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ORIGINAL ARTICLE

The effect of sinomenine on ERK1/2, JNK and p38 phosphorylation in LPS-stimulated endothelial cells.

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Abstract

This study was to investigate the effect of sinomenine by LPS-induced MAPK phosphorylation in endothelial cells. Endothelial cells were challenged with different doses LPS and/or treated with sinomenine at three concentrations (1, 5, or 10 μg/mL) in pathological model, drug safety, treatment and prevention experiments. The cells were incubated at 37 °C in a cell incubator total for 24 h. The lysate cells were collected and analyzed the phosphorylation of ERK1/2, JNK and p38 by MAPK phosphoprotein assay whole cell lysate kit. As expected, LPS could significantly elevated phosphorylation of ERK1/2, JNK and p38, but sinomenine not. The results revealed that sinomenine significantly reduced the phosphorylation of ERK1/2 and p38 in treatment experiment, and inhibited phosphorylation of ERK1/2, JNK and p38 in prevention experiment. Our findings demonstrated that sinomenine protects endothelial cells from LPS-induced inflammation, which might be associated with depressing MAPK signaling pathway.

Abbreviations:

LPS	- Lipopolysaccharide
PIEC	- Porcine iliac artery endothelial cells
MSD	- MESO SCALE DISCOVERY'S
MAPK	- Mitogen-activated protein kinase
GPCRs	- G-protein coupled receptors
ERK1/2	- Extracellular signal-regulated kinase 1/2
JNK	- c-Jun N-terminal kinases
DMEM	- Dulbecco's modified Eagle's medium
FBS	- Fetal bovine serum
RIMECs	- Rat intestinal microvascular endothelial cells

INTRODUCTION

Lipopolysaccharide (LPS), an endotoxin released by Gram-negative bacteria, is the main pathogenic factor of colibacillosis and is largely responsible for morbidity/mortality associated with the disease (Thorgersen *et al.* 2008). LPS activates a series of signal transduction pathways in cells and causes pathological effects (Mackman 2000). LPS can injure the microcirculation and vascular endothelial cells by promoting excessive secretion of immune-associated cytokines, thereby contributing to the inflammatory cascade response that ultimately triggers multiple organ dysfunction

syndromes (Dauphinee & Karsan 2006). Endotoxin is a significant contributing factor underlying the occurrence of fever, diarrhea, inflammation, edema, coagulation, shock, and other syndromes associated with Gram-negative bacterial infections (Qin *et al.* 2016). To date, there is no effective treatment for endotoxemia.

Many Chinese herbal medicines can effectively suppress and kill bacterial pathogens. They are widely used to prevent and cure infectious diseases, often with a high efficacy, low toxicity, less occurrence of resistance, and lower residual levels remaining in a body than many common drugs. Along these lines, many studies have focused on use of Chinese herbal medicines to mitigate/prevent LPS-induced damage (Gao *et al.* 2006). Sinomenine, a pure compound extracted from the *Sinomenium acutum* plant, has been shown to impart an anti-inflammatory effect in the treatment of immune-related disorders in experimental animal models and in some clinical applications (Wang & Li 2011). Further investigations using isolated mouse neutrophils confirmed there was indeed an up-regulation of AR increased by sinomenine and that AR-cAMP-PKA signaling was involved in this induced anti-inflammatory effect by this alkaloid (Li *et al.* 2013).

Endothelial cells play important roles in a number of physiologic and pathologic processes, such as inflammation, fever, diarrhea, coagulation, and shock (Pate *et al.* 2010). Regarding initiation/development of immune responses, endothelial cells can be activated by pathogens, leading to their release of various endogenous compounds that modulate vascular relaxation/constriction, including some cytokines. As it has been confirmed that endothelial cells are important primary targets for systemic diseases (Lehle *et al.* 2010), iliac artery endothelial cells (including those from pigs, i.e., PIEC) represent good models for such studies of induced dysfunction.

MESO SCALE DISCOVERY'S (MSD) unique spot patterns are a hallmark of MULTI-ARRAY® technology, which enables the measurement of biomarkers utilizing the next generation of electro chemiluminescent detection. In an MSD assay, specific capture antibodies for the analytes are coated in arrays in each well of a 96-well carbon electrode plate surface. The detection system uses patented SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of the MULTI-ARRAY and MULTI-SPOT® plates. MSD labels can be conveniently conjugated to biological molecules, are stable, and are non-radioactive. Additionally, only labels near the electrode surface are detected, enabling non-washed assays.

Mitogen-activated protein kinase (MAPK) is a family of evolutionarily conserved eukaryotic serine/threonine protein kinases which link receptors on the cell surface to important intracellular regulatory targets (Keshet & Seger 2010). The MAPK is activated by phosphorylation in response to growth factors, mitogens, inflammatory cytokines, G-protein coupled receptors (GPCRs) or stress. And the cell regulates responses leading to cell proliferation and differentiation, development, inflammation, and cell survival or apoptosis. Extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinases (JNK) and p38 are all MAPKs, which offer desirable targets for the development of anti-inflammatory therapeutics (Zhang & Dong 2005).

However, there is no information on the molecular targets of sinomenine in the inflammatory cascades, the cellular signaling pathways involved in cytokine production and the underlying mechanisms of action. To investigate whether the inhibition of inflammatory mediator secretion by sinomenine is mediated through the MAPK pathway, we examined the effect of sinomenine on LPS-induced phosphorylation of ERK1/2, JNK and p38 by electro chemiluminescent immunoassay using MAPK phosphoprotein assay whole cell lysate kit. It was hoped these studies would provide a theoretical basis for further research on the potential for certain types of alkaloids to be used as novel anti-endotoxin/-inflammatory agents.

MATERIALS AND METHODS

Reagents

Pure sinomenine (at 20 mg/vial; lot #110774) were purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Sinomenine was diluted to 1 mg/ml with Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) containing 10% fetal bovine serum (FBS; Sigma, St. Louis, MO), 100 U/mL penicillin and 10 μg/ml streptomycin (Gibco, Grand Island, NY). The diluted solutions were then filtered through a 0.22-μm membrane and stored at 4°C. Before use, the agents were diluted 10-fold with medium. LPS from Escherichia coli (Type O_{55}:B_5; Sigma, St. Louis, MO) was prepared following the same procedure as above. Porcine iliac artery endothelial cells (PIECs) were obtained from the Committee on Type Culture Collection of Chinese Academy of Sciences (Shanghai, China), and the cell line was developed from a single host animal.

Experimental protocol

When cultured single-layer endothelial cells reached confluence in a cell incubator (BB15; Thermo Scientific, Chicago, IL), they were allocated into four experiments. In pathological model experiment, cells were challenged with LPS at three concentrations (0.1, 0.5, or 1 μg/mL) for 24 hr. In drug safety experiment, cells were treated with sinomenine at three concentrations (1, 5, or 10 μg/mL) for 24 hr. In treatment experiment, cells were pre-treated with 1 μg/mL LPS for 12 hr, followed by sinomenine at three concentrations (1, 5, or 10 μg/mL) incubation for a further 12 hr. In prevention experiment, cells were treated with sinomenine at three concentrations (1, 5, or 10 μg/mL) for 12 hr, then challenged with 1 μg/mL LPS for a further

Tab. 1. Mean values (± SD) of ERK1/2, JNK and p38 phosphorylation levels in model experiment

Groups	Lipopolysaccharide concentration			Blank control
	1 μg/ml	0.5 μg/ml	0.1 μg/ml	0 μg/ml
ERK1/2	45.50 ± 7.42 *	46.33 ± 7.34 *	37.67 ± 7.76	36.17 ± 7.83
JNK	453.17 ± 67.70 *	421.17 ± 76.98	399.00 ± 70.09	370.50 ± 47.28
p38	79.17 ± 11.02 **	73.17 ± 10.11 *	54.00 ± 9.47	58.33 ± 10.50

Data shown are mean (± SD)
Value significantly different from Blank control group at *$p < 0.05$ or ** $p < 0.01$.

Tab. 2. Mean values (± SD) of ERK1/2, JNK and p38 phosphorylation levels in safety experiment

Groups	sinomenine concentration			Blank control
	10 μg/ml	5 μg/ml	1 μg/ml	0 μg/ml
ERK1/2	39.67 ± 6.19	39.83 ± 6.74	41.00 ± 5.93	35.67 ± 8.02
JNK	379.83 ± 59.51	342.50 ± 50.47	358.17 ± 52.67	365.50 ± 56.86
p38	53.33 ± 9.56	60.17 ± 9.43	60.50 ± 10.10	57.00 ± 8.05

Data shown are mean (± SD)

12 hr (Tabandeh *et al.* 2022; Xiang *et al.* 2022; Zou *et al.* 2022). Moreover, the cell viability was measured using the Cell Counting kit-8 (Dojindo Molecular Technologies Inc., Kumamoto, Japan). After each group of experiments ended, cells were lysed at concentrations of 1-5 x 10^7 cells per mL of lysis buffer. Clear cellular debris from the lysate were centrifuged greater than or equal to 10000 x g, at 2-8 °C for 10 minutes. Discard the pellet and determine protein concentration in the lysate were used a detergent compatible protein assay such as BCA. Unused lysates were aliquoted and quickly frozen in a dry ice-ethanol bath and stored at ≤ -70 °C.

Assay Protocol

Assay-specific components for the quantitative determination of phospho-ERK1/2 (Thr/Tyr: 202/204; 185/187), phospho-JNK (Thr183/Tyr185) and phospho-p38 (Thr180/Tyr182) in whole cell lysates were provided by MAPK whole cell lysate kit (Meso, Rockville, USA). Firstly, plate was blocked and samples were prepared. Blocking solution (150 μL) was added into each well. The plate was sealed with an adhesive plate seal and incubated for 1 hr with vigorous shaking (300–1000 rpm) at room temperature. Complete lysis buffer was prepared just prior to sample dilution. Positive and negative cell lysates were prepared and kept on ice until use. Secondly, samples were washed and added. The plate was washed three times with 300 μL/well of Tris wash buffer. Samples (25 μL) were added to each well of the MSD plate. The plate was sealed with an adhesive plate seal and incubated for 3 hr with vigorous shaking (300–1000 rpm) at room temperature. Then, plate was washed and detection antibody was added. The plate was washed three times with 300 μL/well of Tris wash buffer. Detection antibody solution (25 μL) was added to each well of the MSD plate. The plate was sealed with an adhesive plate seal and incubated for 1 hr with vigorous shaking (300–1000 rpm) at room temperature. Lastly, plate was washed and read. The plate was washed three times with 300 μL/well of Tris wash buffer. Read buffer (150 μL) was added to each well of the MSD plate. The plate was analyzed on SECTOR Imager within 5 minutes of read buffer addition.

Statistical analyses

Data were from six repeats and expressed as mean values ± SD statistical analysis involved the paired Student's t test and ANOVA with SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was accepted at $p < 0.05$.

RESULTS

The model experiment

The phospho-ERK1/2, phospho-JNK and phospho-p38 levels in model experiment were shown in Table 1. Compared with the blank control, phospho-ERK1/2 level was significantly increased for the high- and middle-dose LPS groups. These effects were increased by 25.8 and 28.1% with, respectively, the high-dose and middle-dose treatments. Compared with the blank control, phospho-JNK level was significantly increased for the high-dose LPS group. This effect was elevated by 22.3% with the high-dose treatment. Compared with the blank control, phospho-p38 level was significantly increased for the high- and middle-dose LPS groups. These effects were increased by 35.7 and 25.4% with, respectively, the high-dose and middle-dose treatments.

The safety experiment

The phospho-ERK1/2, phospho-JNK and phospho-p38 levels in safety experiment were shown in Table 2. Compared with the blank control, there were no significant differences between the values for

Tab. 3. Mean values (± SD) of ERK1/2, JNK and p38 phosphorylation levels in treatment experiment

Groups	sinomenine concentration			Lipopolysaccharide control
	10 μg/ml	5 μg/ml	1 μg/ml	1 μg/ml
ERK1/2	38.17 ± 6.97 *	39.00 ± 5.90 *	45.83 ± 7.14	47.00 ± 5.37
JNK	414.50 ± 50.04	437.00 ± 36.57	395.83 ± 65.65	446.33 ± 59.76
p38	63.17 ± 9.70 *	64.33 ± 8.29 *	69.67 ± 10.29	79.00 ± 12.12

Data shown are mean (± SD)
Value significantly different from LPS control group at *$p < 0.05$.

Tab. 4. Mean values (± SD) of ERK1/2, JNK and p38 phosphorylation levels in prevention experiment

Groups	sinomenine concentration			Lipopolysaccharide control
	10 μg/ml	5 μg/ml	1 μg/ml	1 μg/ml
ERK1/2	35.83 ± 6.18 *	39.67 ± 7.09	40.50 ± 5.89	45.67 ± 6.95
JNK	392.83 ± 38.44 *	443.67 ± 73.40	444.83 ± 52.32	464.83 ± 63.26
p38	60.50 ± 11.81 **	65.00 ± 9.72 *	72.17 ± 10.55	78.17 ± 9.91

Data shown are mean (± SD)
Value significantly different from LPS control group at *$p < 0.05$ or ** $p < 0.01$.

production among the various ERK1/2, JNK, or p38. No doses of sinomenine had any impact on the drug-induced effect on phospho-ERK1/2, phospho-JNK and phospho-p38 levels. In addition, the indicated drugs did not affect viability of endothelial cells.

The treatment experiment

The phospho-ERK1/2, phospho-JNK and phospho-p38 levels in treatment experiment were shown in Table 3. Compared with the LPS control, phospho-ERK1/2 level was significantly decreased for the high- and middle-dose sinomenine groups. These effects were inhibited by 18.8 and 17.0% with, respectively, the high-dose and middle-dose treatments. Compared with the LPS control, no doses of sinomenine had any impact on phospho-JNK levels. Compared with the LPS control, phospho-p38 level was significantly decreased for the high- and middle-dose sinomenine groups. These effects were suppressed by 20.0 and 18.6% with, respectively, the high-dose and middle-dose treatments.

The prevention experiment

The phospho-ERK1/2, phospho-JNK and phospho-p38 levels in prevention experiment were shown in Table 4. Compared with the LPS control, phospho-ERK1/2 level was significantly decreased for the high-dose sinomenine group. This effect was inhibited by 21.5% with the high-dose treatment. Compared with the LPS control, phospho-JNK level was significantly decreased for the high-dose sinomenine group. This effect was inhibited by 15.5% with the high-dose treatment. Compared with the LPS control, phospho-p38 level was significantly decreased for the high- and middle-dose sinomenine groups. These effects were suppressed by 22.6 and 16.8% with, respectively, the high-dose and middle-dose treatments.

DISCUSSION

The activation of the different MAPK signaling cascades is believed to control different steps in the pro-inflammatory cytokine production process (Xagorari *et al.* 2002).The downstream targets of LPS-induced inflammatory cascades in cells are MAPK family members such as ERK1/2, JNK and p38 (Kaminska 2005). ERK-related pathways, which are involved in signal transduction from receptor tyrosine kinases, growth factor receptors, some G-protein-coupled receptors and TLRs and which promote cell proliferation, differentiation and particularly, macrophage activation and polarization (Zhang & Dong 2005). JNK pathway is activated in response to cytokines such as TNF-α, which likely induces the activation of macrophages towards phagocytic and migratory activities (Huang *et al.* 2004). The p38 pathway, which is also related to the secretion of cytokines such as TNF-α and IL-1β, could be related to the activation of other TLRs, the IL-1β receptor or the TNF-receptor family (Reuter *et al.* 2000). In summary, we observed that LPS increased the activation of classically activated endothelial cells via MAPK phosphorylation, which might result in an increased pro-inflammatory response. Additionally, activated MAPKs might facilitate cell migration and phagocytosis by modulating actin microfilament dynamics and cell adhesion as has been previously reported (Beggs *et al.* 2015). The elevated phosphorylation levels of ERK1/2, JNK and p38 MAPK were observed in high-dose LPS group. So, we decided to select 1 μg/mL as negative

standard for subsequent experiments. In the previous study, the ERK1/2 and JNK1/2 phosphorylation were determined in neonatal mouse cardiomyocytes exposed to 10 μg/ml LPS, whereas neonatal rat cardiomyocytes were stimulated with 1 μg/ml LPS (Geoghegan-Morphet *et al.* 2007; Yu *et al.* 2014). Although it is difficult to explain this inconsistency, it is reasonable to speculate that some factors, such as LPS concentration and species, may contribute to these discrepant results.

One of the advantages of MSD assays is the minimal sample volume required as compared to a traditional Western blot, which is also limited by its inability to measure more than a single analyte. With an MSD assay, up to ten different biomarkers can be analyzed simultaneously using as little as 10-25 μL of sample. We did not observe any change in ERK1/2, JNK, or p38 phosphorylation after sinomenine treatment in normal control cells. Treatment with sinomenine was not cytotoxic as measured by both trypan blue exclusion and tetrazolium salts (MTT) tests. Additionally, sinomenine did not alter the proportion of helper and cytotoxic T lymphocytes, NK cells or macrophages. These were proved that sinomenine was safe without obvious toxicity and side effects. Previous studies reported that MAPKs especially p38/JNK have been implicated in the regulation of inflammatory mediators, including the pro-inflammatory cytokines, which make them potential targets for anti-inflammatory therapeutics (Peng *et al.* 2009; Coronella-Wood *et al.* 2004). Recently, there is increasing evidence indicating a possible association of sinomenine with several processes involved in several inflammatory diseases.

Sinomenine after-treatment resulted in the inhibition of LPS-induced phosphorylation of ERK1/2 and p38, whereas it did not affect JNK phosphorylation in this experiment. The concentration dependent inhibition of sinomenine on ERK1/2 and p38 secretion was observed. This could provide a molecular basis for the therapeutic use of sinomenine against various inflammatory diseases. In support of these observations, other studies have also demonstrated that. Sinomenine has a non-specific but comprehensive protective function for the body, which could simultaneously reduce inflammatory cytokine expression, suppress the transcription of apoptosis genes, accelerate the synthesis of protein, and enhance the energy metabolism of cells to play the anti-endotoxin effects (Hu *et al.* 2018). The combined treatment of sinomenine and acupuncture on collagen-induced arthritis takes effect through the nuclear factor κB and MAPK signaling pathway (Xu *et al.* 2018). Sinomenine induced breast cancer cell death through ROS-dependent and -independent pathways with an upregulation of MAPKs, indicating that sinomenine may be a potential anti-tumor drug for breast cancer treatment (Li *et al.* 2014). Sinomenine might suppress the antigen-induced activation of RBL-2H3 cells via a Ca2+ independent pathway (Huang *et al.* 2008). Our results showed that sinomenine significantly attenuated LPS-induced ERK1/2 and p38 phosphorylation. These data suggest that sinomenine induces the classical activation pathway in various cells via the phosphorylation of MAPK thus activating the adaptive immune system. Above inconsistency on MAPK activation may be largely as a result of the different time-point of MAPK phosphorylation determination, which needs to be further investigated.

At higher sinomenine (10 μg/ml), the pretreatment resulted in the blockade of LPS-induced phosphorylation of ERK1/2, JNK and p38, while 5 μg/ml sinomenine could inhibit LPS-induced p38 phosphorylation. These results showed that sinomenine as a preventive agent against endotoxin, the pharmacological effect is more extensive compared with therapy. It has a clear repair and positive effect for antimicrobial, detumescence and wound healing on pathological damage. Protective effect of sinomenine on the LPS-induced phosphorylation of MAPK was only observed at 10 μg/mL, and it was p38-dependent. Since p38 activation in the present study was more responsive to the inhibitory effects of lower concentration of sinomenine than the other MAPKs that require higher concentration of sinomenine, it is assumed that the anti-inflammatory effect of sinomenine in endothelial cells may depend primarily on the inhibition of the p38 MAPK activation. These results suggested that the suppression of phosphorylation of ERK1/2, JNK and p38 might be implicated in the inhibition of pro-inflammatory mediators in LPS-stimulated cells and that different concentrations of sinomenine would be required to target each signaling molecule. Therefore, multiple signaling pathways are involved in the anti-inflammatory effects of sinomenine in an endothelial cells model of inflammatory injury. However, the precise mechanism of the signaling pathway is still unclear and conduct of further studies is needed.

The present results suggest that sinomenine might potentially have a use as part of a therapeutic regimen to treat toxicities from endotoxin exposure, as demonstrated by animal in vivo experiments (Hu *et al.* 2016) and cell in vitro experiments (Hu *et al.* 2015) in our laboratory. Sinomenine could reduce the incidence/severity of certain LPS-induced toxicities, e.g., body temperature elevation, cell adhesion, systemic inflammation, and multiple organ dysfunction. Sinomenine can downregulate the NF-κB-mediated pro-inflammatory cytokines (Hu *et al.* 2012) and their gene expression. IL1A and FMO3, were considered as potent target genes (Hu *et al.* 2018). As it has been confirmed that endothelial cells are important primary targets for systemic diseases, they represent good models for drug research and development. We compared simultaneously six alkaloids before, and the effect of sinomenine on antiendotoxin was the best. So, we think different genes can be induced or inhibited when treated with another anti-inflammatory agent. Rat intestinal microvascular endothelial cells (RIMECs) have been used

before for antiendotoxin research by author from rat genome array. Up-regulation and down-regulation of differentially expressed genes were significantly less than results of this study (Hu *et al.* 2009).

CONCLUSIONS

The present results suggest that sinomenine might potentially have a use as part of a therapeutic regimen to treat toxicities from endotoxin exposure. Most of these effects could be attributed to its anti-endotoxin and anti-inflammatory effects, which might be associated with depressing MAPK signaling pathway. Clearly, more detailed studies, and pharmacologic (PK/PD) studies are needed to better clarify if there will ever be any potential utility of sinomenine in clinical settings.

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DECLARATION OF INTEREST

The authors declare no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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