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Research Article

Mitogen-activated Protein Kinase p38, not ERK1/2 and JNK, Regulates Nitric Oxide Response to *Salmonella* Heidelberg Infection in Chicken Macrophage HD11 Cells

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Abstract

Salmonella Heidelberg is one of the most prevalent poultry *Salmonella* strains. Infection with *S.* Heidelberg strongly stimulates nitric oxide (NO) response in the chicken macrophage HD11 cells. In the present study, regulation of NO response to *S.* Heidelberg infection by the host mitogen-activated protein (MAP) kinases (p38, ERK1/2, and JNK) was investigated using the selective pharmaceutical inhibitors, SB203580, PO98059, and SP600125, respectively. At the concentrations used, the p38 inhibitor strongly inhibited the NO productions in *S.* Heidelberg infected cells (61-80% reduction; $p \leq 0.05$); whereas ERK1/2 inhibitor produced a much less inhibition (14-20% reduction; $p \leq 0.05$) and JNK inhibitor showed no inhibitory effect (4-6% increase). The total MAP kinases p38 and JNK were not changed by *Salmonella* infection, with mild increase detected in ERK1/2. However, large changes were observed in phopho-p38 (100% increase) and JNK (45% increase), with a minor change detected in phospho-ERK1/2 (9% increase). Although both p38 and JNK phosphorylation can be significantly inhibited ($p \leq 0.05$) by their inhibitors, only inhibition of p38 produced a significant reduction of NO. Together, these results indicated that p38 MAP kinase, not JNK and ERK1/2, plays a significant role in regulating the NO response of HD11 cells to *Salmonella* infection.

Keywords: Chicken; Macrophage cell; MAP kinase; Nitric oxide; *Salmonella*

Introduction

Macrophages are phagocytic mononuclear cells, playing a central role in the host innate immune system where they detect, phagocytize and eradicate invading pathogens by creating an inflammatory milieu through secretion of various proinflammatory cytokines, reactive radical oxygen species (ROS), nitric oxide (NO), lysozyme, and proteolytic enzymes [1,2]. In macrophages, NO is catalyzed by the inducible NO synthase (iNOS) when the cells are stimulated by pathogens, bacterial lipopolysaccharides (LPS), and the cytokine interferon-γ (IFN-γ) [3]. Production of NO by activated macrophages is an important innate immune response of cellular antimicrobial function [4,5]. As precursor to reactive nitrogen species (RNS), NO is actively involved in the host defense against intracellular pathogenic microorganisms such as *Salmonella* [6,7]. It is well established that chicken macrophages are activated to produce NO when exposed to pathogens [8-12] and pathogen associated molecules [13,14].

Salmonella Heidelberg is one of the top prevalent *Salmonella* strain in the poultry and has often been associated with human salmonellosis [15]. It has been shown that the strain stimulates a strong NO response in chicken macrophage cells [12]. As NO playing an important role in controlling intracellular pathogens like *Salmonella*, identifying cellular signaling mechanism that controls NO production is needed for a better understanding of the host-pathogen interaction. In mammalian cells, members of mitogen-activated protein (MAP) kinase family (p38, ERK1/2, and JNK) are known to regulate iNOS activity [16,17]; however, information regarding the role of MAP kinases in chicken macrophage iNOS activation during *Salmonella* infection remains

unclear. Therefore, the present study was to identify the regulatory role of MAP kinases (p38, ERK1/2, and JNK) in NO response of chicken macrophage (HD11) to *S.* Heidelberg infection.

Materials and Methods

Reagents

Cell culture medium and reagents were obtained from Sigma (St. Louis, MO). The inhibitors PD98059 (ERK1/2 inhibitor), SB203580 (p38 MAPK inhibitor), SP600125 (JNK inhibitor) were obtained from Santa Cruz Biotechnology (Dallas, TX). Ultra-pure LPS from *Salmonella* Minnesota was obtained from InvivoGen (San Diego, CA). MAP kinase (p38, ERK1/2, and JNK) activation assay kit (RayBio® Cell-based ERK1/2 (activated) ELISA Sample kit) was obtained from RayBiotech (Norcross, GA).

Cell Line

The MC29 virus-transformed chicken macrophage cell line HD11 [18] was maintained in a complete Dulbecco's Modified Eagles Medium (DMEM) containing 10% chicken serum, antibiotics (100 U penicillin/ml and 100 µg streptomycin/ml), and 1.5 mM L-glutamine at 39°C, 5% CO_2, and 95% humidity. Aliquots of cell suspension ($2x10^6$ cells/ml) was seeded into each well at 500 µl/well for 24-well plate (BD Biosciences, Bedford, MA) and allowed to grow to about 85% confluence (~ 36 h) before used for infection.

Bacterium

The strain of *S.* Heidelberg used in the present study was a field isolates from a poultry farm and was serotyped by the National Veterinary Services Laboratory (Ames, IA). The bacterium was kept at -80°C in glycerol stocks, an aliquot of the stocks was cultured overnight at 41°C in BD's Tryptic Soy Broth (TSB), an aliquot of the overnight cultures was transferred to a fresh TSB and cultured at 41°C for 4 h to reach exponential growth phase, and the bacterium was collected, washed, and suspended in PBS buffer at a final concentration of ~$1x10^9$(cfu, colony-forming unit)/ml [12].

Cell Infection with *S.* Heidelberg

Prior to infection, the culture medium was removed, and the cells were washed once and replaced with 200 µl of plain DMEM (without chicken serum and antibiotics). Aliquots of 50 µl of *Salmonella* suspensions (~$1x10^9$cfu/ml) were added to each well at 20 ~25 MOI (multiplicity of infection) with four replicates and incubated for 1 h at 39°C in a 5% CO_2 humidified incubator. At 1-hour post infection (hpi), the infection medium was removed, and the cells were washed once with plain DMEM, treated with 100 µg/ml of gentamicin sulfate in complete DMEM for 1h to kill extracellular bacteria. After gentamicin-treatment, infected cells were washed twice and continued the culture in complete DMEM containing 25 mg/ml of gentamicin sulfate for 24 h.

Nitrite Assay

Nitrite, a stable metabolite of NO, produced by activated macrophages was measured by the Greiss assay [19]. HD11 cells in 24-well plates were pretreated with or without kinase inhibitor in 4-replicates for 30 min and proceeded with *S.* Heidelberg infection as described above or with LPS stimulation at concentration of 1 µg/ml. Cells were continued to culture at the absence or presence of various inhibitors at the indicated concentrations. After 24 hpi with *S.* Heidelberg or LPS stimulation, aliquots of 100 µl culture supernatant from each well were transferred to the wells of a new flat-bottom 96-well plate and mixed with 50 µl of 1% sulfanilamide and 50 µl of 0.1% naphthylenediamine (both were prepared in 2.5% phosphoric acid solution) sequentially. After 10 min incubation at room temperature, the nitrite concentration was determined by measuring optical density (OD_{595}) of each well using a SPECTRA MAX microplate reader (Molecular Devices, Sunnyvale, CA). Sodium nitrite (Sigma) was used as a standard to determine nitrite concentrations in the cell-free medium.

MAP Kinase Activation Assay

For cell-based MAP kinase activation assays, 100 µl aliquots of cell suspension ($2x10^6$ cells/ml) was seeded into each well of a flat-bottom 96-well plate and allowed to grow to about 85% confluence (~ 36 h). Cells were infected with *S.* Heidelberg for 1 hr and then proceeded with the MAP kinase (p38, ERK1/2, and JNK) activation assay using a RayBio® Cell-based ERK1/2 (activated) ELISA Sample kit (RayBiotech, GA, USA) according to the manufacture's instruction. Relative amounts of total or phospho-MAP kinases were detected with specific antibodies and measured spectrophotometrically at OD_{450}.

Data Analysis

Two independent experiments were conducted at different times. Within each experiment, four replicates were measured for each treatment. Data were analyzed by One Way ANOVA followed by multiple comparisons (Tukey test) using SigmaStat® software (Jandel Scientific, San Rafael, CA). The value of $p< 0.05$ is considered to be significant.

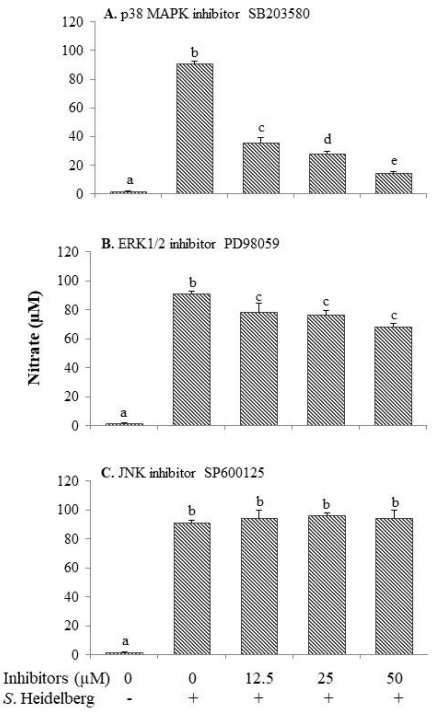
Results and Discussion

In macrophages, NO is synthesized from L-arginine, oxygen and NADPH by iNOS in response to stimulation by microbial products [3]. NO exerts its antimicrobial function by disrupting respiration and DNA synthesis and repair, and by producing reactive nitrogen species (RNS) which cause oxidative and nitrosative stress to intracellular pathogens [20,21]. As a result, NO response to microbial stimulation is an important innate immune function of macrophages and has been shown to play a critical role in control of the proliferation of intracellular bacterial pathogens such as *S.* Typhimurium [22,23].

There are three major pathways, including NF-κB, AP-1 and JAK/STAT, that play a critical role in the regulation of iNOS activity which is responsible for the production of NO in macrophages [24]. The MAP kinase family is directly involved in activation of NF-κB and AP-1 [25-27] and there is plethora of evidence indicating the involvement of MAP kinase family in activation of iNOS in various cell types [17]. However, information regarding regulation of NO production in avian macrophage cells in response to microbial stimulation is limited [13,28-30].

In this study, infection with *S.* Heidelberg stimulates a strong NO response in chicken macrophage HD11 cells. Using selective MAP kinase inhibitors, SB203580 (p38), PD98059 (ERK1/2), and SP600125 (JNK), we have demonstrated a differential role of MAP kinases in regulating NO response to *S.* Heidelberg infection in HD11 cells (Figure 1).

Fig. 1

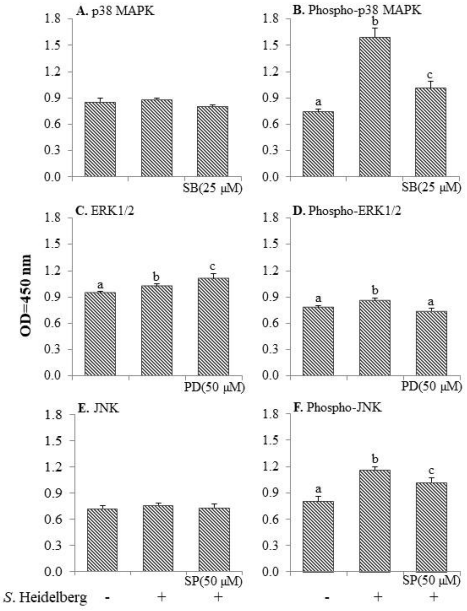


Figures 1(A-C): Effect of selective MAP kinase inhibitors on production of nitric oxide (NO) in chicken macrophage cell line HD11 cells infected with *S.* Heidelberg. Cells were infected with *S*. Heidelberg in the absence or presence of specific inhibitors at the indicated concentrations. Nitrite of the culture media was measured at 24 h post infection (hpi). **A**. p38 inhibitor SB203580; **B**. ERK1/2 inhibitor PD98059; and **C**. JNK inhibitor SP600125. Different letters indicate that the difference between the treatments is statistically significant ($p \leq 0.05$).

At 12.5, 25, and 50 μM concentrations, the p38 inhibitor, SB203580, strongly inhibited the NO productions (61%, 70%, and 80% reduction (Figure 1A); whereas the ERK1/2 inhibitor, PD98059, was shown to be less effective (14%, 16%, and 20% reduction (Figure 1B) and the JNK inhibitor, SP600125, had no inhibitory effect at all (4%. 6%, and 4% increase (Figure 1C). These results suggest that p38 MAP kinase have a more influential role in regulating chicken macrophage cell iNOS activity in responding to *S.* Heidelberg infection than its counterparts ERK1/2 and JNK.

To validate the observation, a cell-based MAP kinase phosphorylation assay was conducted to examine the activation of MAP kinases in HD11 cells when infected with *S.* Heidelberg. There was no change in total protein contents of p38 (Figure 2A) and JNK (Figure 2E), with a slight increase of ERK1/2 (Figure 2C) when cells were infected by *S*. Heidelberg. However, *S*. Heidelberg infection significantly increased the activated state phosphor-p38 (100%, Figure 2B) followed by JNK with 45% increase (Figure 2F), with ERK1/2 the least with 9% increase (Figure 2D). The increase of phospho-p38 MAP kinase was effectively inhibited by SB203580 (Figure 2B), which corresponded well with the strong inhibitory effect of SB203580 on NO production (Figure 1A). These results confirmed the critical role of p38 MAP kinase in chicken macrophage NO response to *S.* Heidelberg infection. The phosphor-ERK1/2 proteins were little changed (Figure 2D) whether or not cells were infected by *S.* Heidelberg and which together with that the production of NO was mildly affected by the inhibitor PD98059 (Figure 1B) suggest that ERK1/2 may not have a significant role in regulating the NO response to *S.* Heidelberg infection.

Fig. 2



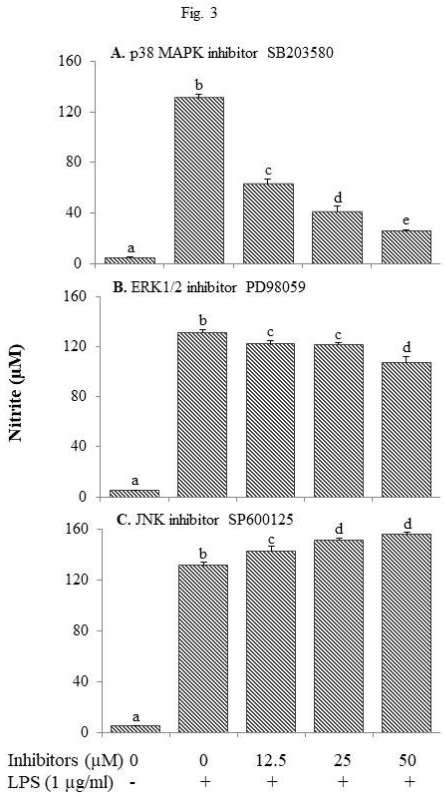
Figures 2(A-F): Phosphorylation of MAP kinases induced by *S.* Heidelberg infection in chicken macrophage HD11 cells. Cells were pretreated with specific inhibitors at the indicated concentrations for 30 min and then infected with *S.* Heidelberg for 1 h. The protein contents of

total and phosphorylated MAP kinase were detected by specific antibodies using the ELISA kit from Ray Biotech (GA, USA). **A**. Total p38 MAP kinase; **B**. Phospho-p38 MAP kinase; **C**. Total ERK1/2; **D.** Phospho-ERK1/2; **E**. Total JNK; and **F.** Phospho-JNK. Different letters indicate that the difference between the treatments is statistically significant ($p \leq 0.05$).

Although *S.* Heidelberg infection induced increase of phospho-JNK can be significantly inhibited by its selective inhibitor, SP600125 (Figure 3F), treatment with the inhibitor resulted in no reduction, rather a slightly increased production of NO in HD11 cells (Figure 1C). The results clearly indicate that JNK is less likely involved in the activation of iNOS in chicken macrophages in response to *S.* Heidelberg infection.

Lipopolysaccharides from the outer membrane of Gram-negative bacteria, including *Salmonella*, are known to strongly stimulate NO production in the chicken macrophage HD11 cells. The same inhibitors were evaluated to verify whether MAP kinases (p38, ERK1/2, and JNK) play the similar role in LPS-stimulated NO production in chicken macrophage cells. Indeed, an identical pattern of inhibitory effects on the NO production emerged: the p38 inhibitor strongly abolished, in a dose-dependent manner, the LPS-stimulated NO production (Figure 3A), while inhibition of ERK1/2 and JNK produced less or no inhibitory effects (Figure 3B and Figure 3C) respectively. These results confirmed the importance of p38 MAP kinase in regulation of chicken macrophage NO immune response.

In conclusion, we have demonstrated that *S.* Heidelberg infection induces a strong NO production in the chicken macrophage cell line HD11. The p38 MAP kinase, but not ERK1/2 and JNK, plays a critically important regulatory role in the NO response to *S.* Heidelberg infection and LPS stimulation in chicken macrophage HD11 cells. These results demonstrate a unique aspect of MAP kinase family in regulating iNOS activity in chicken macrophages.



Figures 3(A-C): Effect of selective inhibitors on production of nitric oxide (NO) in chicken macrophage cell line HD11 cells stimulated with lipopolysaccharide (LPS). Cells were stimulated with LPS at 1 µg/ml in the absence or presence of specific inhibitors at the indicated concentrations. Nitrite of the culture media was measured at 24 h post stimulation. **A**. p38 inhibitor SB203580 9SB); **B.** ERK1/2 inhibitor PD98059 (PD); **C**. JNK inhibitor SP600125 (SP). Different letters indicate that the difference between the treatments is statistically significant ($p \leq 0.05$).

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Mention of commercial or proprietary products in this paper does not constitute an endorsement of these products by the USDA, nor does it imply the recommendation of products by the USDA to the exclusion of similar products.

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