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Full Length Research Paper

Apoptosis induced by *Staphylococcus aureus* in human monocytic U937 cells involves Akt and mitogen-activated protein (MAPK) phosphorylation

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***Staphylococcus aureus* (*S. aureus*) is a leading etiologic agent of nosocomial and community-acquired infectious diseases. Numerous studies have shown that, *S. aureus* could promote apoptosis in host cells. Unfortunately, the cellular and molecular mechanisms responsible for this phenomenon are still poorly understood. The present study aims to elucidate the signaling mechanisms involved in *S. aureus*-induced U937 cells apoptosis by investigating the role of phosphatidylinositol 3-kinase/Akt (PI3K/Akt), mitogen-activated protein (MAPK) and caspases. Our results showed that, *S. aureus* decreased the expression of phosphorylation-Akt. In contrast, *S. aureus* increased phosphorylation-JNK1/2, phosphorylation-ERK1/2 and phosphorylation-p38 MAPK. Treatment of U937 cells with *S. aureus* resulted in the activation of caspase-3 and -9. Furthermore, caspases inhibitors, SP600125 (JNK inhibitor), SB203580 (p38MAPK inhibitor) and PD98059 (ERK inhibitor) decreased apoptosis in U937 cells. However, LY294002 (Akt inhibitor) increased U937 cells apoptosis. Taken together, our study for the first time suggest that *S. aureus* is able to enhance apoptosis of U937 cells through inhibition of PI3K/Akt and activation of MAPK signaling pathways.**

Key words: *Staphylococcus aureus*, mitogen-activated protein (MAPK), apoptosis, U937.

INTRODUCTION

Staphylococcus aureus is a major human pathogen causing significant morbidity and mortality due to both community- and hospital-acquired infections. This pathogen causes a variety of diseases, including impetigo, cellulitis, food poisoning, toxic shock syndrome, necrotizing pneumonia, and endocarditis (Szabados et al., 2010; Momtaz et al., 2010). During the disease process, the primary host cells are thought to be macrophages and monocytes, which are necessary for the innate immune response to bacterial infections.

Apoptosis or programmed cell death is an important physiological mechanism, through which the human immune system regulates homeostasis and responds to diverse forms of cellular damage. Apoptosis may also be involved in immune counteraction to microbial infection (Baines and Molkentin, 2005). There is growing evidence that bacteria or their products can induce apoptosis in host cells, including monocytes/macrophages and it has been suggested that, bacterium induced apoptosis of monocytes/ macrophages promotes an inflammatory response that causes tissue damage. Several studies showed that, whole live *S. aureus* induces apoptosis in monocytes, chondrocytes, keratinocytes, endothelial cells, epithelial cells and osteoblasts (Weglarczyk et al., 2004; Lee et al., 2001; Mempel et al., 2002; Menzies and Kourteva, 2000; Wesson et al., 2000). In both of cell types, *S. aureus* promptly escapes from the endosomes/ phagosomes and proliferates within the cytoplasm, which quickly leads to host cell death (Malgorzata et al., 2008). Despite the emerging evidence of its importance, no studies have been reported to date to elucidate the effect of *S. aureus* on human monocytic U937 cells. Therefore, this study sought to investigate whether treatment with *S. aureus* modulates cell apoptosis and the signaling pathway events in U937 cells. In the present study, results provide novel insights into the intracellular mechanism of *S. aureus* on U937 cell apoptosis.

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Apoptosis is highly regulated and consists of diverse upstream pathways for the transmission of extracellular death signals into intracellular events (Koziel et al., 2009). The PI3K/Akt and mitogen-activated protein (MAPK) signaling pathways are known to play pivotal roles in mediating cell growth and apoptotic responses (Wei et al., 2010; Zhang, et al., 2010). In recent years, Akt has been shown to impact microbial pathogenesis. A number of bacterial pathogens are able to modulate host cell survival via effects on Akt (Wiles et al., 2008). Among these are *Salmonella enterica*, *Shigella flexneri* and *Neisseria gonorrhoeae*, which were inject into their host cells effector molecules that can activate Akt and thereby inhibit apoptosis during infection (Chiu et al., 2009; Edwards, 2010). MAPK family includes extracellular signal- regulated kinase (ERK), p38 MAPK and c-Jun N-terminal kinase (JNK) (Singh et al., 2010). In many cell types, the ERK cascade appears to mediate specifically with cell growth and survival signals. In contrast, the p38 and JNK families appear to be proapoptotic effects (Zhao et al., 2007). As a result of the involvement of Akt and MAPK in other bacterial invasion systems, the role of Akt and MAPK in the invasion of human U937 cells by *S. aureus* strain Wood 46 was examined.

In our previous study, we found that *S. aureus* strain Wood 46, a capsular strain encoding α-hemolysin, can induce apoptosis in U937 cells by inhibiting Akt-regulated NF-κB. Moreover, the expression of Bax and phospho-JNK significantly increased in *S. aureus*-treated with U937 cells (Wang et al., 2009a, b). We report herein that, *S. aureus* induced a time-dependent and dose-dependent activation of several members of the MAPK family, including ERK 1/2 and p38 MAPK, upon association with U937 cells. Moreover, the triggering of caspase-3 and -9 activation mediated apoptotic induction. These studies are the first to examine intracellular signaling in U937 cells in response to *S. aureus* infection.

MATERIALS AND METHODS

Reagents

Monoclonal anti-β-actin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). They include: Phospho-specific p38 MAPK and p38 MAPK; phospho-specific ERK1/2 and ERK1/2 (New England Biolabs, Bevely, MA); stocks of the selective PI3K/Akt inhibitor LY294002; stocks of the selective p38 MAPK inhibitor SB203580; stocks of the selective JNK1/2 inhibitor SP600125; stocks of the selective ERK1/2 inhibitor PD98059 (Calbio-chem-Behring, Za Jolla, CA). RPMI-1640 and fatal bovine serum (FBS) were purchased from Gibco BIL Company (Gibco, NY, USA). An annexin V apoptosis detection kit was purchased from RD Systems (Abingdon, U.K.). Cell isolation and tissue culture reagents were obtained from Invitrogen life Technologies (Lidingo, Sweden). All other reagents were obtained from Sigma-Aldrich (St. Lowis, MO).

Preparation of bacteria

S. aureus strain Wood 46 (from American Type culture collections, Manassas, VA) were cultured for 18 h and then washed and resuspended in RPMI 1640 medium containing 5% fetal calf serum and 2 Mm L-glutamine (RPMI medium). For heat inactivation, *S. aureus* were incubated at 60°C for 30 min.

Induction of U937 cells apoptosis

Human monocytic U937 cells were used in all experiments and they were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutanine, 100 U/ml penicillin and 100 g/ml streptomycin (RPMI medium) incubating at 37°C in a humidified air containing 5% CO_2. Before infection with *S. aureus*, the cells were washed once with PBS to remove dead cells, resuspended in RPMI medium without the antibiotic and incubated in tissue-culture plates for 30 min at 37°C. U937 cells in multiwall tissure-culture plates were incubated with *S. aureus* at different multiplicity of infections (MOI of 0, 5:1, 10:1, 20:1, 40:1 CFU per cell) for 30 min at 37°C or at an MOI of 20:1 for 0, 15, 30, 60 and 90 min, respectively. In some experiments, different doses of PI3K inhibitor LY294002, ERK 1/2 inhibitor PD98059, JNK inhibitor SP600125 and p38 MAPK inhibitor SB203580 were used at 30 min before *S. aureus* infection.

Cell viability

To assess the overall viability of U937 cells following *S. aureus* infection, the cells were infected as described earlier. At a particular points in time or different MOI for 30 min after infection with bacteria, the U937 cells were washed two times with PBS and treated with a 0.4% solution of trypan blue and be visualized as clear cells under the microscope. U937 cells that are no longer viable have damaged membranes that allow entry of the dye, stain blue. Assays were performed in triplicate and repeated at least three times. The number of intact viable cells was expressed as a percentage of total cells and was assessed at different times post-infection.

Giemsa staining

At 0, 15, 30, 60 and 90 min after infection with the bacteria, the cells were collected and washed twice with PBS and fixed for 5 to 7 min with methanol at room temperature. The cells were air dried and stained for 15 min with giemsa stain prepared as instructed by the manufacturer. After the cells were washed three times with distilled water, they were air dried and observed microscopically under oil immersion.

Acridine orange staining

Incubate 25 µl of cell suspension (0.5×10^6 to 2.0×10^6 cells/ml) with 1 µl of AO/EB solution, mix gently. Each sample was mixed just prior to microscopy and quantification. 10 µl of cell suspension was place onto a microscopic slide, cover with a glass coverslip and examine at least 200 cells in a fluorescence microscope using a fluorescein filter.

Flowcytometry analysis

U937 apoptosis was quantified by flowcytometry using FITC-conjugated annexin V and PI. Specific binding of annexin V was achieved by incubating 10^6 cells in 60 µl of binding buffer saturated with annexin V for 15 min at 4°C in the dark. To discriminate between early apoptosis and necrosis, the cells were simultaneously stained with annexin V and PI before analysis. The binding of annexin V-FITC and PI to the cells was measured by flow cytometry

(FACS Calibur, BD Biosciences) using CellQuest software. At least 10 000 cells were counted in each sample. Experiments were performed and interpreted as follows: cells that were annexin V (-)/PI (-) (lower left quadrant) were considered as living cells, the annexin V (+)/PI (-) cells (lower right quadrant) as apoptotic cells, annexin V (+)/PI (+) (upper right quadrant) as necrotic or advanced apoptotic cells and annexin V (-)/PI (+) (upper left quadrant) may be bare nuclei cells in late necrosis or cellular debris.

Western blot analysis

After treatment, briefly, cells were washed once with ice-cold phosphate buffered saline containing 1 mM Na_2VO_4 and extracted with lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 5% Glycerol, 1% Triton X-100, 25 mM NaF, 2 mM Na_2VO_4, 10 µg/ml of each aprotinin, leupeptin and pepstatin). The preparation of cyto-plasmic was conducted using the NE-PER cytoplasmic extraction reagents (Pierce). The cell lysates were frozen and thawed three times and were further centrifuged at 14 000×g for 10 min at 4°C to pellet insoluble material. The supernatant of cell extracts was analyzed for protein concentration by a DC protein assay kit based on the Lowry method (Bio-Rad, Hercules, CA). Equal amounts of protein (50 µg) from each sample were separated on 10% sodium dodecyl sulfate-polyacrylamide gels and transferred to PVDF membranes (MSI, Westborough, MA). Membranes were blocked in 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween-20 (TBST) and then incubated with rabbit polyclonal for Phospho-specific p38 MAPK and p38 (1:2000 dilution); phospho-specific ERK1/2 and ERK1/2 (1:2000 dilution) overnight at 4°C. β-Actin (1:2000) was used to control for equal protein loading. The immunoblots were then washed three times with TBS-T buffer, incubated with a horseradish peroxidase conjugated secondary antibody (goat anti-rabbit IgM, Santa Cruz, CA) and developed using chemiluminescent substrate (PIERCE, Rockford, IL). To quantify and compare levels of proteins, the density of each band was measured by densitometry.

Measurement of caspase-3 and -9 activities

U937 cells were harvested and centrifuged at 1500 rpm for 10 min. Cells were washed two times with PBS (pH 7.4) and then resuspended with 50 µl lysis buffer at 4°C and incubated on ice for 10 min. All subsequent steps were performed on ice. After centrifugation, cell extracts were transferred to fresh tubes and protein con- centrations were measured. Each 50 µl cell extract containing 100 µg of protein were combined with equal volumes of 2 × reaction buffer in a microplate followed by the addition of 5 µl of peptide substrates of caspase-3 and -9. After overnight incubation in dark at 37°C, samples were read in a microplate reader at 405 nm. Caspase-3 and -9 activity were evaluated by the absorbance ratio of treated/control samples.

Statistical analysis

Each experiment was carried out in duplicate or triplicate and three or four independent experiments were performed. Results are expressed as means ± standard deviation (SD) and analyzed with SPSS 11.5 software. Results were compared using analysis of variance (ANOVA). When ANOVA showed a statistically significant difference, a group-by-group comparison was performed using a t-test with Tukey's correction for multiple comparisons. Statistical significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

S. aureus induces apoptosis in human U937 cells

We determined that *S. aureus* can induce apoptosis in U937 cells. In order to distinguish primary U937 cells apoptosis from cell necrosis, a trypan blue exclusion assay was performed. Trypan blue staining showed that, 98.03 ± 0.46% of the cells incubated with medium retained an integrated cell membrane (resisted trypan blue staining) (means ± standard errors of the means: n= 5). The percentage of necrosis cells rose with the increase of time and concentrations of *S. aureus* (Figure 1). The change in morphology of cells that were treated with *S. aureus* was microscopically examined. Chromatin condensation, one of the characteristics of apoptotic cells and fragmented nuclear fluorescence in treated cells were observed under a microscope after Giemsa and Acridine orange staining. Moreover, apoptotic bodies were observed (Figure 2).

Flowcytometry using FITC-conjugated annexin V revealed that, U937 cells exposed to *S. aureus* underwent rapid apoptosis. This effect was positively correlated with exposure time and excessive apoptosis was associated with loss of membrane integrity in an increased portion of U937, which indicates necrosis or late apoptosis (Data not shown). The apoptosis rate was positively correlated with the concentrations of *S. aureus* (Figure 3). In contrast, apoptosis rate was similar to that of control if bacteria were heat-inactivated before the infection.

S. aureus induces U937 apoptotic cell death via modulation of p38 MAPK and ERK1/2 signaling pathways

The MAPK signaling pathways are among the most ancient signal transduction pathways and the highly evolutionary conserved signaling regulators that are activated in response to infectious agents and innate stimulators, mediating important cellular responses such as proliferation, differentiation and apoptosis. P38 MAPK is activated by a variety of cellular stresses including ultraviolet light, infection, hyperosmolarity, heat shock and proinflammatory cytokines, and acts at early step prior to dysfunction of mitochondria and caspase activation (Ghatan et al., 2000; Chang and Karin, 2001; Grethe et al., 2004; Cowan and Storey, 2003). As MAPK pathways are reported to be required for cell growth and apoptosis, we hypothesized that, the U937 apoptosis induced by *S. aureus* was due to the mechanism of MAPK signaling cascades. The mechanism by which *S. aureus* exerts its effect on host cells is still controversial. Recent data suggested that, phosphorylation of p38 MAPK appears to be a conserved response of epithelial cells to subcytolytic concentrations of pore-forming toxins from *S. aureus* (Matthias et al., 2006). Some *S. aureus* strains enhanced

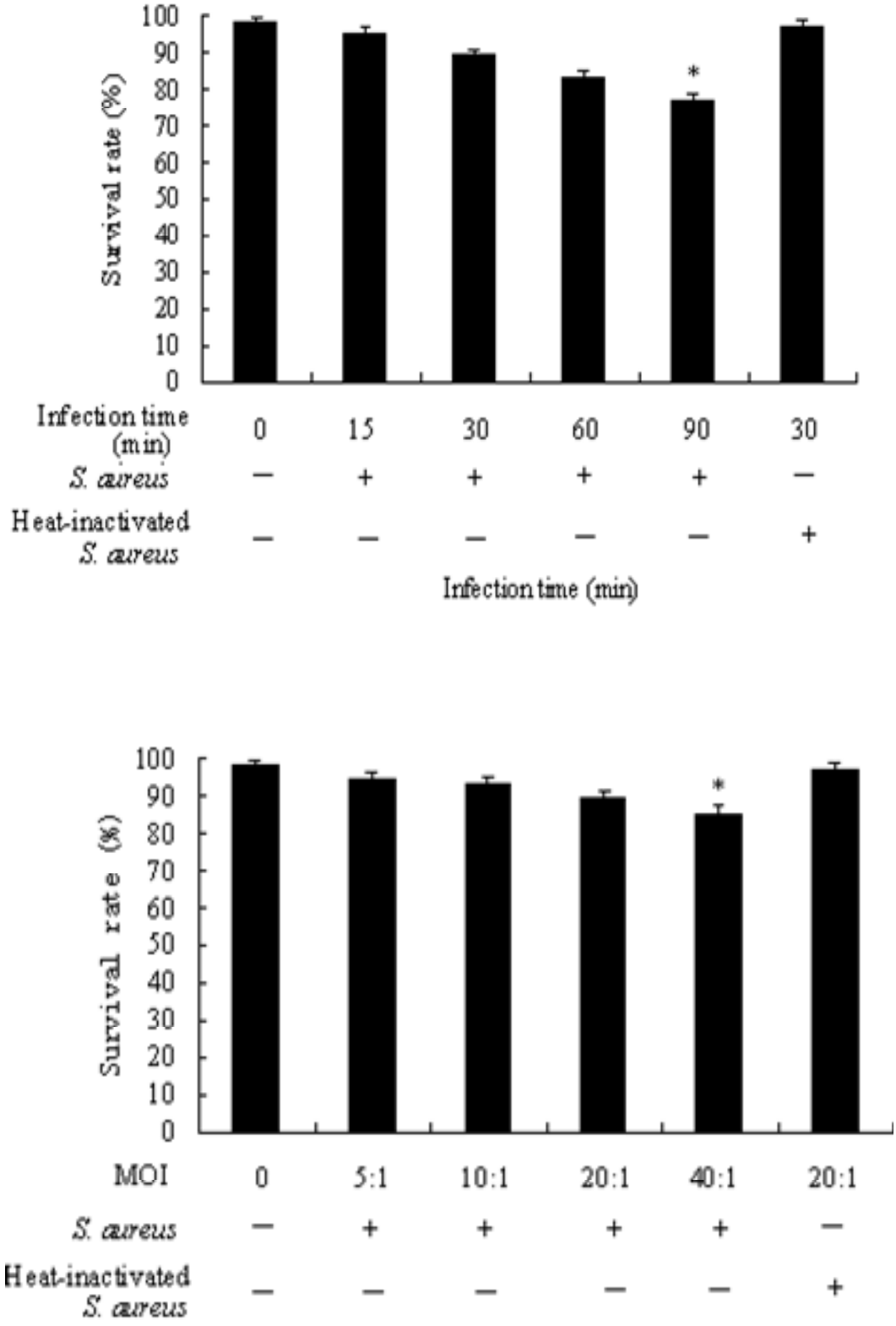

Figure 1. Effect of *S. aureus* on cell cytotoxicity of human monocytic U937 cells. U937 cells were incubated with different concentrations of *S. aureus* or heat-inactivated *S. aureus* for 30 min or at an MOI of 20:1 for different time points. Viability of U937 cells was determined by trypan blue assay. The number of dead cells was increased with *S. aureus* concentration and infection time. Data represent means ± SD of three determinations *$P < 0.05$ compared with that of 0 min.

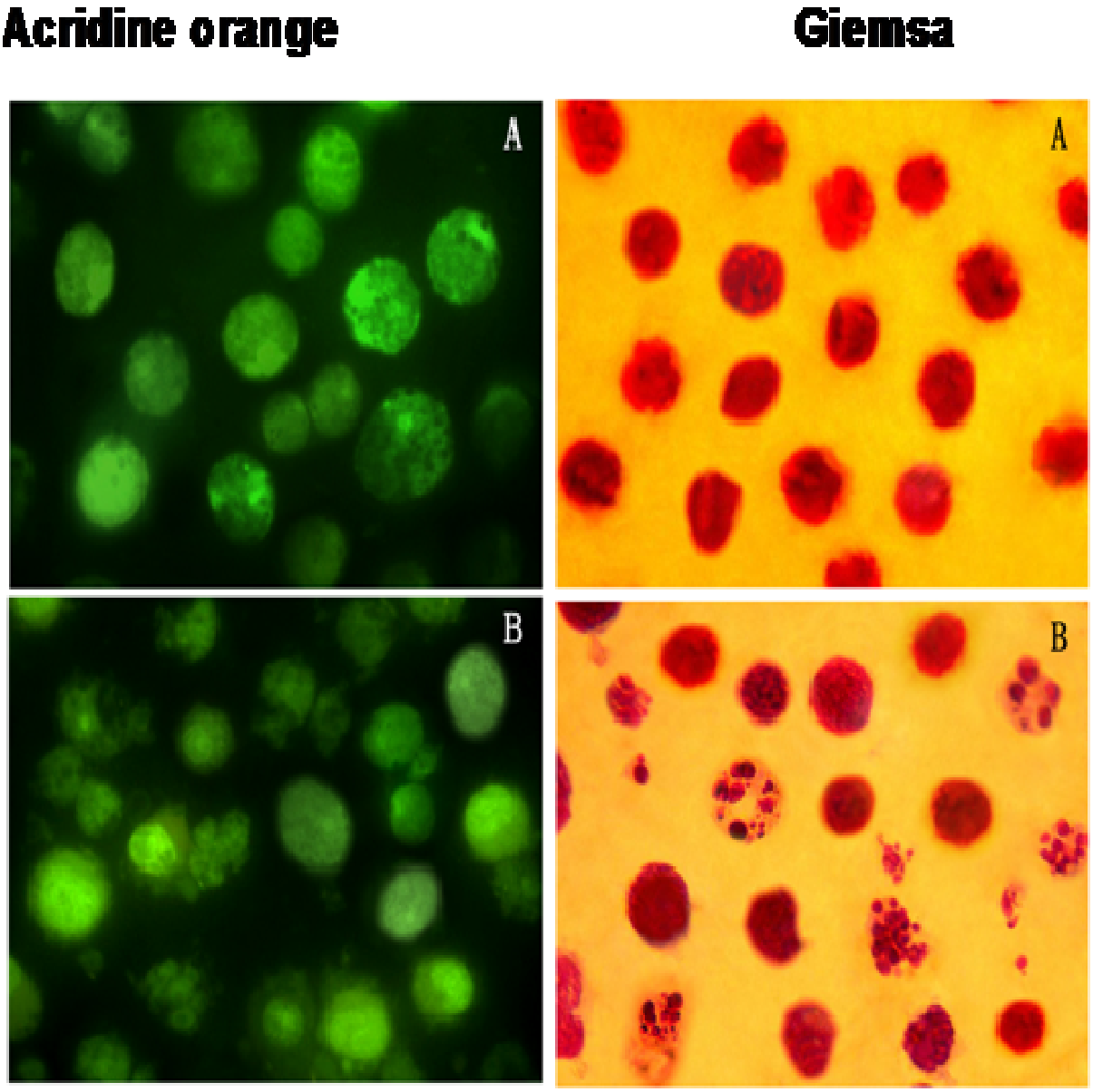

Figure 2. Morphology of U937 cells undergoing apoptosis. U937 cells were cultured without or with *S. aureus* strain Wood46. U937 cells in control (A) and treated (B), harvested for 30 min at an MOI of 20:1) were stained with Giemsa dye and Acridine orange dye

the oxidative reaction and this priming capacity was linked to p38-MAPK activation and induction of apoptosis in human neutrophils (Nilsdotter-Augustinsson et al., 2004).

In this study, after U937 cells were exposed to *S. aureus* at an MOI of 20:1, active p38 was detected in unstimulated U937 cells and induced further by *S. aureus*, appearing at 15 min, peaked at 30 min and returning to baseline levels after 90 min. However, the level of phosphorylated ERK (Figure 4) and JNK (Data not shown) increased at 15 min and peaked at 90 min after infection. The results were similar to that of control if bacteria were heat-inactivated before the infection (Figure 4). Although, SB203580 and PD98059 were able to inhibit phosphorylation of their specific proteins, the expressions of p38 MAPK and ERK1/2 protein in a whole cell lysate did not change.

Effects of inhibitors of Akt, p38 MAPK, ERK1/2, JNK on *S. aureus*-induced U937 apoptosis

To identify the relevance of Akt, p38 MAPK, ERK1/2 and JNK signaling pathways in controlling the apoptotic cell death by *S. aureus*, inhibition assays were performed with

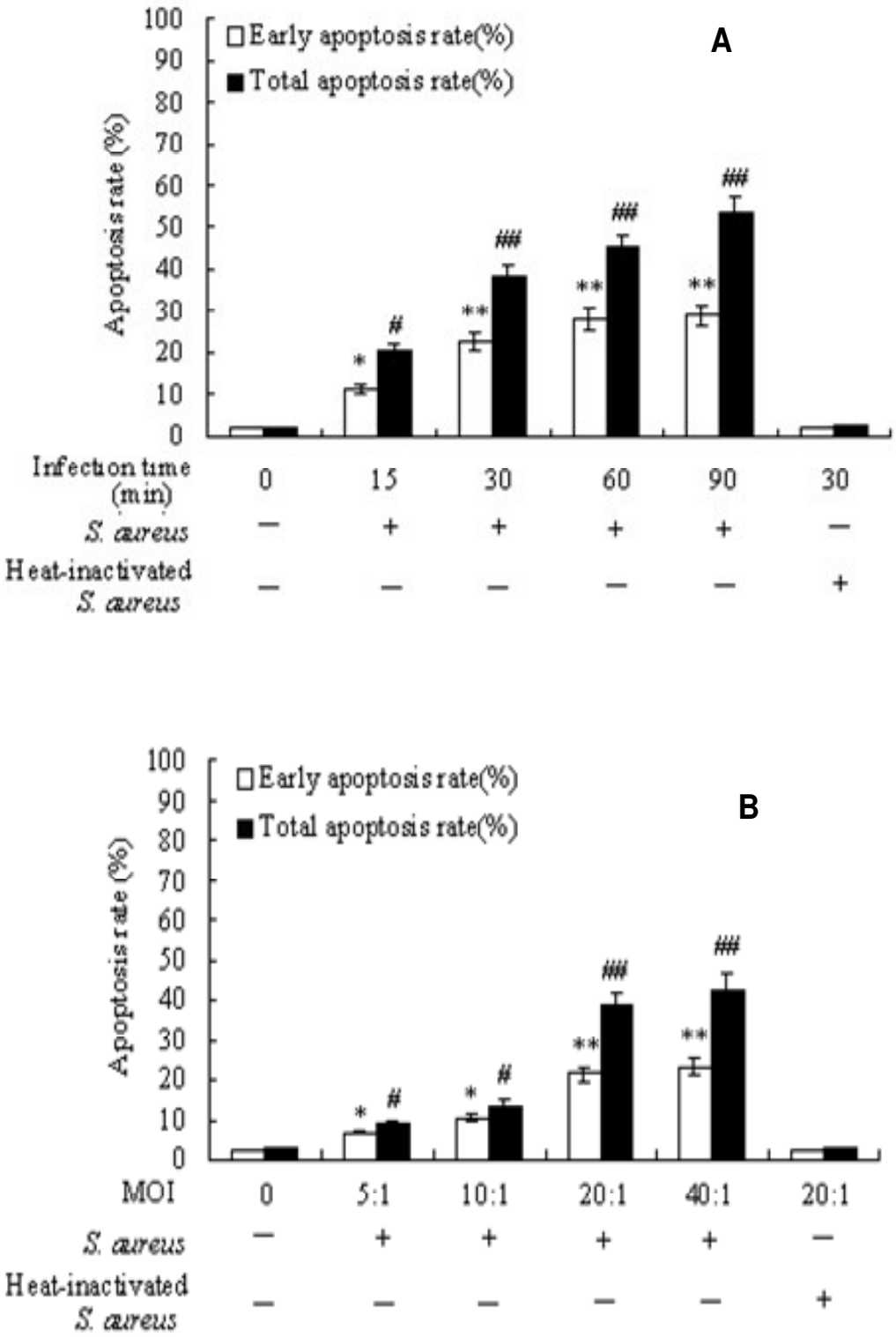

Figure 3. *S. aureus*-induced apoptosis in U937 cells. U937 cells were cultured without or with *S. aureus* strain Wood 46. U937 cells were harvested (A) at an MOI of 20:1 for different time points or for (B) 30 min at different MOI after *S. aureus* infection or and incubated with FITC-conjugated annexin V (AV) and propidium iodide (PI) double staining. Flow cytometric analysis was performed, and the data shown are representative of three separate experiments. The lower right quadrants represent early apoptotic cells that were stained by annexin V but not by propidium iodide. The upper right quadrants represent late apoptotic cells that were stained by both annexin V and propidium iodide. $^{*,\#}P < 0.05$; $^{**,\#\#}P < 0.001$, compared with that of U937 alone.

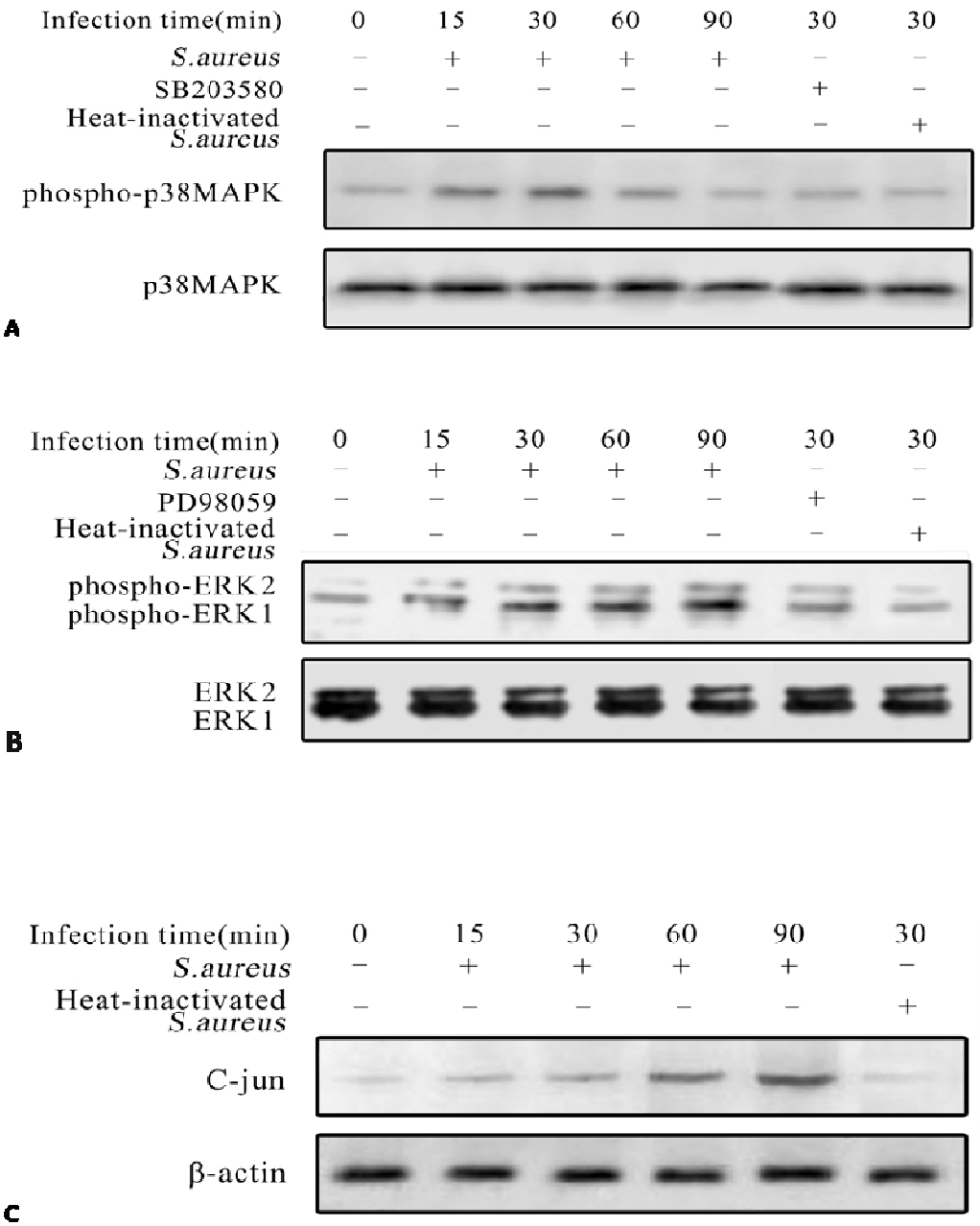

Figure 4. Involvement of p38 MAPK, ERK1/2 and c-Jun activation during *S. aureus* induced U937 cell apoptosis. The cells were pretreated with 20 μM SB203580 (A) and 20 μM PD98059 (B) and then incubated with *S. aureus* for the indicated time period. Cell lysate was separated by 12% SDS-PAGE; the phosphor-p38 MAPK and phosphor-ERK1/2; total p38 MAPK (A); ERK1/2 (B); c-Jun (C) band were detected by western blot analysis. Triplicate experiments gave similar results.

LY294002, SB203580, PD98059 and SP600125. The percentage of apoptosis was determined by flow cytometery. U937 cells were pretreated with different doses of LY294002, SB203580, PD98059 and SP600125 for 30 min and then cultured with *S. aureus* for 30 min. The results showed that, SB203580, PD98059 and SP600125 significantly reduced the apoptosis rate. However, LY294002 induced the apoptosis rate (Figure 5).

Expression of caspases activity

The expression of caspase-3 and -9 activity in U937 cells incubated in the presence of *S. aureus* is presented in Figure 6. Treatment of U937 cells with *S. aureus* showed a marked increase of caspase-3 and -9 activations. Activities of caspase -3 and -9 in U937 cells with *S. aureus* treatment showed time-dependent up-regulation.

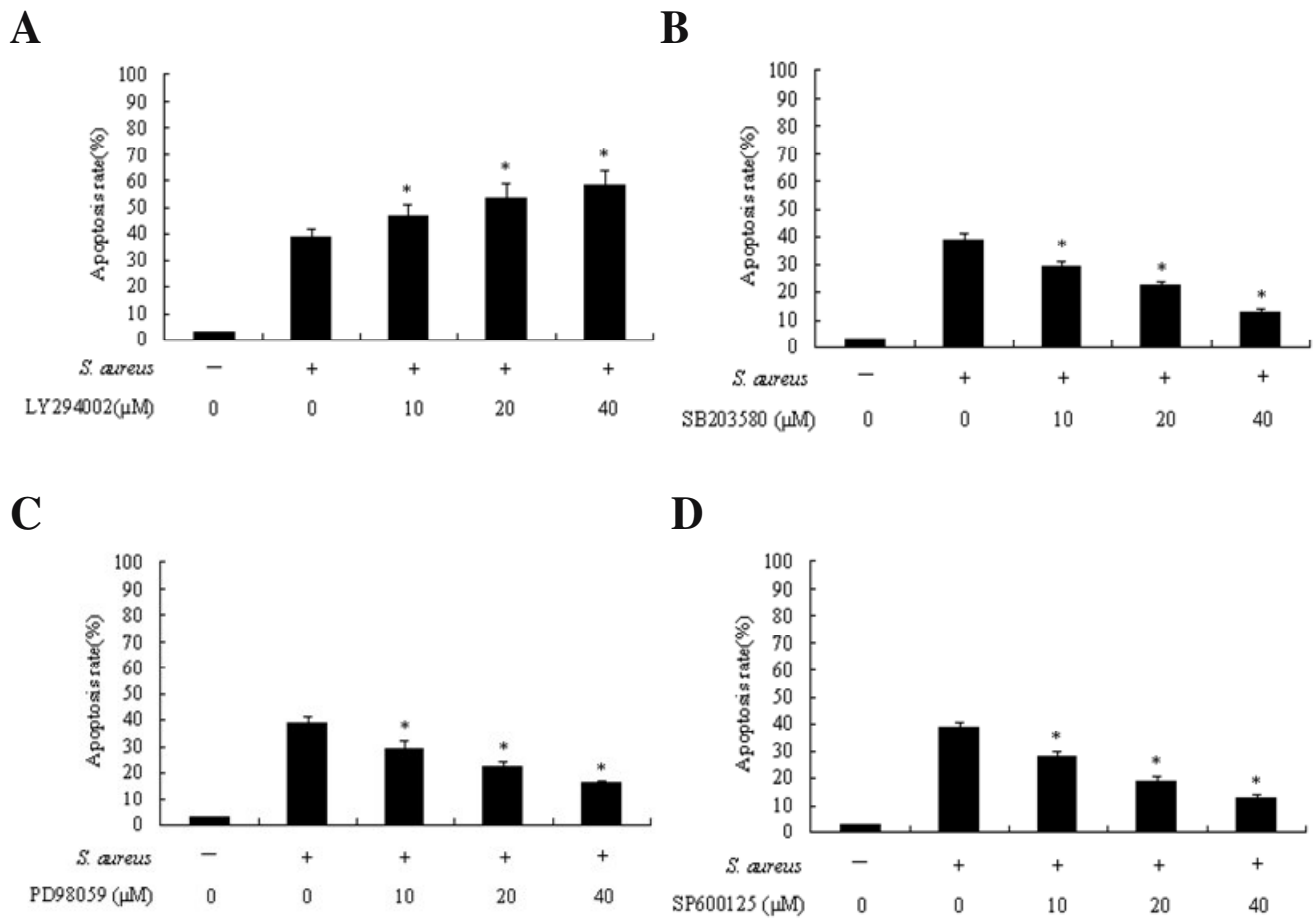

Figure 5. Effect of Akt or MAPK inhibitors on *S. aureus*-induced U937 cell apoptosis. Human monocytic U937 cells were treated with *S. aureus* at an MOI of 20:1 and incubated for 30 min with the indicated concentrations of Akt or MAPK inhibitors: (A) LY294002; (B) SB203580; (C) PD98059; (D) SP600125 for 30 min. Values represent means ± SD of five experiments performed in duplicate. *$P < 0.05$ compared with that of *S. aureus* alone.

Inhibition of PI3K/Akt pathway with LY294002 potentiated the *S. aureus* -induced caspase-3 and -9 activity, while the SB203580, PD98059 and SP600125, caused decline in the *S. aureus*-induced expression of caspase-3 and -9 activity. To characterize the pathway of apoptosis execution, experiments were carried out using the caspase inhibitors Z-DEVD-FMK (specific for caspase-3) and Z-LEDH-FMK (specific for caspase-9). Apoptosis was greatly reduced by Z-LEDH and Z-DEVD (Data not shown). Together, our data demonstrated that caspase-3 and -9 are caspases mediating *S. aureus* -induced U937 cell apoptosis.

The present study have found that the pharmacological inhibitor of PI3K, LY294002, dramatically exert caspase-3 and -9 activity under *S. aureus* treatment condition. Caspases, a family of cysteine proteases, are integral parts of the apoptotic pathway; caspase-3 in particular, when activated, has many cellular targets that, when severed and/or activated, produce the morphologic features of apoptosis (Cohen, 1997). In this study, apoptosis induced by treating cells with *S. aureus* was proof that *S. aureus* dose- and time-dependent up-regulated caspase-3 and -9 activities in U937 cells. The inhibitor of caspase-9 or caspase-3 could markedly block the *S. aureus*–triggered apoptosis.

Taken together, these results demonstrate for the first time that *S. aureus* induces U937 cell death via activation of p38MAPK, ERK1/2 and JNK, whereas activation of PI3K/Akt exerts an opposing action. These results may contribute to a better understanding of the signals involved in the phagocytic process of monocytic cells against *S. aureus*, which may be important in order to develop new antibacterial therapies. However, the overall mechanism of *S. aureus* invasion of U937 cells clearly involves a variety of converging signal transduction pathways. *S. aureus* invasion of U937 cells is a complex process, in which the bacterium exploits the host cell machinery and escapes the extracellular environment and humoral immune response. Moreover, the *in vivo* relevance of these results obtained from *in vitro* cell.

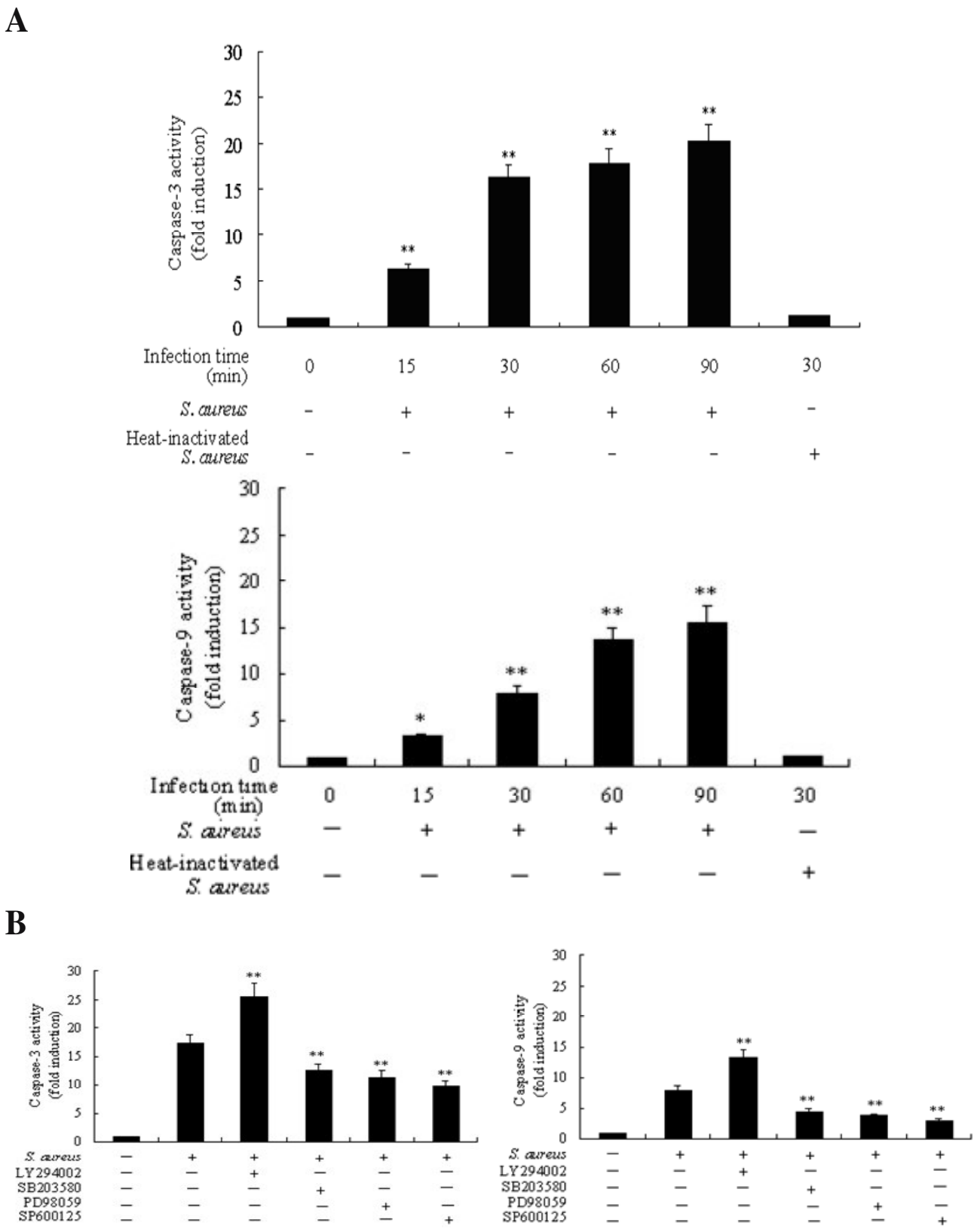

Figure 6. Effect of *S. aureus*, Akt inhibitor, MAPK inhibitors or caspase 3 and 9 inhibitors Z-DEVD-FMK and Z-LEHD-FMK on the activity of caspase-3 and 9. (A) Time-dependency of *S. aureus*-induced caspase-3 and 9 activity; (B) U937 cells were treated with *S. aureus* at an MOI of 20:1 and incubated for 30 min, with 20 μM of LY294002, PD98059, SP600125 or SB203580. Values represent means ± SD of five experiments performed in duplicate. $^{*}P < 0.05$, $^{**}P < 0.001$ compared with that of control.

culture needs to be verified in animal models

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