphorylation of several cellular proteins [39]. Therefore, it seems that several superficial bacterial components are able to activate cells with similar but not identical mechanisms.

We are currently working on the identification of the proteins phosphorylated during porin stimulation. Among the most prominent tyrosine phosphorylated bands in porin stimulated cells there is a number of proteins with a molecular mass that is similar to that of the family of tyrosine/serine/threonine protein kinases: cells or lysates of *S. typhimurium* cells induce tyrosine phosphorylation of p44 and p42 MPK [32].

In conclusion, although the pattern of porin inducing signal transduction is still unknown, our results indicate that protein tyrosine phosphorylation is a rapid and important signalling event for porins as well as for LPS [40] and other bacterial molecules [41]; excessive stimulation of macrophages by bacterial components, through several routes of signal transmission occurring during bacterial infections, can lead to extensive tissue damage and septic shock. Investigation of the early biochemical events that are triggered in macrophages by LPS [16,17], porins [42], peptidoglicans [39] and other bacterial molecules could provide important insight into the regulation of the host response to maximal bacterial invasion.

References

[1] Morrison, D.C., Danner, R.L., Dinarello, C.A., Munford, R.S., Natason, C., Pollack, M., Spitzer, J.J., Ulevitch, R.J., Vogel, S.N. and McSweegan, E. (1994) Bacterial endotoxins and pathogenesis of Gram-negative infections: current status and future direction. J. Endotoxin Res. 1, 71–83.

[2] Zhang, H., Niesel, D.W., Peterson, J.W. and Klimpel, G.R. (1998)

Lipoprotein release by bacteria: potential factor in bacterial pathogenesis. Infect. Immun. 66, 5196–5201.
[3] Kuusi, N., Nurminen, M., Saxen, H., Valtonen, M. and Makela, P.H. (1979) Immunization with major outer membrane proteins in experimental salmonellosis in mice. Infect. Immun. 25, 857–862.
[4] Munkley, A., Tinsley, C.R., Virji, M. and Heckels, J.E. (1991) Blocking of bactericidal killing of *Neisseria meningitidis* by antibodies directed against class 4 outer membrane protein. Microb. Pathog. 11, 447–452.
[5] Sengupta, D.K., Sengupta, T.K. and Ghose, A.C. (1992) Major outer membrane proteins of *Vibrio cholerae* and their role in induction of protective immunity through inhibition of intestinal colonization. Infect. Immun. 60, 4848–4855.
[6] Srikumar, R., Dahan, D., Gras, M.F., Saarinen, L., Kayhty, H., Sarvas, M., Vogel, L. and Koulton, J.W. (1993) Immunological properties of recombinant porin of *Haemophilus influenzae* type b expressed in *Bacillus subtilis*. Infect. Immun. 61, 3334–3341.
[7] Wetzler, L.M., Blake, M.S. and Gotsch-lich, E.C. (1988) Characterization and specificity of antibodies to protein I of *Neisseria gonorrhoeae* produced by injection with various protein I-adjuvant preparations. J. Exp. Med. 168, 1883–1897.
[8] Buchanan, T.M., Eschenbach, D.A., Knapp, J.S. and Holmes, K.K. (1980) Gonococcal salpingitis is less likely to recur with *Neisseria gonorrhoeae* of the same principal outer membrane antigen type. Am. J. Obstet. Gynecol. 138, 978–980.
[9] Virji, M., Zak, K. and Heckels, J.E. (1986) Monoclonal antibodies to gonococcal outer membrane protein IB: use in investigations of the potential protective effects of antibodies directed against conserved and type-specific epitopes. J. Gen. Microbiol. 132, 1621–1629.
[10] Galdiero, F., Sommese, L., Scarfogliero, P. and Galdiero, M. (1994) Biological activities – lethality, Shwartzman reaction and pyrogenicity – of *Salmonella typhimurium* porins. Microb. Pathog. 16, 111–116.
[11] Galdiero, F., Tufano, M.A., Sommese, L., Folgore, A. and Tedesco, F. (1984) Activation of complement system by porins extracted from *Salmonella typhimurium*. Infect. Immun. 46, 559–563.
[12] Galdiero, F., Cipollaro de l'Ero, G., Benedetto, N., Galdiero, M. and Tufano, M.A. (1993) Release of cytokines by *Salmonella typhimurium* porins. Infect. Immun. 61, 155–161.
[13] Galdiero, M., Cipollaro de l'Ero, G., Donnarumma, G., Marcatili, A. and Galdiero, F. (1995) Interleukin-1 and interleukin-6 gene expression in human monocytes stimulated with *Salmonella typhimurium* porins. Immunology 86, 612–619.
[14] Henderson, B., Poole, S. and Wilson, M. (1996) Bacterial modulins: novel class of virulence factors which cause host tissue pathology by inducing cytokine synthesis. Microbiol. Rev. 60, 316–341.
[15] Donnarumma, G., Brancaccio, F., Cipollaro de l'Ero, G., Folgore, A., Marcatili, A. and Galdiero, M. (1996) Release of GM-CSF, sE-selectin, and sICAM-1 by human vascular endothelium stimulated with Gram-negative and Gram-positive bacterial components. Endothelium 4, 11–22.
[16] Weinstein, S.L., Gold, M.R. and DeFranco, A.L. (1991) Bacterial lipopolysaccharide stimulates protein tyrosine phosphorylation in macrophages. Proc. Natl. Acad. Sci. USA 88, 4148–4152.
[17] Weinstein, S.L., Sanghera, J.S., Lemke, K.A., DeFranco, L. and Pelech, S.L. (1992) Bacterial lipopolysaccharide induces tyrosine phosphorylation and activation of mitogen-activated protein kinases in macrophages. J. Biol. Chem. 267, 14955–14962.
[18] Jakway, J.P. and DeFranco, A. (1986) Pertussis toxin inhibition of B cell and macrophage responses to bacterial lipopolysaccharide. Science 234, 743–745.
[19] Evans, D.J., Frank, D.W., Finck-Barbançon, V., Wu, C. and Fleiszig, S.M.J. (1998) *Pseudomonas aeruginosa* invasion and cytotoxicity are independent events, both of which involve protein tyrosine kinase activity. Infect. Immun. 66, 1453–1459.
[20] Kenny, B. and Finlay, B.B. (1997) Intimin-dependent binding of enteropathogenic *Escherichia coli* to host cells triggers novel signaling events, including tyrosine phosphorylation of phospholipase C-γ1. Infect. Immun. 65, 2528–2536.
[21] Rosenshine, I., Donnenberg, M.S., Kaper, J.B. and Finlay, B.B. (1992) Signal transduction between enteropathogenic *Escherichia coli* (EPEC) and epithelial cells: EPEC induces tyrosine phosphorylation of host cell proteins to initiate cytoskeletal rearrangement and bacterial uptake. EMBO J. 11, 3551–3560.
[22] Nikaido, H. and Vaara, M. (1987) Outer membrane. In: *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology, Vol. 1 (Neidhart F.C., Ed.), American Society for Microbiology, Washington, DC.
[23] Nurminem, M. (1985) Isolation of porin trimers. In: Enterobacterial Surface Antigens. Methods for Molecular Characterization. FEMS Symposium 25 (T.K. Korhonen, Dawes, E.A. and Makela, P.H., Eds.), p. 294. Elsevier, Amsterdam.
[24] Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 277, 680–685.
[25] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.
[26] Tsai, C.M. and Frasch, C.E. (1982) A sensitive silver-stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. 119, 115–119.
[27] Thye Yin, E., Galanos, C., Kinsky, S., Bradshaw, R.A., Wessler, S., Luderity, O. and Sarmiento, M.F. (1972) Picogram sensitive assay for endotoxin: gelation of *Limulus polyphemus* blood cell lysate induced by purified lipopolysaccharide and lipid A from Gram negative bacteria. Biochim. Biophys. Acta 9, 245–249.
[28] Blanchard, D.K., Djeun, J.Y., Klein, T.W., Friedman, H. and Stewart, W.E. (1986) Interferon-gamma induction by lipopolysaccharide: dependence on interleukin 2 and macrophages. J. Immunol. 136, 963–970.
[29] Morrison, D.C. and Jacobs, D.M. (1976) Binding of polymyxin B to the lipid A portion of bacterial lipopolysaccharides. Immunochemistry 13, 813–818.
[30] Galdiero, F., Tufano, M.A., Galdiero, M., Masiello, S. and Di Rosa, M. (1990) Inflammatory effects of *Salmonella typhimurium* porins. Infect. Immun. 58, 3183–3188.
[31] Galdiero, M., Palomba, E., De Martino, L., Vitiello, M. and Pagnini, P. (1998) Effects of the major *Pasteurella multocida* porin on bovine neutrophils. Am. J. Vet. Res. 59, 1270–1274.
[32] Rosenshine, I., Ruschkowski, S., Foubister, V. and Finlay, B.B. (1994) *Salmonella typhimurium* invasion of epithelial cells: role of induced host cell tyrosine protein phosphorylation. Infect. Immun. 62, 4969–4974.
[33] Galdiero, M., Brancaccio, F., Nazzaro, C. and De Martino, L. (1998) *Salmonella typhimurium* porin internalization by leukocytes. Res. Microbiol. 149, 625–630.
[34] Luchi, M. and Munford, R.S. (1993) Binding, internalization, and deacylation of bacterial lipopolysaccharides by human neutrophils. J. Immunol. 151, 959–969.
[35] Beaty, C., Franklyn, T.L., Uehara, Y. and Wilson, C.B. (1994) Lipopolysaccharide-induced cytokine production in human monocytes: role of tyrosine phosphorylation in transmembrane signal transduction. Eur. J. Immunol. 24, 1278–1284.
[36] Han, J., Lee, J.D., Tobias, P.S. and Ulevitch, R.J. (1993) Endotoxin induces rapid protein tyrosine phosphorylation in 70Z/3 cells expressing CD14. J. Biol. Chem. 268, 25009–25014.
[37] Stefanova, I., Corcoran, M.L., Horak, E.M., Wahl, L.M., Bolen, J.B. and Horak, I.D. (1993) Lipopolysaccharide induces activation of CD14-associated protein tyrosine kinase $p53/56^{lyn}$. J. Biol. Chem. 268, 20725–20728.
[38] Weinstein, S.L., June, C.H. and DeFranco, A.L. (1993) Lipopolysaccharide-induced protein tyrosine phosphorylation in human macrophages is mediated by CD14. J. Immunol. 151, 3829–3838.
[39] Gupta, D., Jin, Y.P. and Dziarski, R. (1995) Peptidoglycan induces transcription and secretion of TNF-α and activation of Lyn, extra-

cellular signal-regulated kinase, and Rsk signal transduction proteins in mouse macrophages. J. Immunol. 12, 2620–2630.
[40] Steven, L., Weinstein, S.L., Gold, M.R. and DeFranco, A.L. (1991) Bacterial lipopolysaccharide stimulates protein tyrosine phosphorylation in macrophages. Immunology 88, 4148–4152.
[41] Scholl, P.R., Trede, N., Chatila, T.A. and Geha, R.S. (1992) Role of protein tyrosine phosphorylation in monokine induction by the staphylococcal superantigen toxic shock syndrome toxin-1. J. Immunol. 148, 2237–2241.
[42] Gupta, S., Kumar, D. and Ganguly, N.K. (1999) Involvement of signal transduction pathways in *Salmonella typhimurium* porin activated gut macrophages. Mol. Cell. Biochem. 194, 235–243.