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## Ampicillin activates Mpk1 phosphorylation in *Saccharomyces cerevisiae* and ERK1/2 phosphorylation in HepG2 cells

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**Abstract:** Ampicillin has been widely used to treat bacterial infections. When we used ampicillin to eliminate bacterial contamination in yeast cultures, we observed induction of phosphorylation of MAP kinase 1 (Mpk1), a previously unknown function of ampicillin. We therefore investigated whether ampicillin activates the signal transduction pathway. Phosphorylation of *Saccharomyces cerevisiae* Mpk1 was induced by ampicillin in a dose- and time-dependent manner through the PKC1-CWI pathway. Mpk1 phosphorylation was maximal after treatment with 3 mM ampicillin for 90 min. Despite activation of Mpk1 phosphorylation, ampicillin did not influence yeast cell growth. Ampicillin reduced miconazole antifungal activity; miconazole had a minimum inhibitory concentration of 3.125 µg/mL against *Candida albicans*, which increased to 25 µg/mL after 48 h of treatment with 3 mM ampicillin. Finally, ampicillin activated phosphorylation of ERK1/2 (a mammalian homolog of Mpk1), with maximum effect at 3 mM ampicillin, in human HepG2 cells, but did not influence cell viability. The results of this study clearly indicate that ampicillin activated Mpk1 phosphorylation in yeast and ERK1/2 phosphorylation in HepG2 cells. In addition to its clinical application to eliminate bacteria, ampicillin could also be used to activate Mpk1 or ERK1/2 in the laboratory.

**Key words:** Ampicillin, CWI pathway, Mpk1, *Saccharomyces cerevisiae*, ERK1/2, HepG2, *Candida albicans*

### 1. Introduction

Ampicillin is a broad-spectrum antibiotic compound that is used for the treatment of many bacterial infections. It is relatively nontoxic and effective against many gram-positive and gram-negative bacteria, including *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Escherichia coli*, *Salmonella*, and *Neisseria meningitidis*. Ampicillin belongs to the family of beta-lactam antibiotics that irreversibly inhibit the transpeptidase activity required for the synthesis of bacteria cell walls (Acred et al., 1962; Kennedy et al., 1963; Seligman et al., 1963; Campos et al., 1992; Lacy et al., 1999; WHO Young Infants Study Group, 1999).

In yeast, the cell wall is the outermost component that protects the cell and receives external signals. The integrity and composition of the cell wall are essential for survival and adaptation to environmental stresses. Two pathways—the high-osmolarity glycerol (HOG1) pathway and the cell wall integrity (CWI) pathway—are important for maintenance of cellular integrity. Of these, the CWI pathway is considered the most essential for recognizing cell wall perturbations in many cell stress conditions (Levin,

2005; Kim et al., 2010; Kim and Levin, 2011; Shin and Kim, 2016). The CWI pathway is composed of cell surface receptors (Wsc1, Wsc2, Wsc3, Mid2, and Mtl1), transducer proteins (Rom2 and Rho1), calcium-dependent protein kinase (Pkc1), mitogen-activated protein (MAP) kinase kinase kinase (Bck1), MAP kinase kinases (Mkk1/2), and MAP kinase (Mpk1). Activation of Mpk1 by signals that are received and transmitted through the CWI pathway activates two downstream transcription factors (Rlm1 and Swi4/6), which are involved in the regulation of the expression of multiple cell wall components. One of the target genes of Swi4/6 is *FKS2*, which encodes the catalytic subunit of 1,3-beta-glucan synthase involved in β-glucan biosynthesis (Kim et al., 2008, 2010; Kim and Levin, 2011). Inhibition of the CWI pathway or mutation of involved proteins may sensitize cells to standard antifungal drugs (Levin, 2005; Pagán-Mercado et al., 2009; Shin and Kim, 2016).

One of the most widely used antifungal agents is miconazole, which inhibits the cytochrome P-450-dependent 14α-demethylation of lanosterol in the sterol biosynthesis pathway (Hitchcock et al., 1990; Ghannoum

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and Rice, 1999). Azole compounds are synergistically active with cell wall inhibitors and increase expression of *FKS2*, which participates in the CWI pathway and is also regulated by the PKC1 pathway (Cornelissen and Van den Bossche, 1983; Lorito et al., 1994; Edlind et al., 2002). The PKC1 pathway is associated with fungal resistance of *Candida albicans*. (LaFayette et al., 2010). Miconazole antifungal function is synergistically activated by inhibition of Mpk1 phosphorylation (Shin and Kim, 2016), suggesting that miconazole activity is influenced by the cell wall.

Several MAP kinase homologs have been identified, including ERK1/2 in humans. ERK1/2 is the effector component of the prototypical ERK1/2 MAP kinase pathway that regulates cell proliferation, differentiation, and survival (Davis, 1995).

When we tried to prevent bacterial contamination in yeast cultures using ampicillin, we unexpectedly found that ampicillin-treated controls showed elevated Mpk1 phosphorylation. Therefore, we aimed to investigate the regulation of MAPK phosphorylation by ampicillin in fungi and human cells. We showed that ampicillin activated Mpk1 phosphorylation in yeast and ERK1/2 phosphorylation in HepG2 cells and reduced miconazole antifungal activity.

## 2. Materials and methods

### 2.1. Strains and growth conditions

BY4742 *Saccharomyces cerevisiae* (*MAT $\alpha$  his3 $\Delta$ 1 leu2 $\Delta$ 0 lys2 $\Delta$ 0 ura3 $\Delta$ 0*), Y595 *bck1* deletion (*MAT $\alpha$  bck1 $\Delta$ ::G418*), and Y698 *mpk1* deletion mutant (*MAT $\alpha$  mpk1 $\Delta$ ::G418*) strains from the Yeast Knockout Collection (GE Healthcare, Aurora, OH, USA) were grown in YPD (1% Bacto yeast extract, 2% Bacto peptone, and 2% glucose) or SD (0.67% yeast nitrogen base and 2% glucose) medium supplemented with the appropriate nutrients to select for yeast strains harboring plasmids (MPK1-FLAG) at 30 °C. *Escherichia coli* DH5 $\alpha$  harboring YEp351-MPK1-FLAG (p2313; Shin and Kim, 2016) was cultivated in Luria broth medium (1% Bacto tryptone, 0.5% Bacto yeast extract, and 1% NaCl) supplemented with ampicillin at 37 °C. Ampicillin-resistant clones were used to propagate the plasmid by standard methods. The human hepatocellular carcinoma cell line HepG2 (Shin and Kim, 2016) was maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 g/L streptomycin, and 100 U/mL penicillin (all from Invitrogen). The cells were cultured in a humidified atmosphere containing 5% CO<sub>2</sub>. All chemicals, including ampicillin (A0166), were purchased from Sigma (St. Louis, MO, USA) or were of the highest available grade.

### 2.2. Immunoblot detection of phosphorylated Mpk1 in yeast cells

Immunoblotting was performed as described by Shin and Kim (2016) with slight modification. Y595 and Y698 strains harboring YEp351-MPK1-FLAG were grown overnight in SD medium. Yeast cells were divided among four or six tubes and continuously grown for 3 h at room temperature. After 1 h of treatment with test compounds at the indicated concentrations, cells were lysed with 200  $\mu$ L of cold lysis buffer (50 mM Tris/HCl (pH 7.5), 10% glycerol, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 50 mM NaF, 1 mM sodium orthovanadate, 50 mM  $\beta$ -glycerol phosphate, 5 mM sodium pyrophosphate, 5 mM EDTA, 1 mM PMSF, and protease inhibitor cocktail (Roche, Basel, Switzerland)) by vigorous vortexing with 0.45-mm glass beads. Protein extracts were collected by centrifugation at 13,000  $\times$  g for 10 min at 4 °C. Protein concentrations were determined by the Bradford assay (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Protein samples (20  $\mu$ g for Mpk1-FLAG detection and 30  $\mu$ g for phosphorylated Mpk1 detection) were separated on 7.5% polyacrylamide SDS-PAGE gels and transferred to nitrocellulose membranes (Amersham Hybond, GE Healthcare). Total Mpk1 was detected with anti-FLAG antibodies (dilution 1:4000; Cell Signaling Technology, Danvers, MA, USA) in the presence of 5% nonfat milk for 1 h followed by horseradish peroxidase-conjugated antimouse secondary antibodies (dilution 1:4000; antimouse IgG, HRP-linked antibody; Cell Signaling Technology) for 1 h. After stringently washing the membrane, an enhanced chemiluminescence (ECL) detection system (Santa Cruz Biotechnology, Houston, TX, USA) was used to detect signals. Phosphorylation of Mpk1 was detected by incubation with rabbit polyclonal anti-phospho-p44/p42 MAPK (Thr<sup>202</sup>/Tyr<sup>204</sup>) antibodies (New England Biolabs, Ipswich, MA, USA) at a dilution of 1:3000 as described previously (Kim et al., 2008; Shin and Kim, 2016) overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (donkey antirabbit antibody; GE Healthcare) at a dilution of 1:4000 for 1 h. Signals were detected using an ECL kit (Bio-Rad). Experiments were performed at least three times, and a representative image was selected. Densitometry analysis of the bands was performed with ImageJ software (NIH, Bethesda, MD, USA). In detail, the blots were scanned and capture at 1200 dpi. Band intensities were quantified using ImageJ (NIH). Pictures were divided by base images of blank membranes with the same exposure length to eliminate the background. Pictures were then inverted, and using the gel analysis features, band intensities were measured by selecting the band with the rectangle tool, graphing the intensity, and calculating the area of the intensity plots. Intensities of the selected bands from three

different experiments were then displayed in Excel and statistical analyses were carried out.

### 2.3. Immunoblot detection of phosphorylated ERK1/2 in HepG2 cells

HepG2 cells were plated at  $1 \times 10^6$  cells per 60-mm culture dish. Before ampicillin treatment, the medium was replaced with fresh medium without antibiotics. After treatment with the indicated concentrations of ampicillin, cells were washed with cold PBS and lysed using lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na<sub>2</sub>EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM  $\beta$ -glycerol phosphate, 1 mM sodium orthovanadate, 1  $\mu$ g/mL leupeptin) containing 1 mM PMSF. Total proteins were collected by centrifugation at  $13,000 \times g$  for 10 min at 4 °C and protein concentration was determined using the Bradford assay (Bio-Rad). Protein samples (50  $\mu$ g for total ERK1/2 detection and 100  $\mu$ g for phosphorylated ERK1/2 detection) were separated on 10% polyacrylamide SDS-PAGE gels and transferred to nitrocellulose membranes (Amersham Hybond). Membranes were incubated with anti-ERK1 (Cell Signaling Technology) or anti-phospho-p44/p42 MAPK (Thr<sup>202</sup>/Tyr<sup>204</sup>) antibodies (New England Biolabs) at a dilution of 1:3000 overnight and then with HRP-conjugated secondary antibodies (donkey antirabbit antibody; GE Healthcare) at a dilution of 1:4000 for 1 h. Proteins were detected using an ECL kit (Bio-Rad). Experiments were performed at least three times, and a representative image was selected. Densitometry analysis of the bands was performed with ImageJ software (NIH).

### 2.4. RT-PCR analysis

RT-PCR analysis was performed as described by Kim and Levin (2011). Briefly, cells growing in log phase were divided with three tubes at  $OD_{600} = 0.05$  and treated with ampicillin for 30 min or 90 min. One of the tubes was used as an untreated control. Total RNA was extracted as described previously (Schmitt et al., 1990; Kim and Levin, 2011). cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions and amplified by PCR using Taq polymerase with 18S rRNA forward primer (ACTTTTCGAATCGCATGGCC) and 18S rRNA reverse primer (GAATCGAACCCCTTATTCCCC), or *FKS2* forward primer (GCAAATTGCCTACCTGGATG) and *FKS2* reverse primer (CTCTTCCAAGTAATTGTCTTG) (Kim and Levin, 2011).

### 2.5. Antifungal susceptibility test

Antifungal activity against *S. cerevisiae* or *C. albicans* was tested using the broth microdilution assay based on the standard NCCLS document M27-A (NCCLS, 1997) as described in Kim et al. (2004) and Shin and Kim (2016). A stock of miconazole (10 g/L) was first serially 2-fold diluted with or without 3 mM ampicillin and dispensed

in duplicate into wells of a 96-well microtiter plate with a total volume of 100  $\mu$ L of RPMI 1640 medium (RPMI tissue culture medium supplemented with glutamine; Sigma) with 2% glucose, buffered to pH 7.0 with 0.156 M 3-*N*-morpholinopropanesulfonic acid (MOPS; Sigma). *S. cerevisiae* or *C. albicans* was inoculated with 100  $\mu$ L of suspension containing  $1 \times 10^4$  cells/mL. Final miconazole concentrations ranged from 200  $\mu$ g/mL to 0.2  $\mu$ g/mL. The last well was used for contamination control. MIC values of miconazole were determined as the lowest concentration of the compound that gave 100% inhibition of cell growth. Tests were performed at least three times.

### 2.6. Cell proliferation assay

*S. cerevisiae* proliferation was evaluated using a cell growth curve. *S. cerevisiae* was inoculated with 3 mL ( $OD_{600} = 0.1$ ) of suspension containing various concentrations of ampicillin (0–3 mM). After the indicated time, the cell proliferation rates were calculated from the optical density ( $OD_{600}$ ) readings. Experiments were performed at least three times.

HepG2 cell proliferation was evaluated using a cell-based MTT assay (Shin and Kim, 2016). Cells were seeded at  $1 \times 10^4$  cells per well in 96-well plates and incubated for 24 h. Cells were washed with PBS and then cultured in DMEM containing various concentrations of ampicillin (0–4 mM). After 48 h of incubation, the cells were washed and MTT solution in PBS was added to a final concentration of 0.5 mg/mL. After incubation for 3 h at 37 °C, absorbance was obtained at 520 nm using a microplate reader (VersaMax, Molecular Devices, Sunnyvale, CA, USA). The cell proliferation rates were calculated from the optical density readings and represented as percentages of those for the vehicle control.

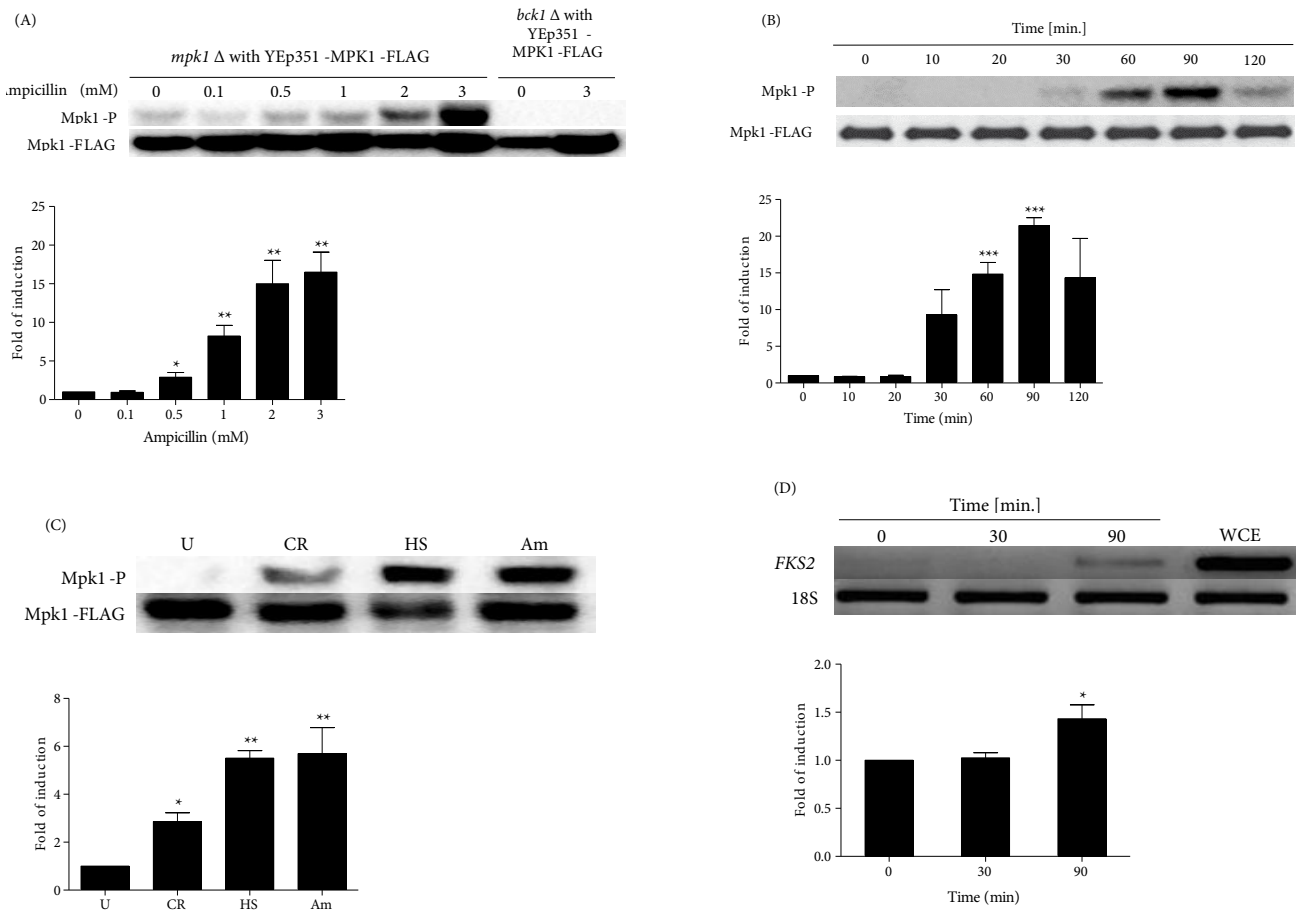
### 2.7. Statistical analysis

Values are expressed as the mean  $\pm$  SD. Differences between the control and samples were evaluated using Student's *t*-test.  $P < 0.05$  was considered statistically significant.

## 3. Results

### 3.1. Ampicillin-induced Mpk1 phosphorylation

To investigate PCK1-CWI pathway regulation in *S. cerevisiae* by ampicillin, we examined the induction of Mpk1 phosphorylation. Mpk1 phosphorylation was increased even from the 0.5 mM treatment, and it was clear above 2 mM ampicillin with a maximal effect at 3 mM. However, increased Mpk1 phosphorylation was not seen after treatment with 3 mM ampicillin in the *bck1* $\Delta$  deletion strain (Figure 1A). To determine the time dependency, we measured phospho-Mpk1 after treatment with 3 mM ampicillin for different times. Mpk1 phosphorylation was induced after 30 min and maximized at 90 min of ampicillin treatment (Figure 1B). Thus, phosphorylation of Mpk1 in



**Figure 1.** Ampicillin activates phosphorylation of Mpk1 in *S. cerevisiae*. (A) Mpk1 phosphorylation is dose-dependently stimulated by ampicillin. Mpk1 phosphorylation was examined after treatment with various concentrations of ampicillin for 1 h in a *bck1Δ* or *mpk1Δ* strain bearing YEp351-Mpk1-FLAG. The densitometry analysis data are expressed as fold relative to Mpk1-FLAG expression. Values are means  $\pm$  SD. Asterisks indicate significant differences (\* $P < 0.05$ , \*\* $P < 0.01$ ) between no treatment and ampicillin treatment. (B) Time course for Mpk1 phosphorylation by ampicillin. Mpk1 phosphorylation was examined after treatment with 3 mM ampicillin for the indicated time (min). The densitometry analysis data are expressed as fold relative to Mpk1-FLAG expression. Values are means  $\pm$  SD. Asterisks indicate significant differences (\*\* $P < 0.001$ ) between 0 min and each time point. (C) Mpk1 phosphorylation is induced by ampicillin. Mpk1 phosphorylation was tested by treatment with 3 mM ampicillin, Congo red, or heat stress for 1 h. U, unstressed (RT); CR, 50 mg/mL Congo red; HS, heat stress at 39 °C; Am, 3 mM ampicillin. The densitometry analysis data are expressed as fold relative to Mpk1-FLAG expression. Values are means  $\pm$  SD. Asterisks indicate significant differences (\* $P < 0.05$ , \*\* $P < 0.01$ ) between U and each stress condition. (D) *FKS2* gene expression is stimulated by ampicillin. Wild-type *S. cerevisiae* cells were treated with 3 mM ampicillin for the indicated time (min). Total RNA was used for RT-PCR analysis of *FKS2* mRNA and 18S RNA. Whole-cell extract was used to confirm RT-PCR product size. The densitometry analysis data are expressed as fold relative to 18S expression. Values are means  $\pm$  SD. Asterisks indicate significant differences (\* $P < 0.05$ ) between 0 min and 90 min.

*S. cerevisiae* is dependent on the PKC1-CWI pathway and maximal after treatment with 3 mM ampicillin for 90 min.

To assess the efficiency of ampicillin for the induction of Mpk1 phosphorylation compared with Congo red and heat stress, both of which induce cell wall stress (Kim and Levin, 2011), Mpk1 phosphorylation was examined after 1 h under each stress condition. Induction of Mpk1 phosphorylation by ampicillin was more effective than that with Congo red, but similar to the effect of heat stress (Figure 1C).

*FKS2* gene expression is induced by activated Mpk1 in response to various cell wall stresses (Kim et al., 2008). Therefore, we measured *FKS2* gene expression after 30 min and 90 min of 3 mM ampicillin treatment and showed that *FKS2* mRNA expression was induced after 90 min of treatment with 3 mM ampicillin (Figure 1D).

**3.2. Ampicillin inhibited miconazole antifungal activity**  
Based on activation of Mpk1 phosphorylation by ampicillin, we speculated that ampicillin might suppress miconazole antifungal activity because miconazole

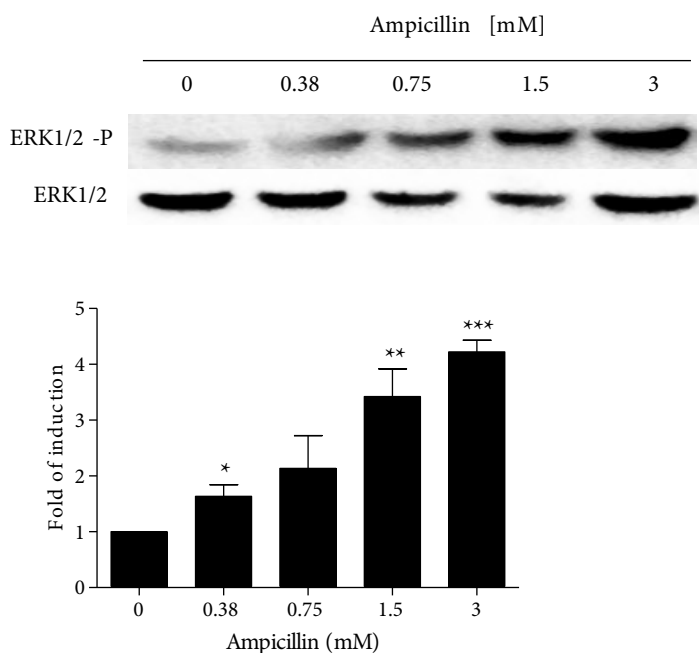
inhibits ergosterol biosynthesis. In addition, the PKC1-CWI pathway is related to azole resistance and Mpk1 inhibition has a synergistic effect on the antifungal activity of miconazole (Edlind et al., 2002; LaFayette et al., 2010; Shin and Kim, 2016). We performed a broth microdilution assay to determine the effect of ampicillin on the MIC of miconazole. For this, we used 3 mM ampicillin and the control was treated with the same volume of vehicle. After ampicillin treatment for 24 h and 48 h, the MIC values of miconazole against *C. albicans* increased from 1.56 to 25  $\mu\text{g}/\text{mL}$  and from 3.125 to 25  $\mu\text{g}/\text{mL}$  after incubation with ampicillin, respectively. MIC values of miconazole against *S. cerevisiae* also increased from 3.125 to 6.25  $\mu\text{g}/\text{mL}$  after 24 or 48 h of incubation in the presence of ampicillin (Table).

### 3.3. Ampicillin activated ERK1/2 phosphorylation

ERK MAPKs are human homologues of Mpk1 that participate in cell proliferation. We examined ERK1/2 phosphorylation in HepG2 cell lines (Figure 2) and found that ampicillin induced ERK1/2 phosphorylation with a maximum effect at 3 mM ampicillin. This result is consistent with the induction of Mpk1 phosphorylation in yeast. Normally, about 0.3 mM ampicillin is used in cell culture media to prevent microbial growth. In Figure 2, phosphorylation of ERK1/2 is seen to be increased in the 0.38 mM treatment. Even though the phosphorylation level of ERK1/2 in 0.3 mM ampicillin was not high enough, researchers need to consider the effect of ampicillin on MAPK signal transduction.

**Table.** Ampicillin suppresses miconazole antifungal activity. MIC, minimum inhibitory concentration ( $\mu\text{g}/\text{mL}$ ).

	MIC without ampicillin		MIC with ampicillin	
	24 h	48 h	24 h	48 h
<i>Candida albicans</i>	1.56	3.125	25	25
<i>Saccharomyces cerevisiae</i>	3.125	3.125	6.25	6.25



**Figure 2.** Ampicillin activates ERK1/2 phosphorylation in HepG2 cells. HepG2 cells were subjected to western blotting with antibodies specific for ERK1/2 or phosphorylated ERK1/2. The densitometry analysis data are expressed as fold relative to ERK1/2 expression. Values are means  $\pm$  SD. Asterisks indicate significant differences (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ) between no treatment and ampicillin treatment.

### 3.4. Ampicillin did not influence cell viability

The finding that ampicillin activated Mpk1 and ERK1/2 phosphorylation suggested that ampicillin might influence cell viability or growth. The PKC1-CWI pathway participates in cell wall integrity and ERK1/2 participates in cell proliferation. If treatment with ampicillin reduces cell wall integrity, there may be a delay in cell growth, or the induction of MAPK phosphorylation might increase cell viability. We tested *S. cerevisiae* cell growth and HepG2 cell viability in the presence of various concentrations of ampicillin, but we did not see any growth effects (Figure 3A) in *S. cerevisiae* or any effect on HepG2 cell viability by the MTT assay (Figure 3B). These results suggested that ampicillin increases MAPK phosphorylation without affecting cell viability or growth.

### 4. Discussion

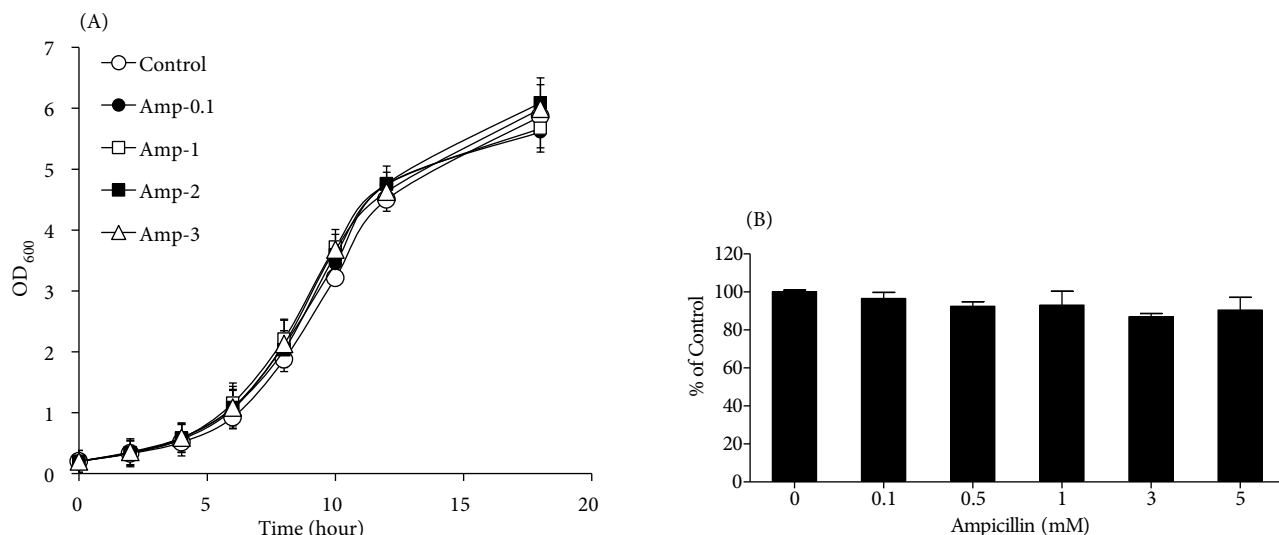
Ampicillin has been widely used to eliminate bacterial infection because of its potent antibacterial activity and low level of side effects on animal cells. When we tried to remove bacterial contamination to study Mpk1 activation in yeast, we observed unusual activation of Mpk1 phosphorylation in the absence of any stress. The finding that ampicillin appeared to activate MAPK was a surprise because it has been used as an antibiotic for a long time. We therefore performed this study to confirm that ampicillin actually activates Mpk1 phosphorylation and investigate the underlying mechanism.

We found that ampicillin maximally activated Mpk1 phosphorylation at concentration of 3 mM for 90 min.

Under these conditions, ampicillin also activated *FKS2* gene expression. Induction of *FKS2* mRNA has been proposed to decrease miconazole antifungal activity. These results are consistent, as the azole increased *FKS2* gene expression and the PKC1 pathway is related with azole resistance. Moreover, miconazole combined with MAPK inhibitors showed synergistic antifungal activity. Together these data indicate that ampicillin activated the PKC1-CWI pathway.

Ampicillin did not influence cell growth based on the MIC value ( $>200 \mu\text{g/mL}$  for *S. cerevisiae* and *C. albicans*) and cell growth analysis. Because Mpk1 phosphorylation is dependent on the PKC1-CWI pathway, we wondered whether ampicillin affects cell wall integrity, but we did not find any differences between treated and untreated cells in microscopic analysis with respect to cell lysis, cell size, and cell shape in log phase (data not shown). ERK1/2 phosphorylation was also induced by treatment with ampicillin but did not influence cell viability of HepG2 cells. These results suggest that activation of MAPK (Mpk1 and ERK1/2) phosphorylation is not related to cell wall stress, but rather occurs through another mechanism. In future studies, we will characterize how ampicillin activates MAPK phosphorylation.

In mammalian cells, the phosphorylation of ERK1/2 was increased in the 0.3 mM treatment, which is normally used in cell culture media to prevent microbial growth. ERK1/2 serves as a signaling conduit between activated growth factor receptors at the plasma membrane and transcription factors in the nucleus, thereby controlling



**Figure 3.** Ampicillin does not inhibit cell viability. (A) Ampicillin did not influence the growth of *S. cerevisiae*. Precultured cells at 30 °C were diluted to an OD<sub>600</sub> = 0.1 and treated with various concentrations of ampicillin. Growth was determined after the indicated time as the OD<sub>600</sub> of each culture. Experiments were repeated three times, and data are presented as the mean and standard deviation of the mean. Amp-0.1, 0.1 mM ampicillin; Amp-1, 1 mM ampicillin; Amp-2, 2 mM ampicillin; Amp-3, 3 mM ampicillin. (B) Ampicillin did not influence HepG2 cell growth. HepG2 cells plated at  $1 \times 10^4$  cells/mL were treated with various concentrations of ampicillin. After incubation for 24 h, cell viability was determined by MTT assay.

transcription of numerous genes implicated in cell cycle, cell survival, and cell motility. ERK1/2 phosphorylation is subject to extensive negative feedback regulation, which allows for fine-tuning of pathway activation and output. For example, the  $G\alpha_{13}$ -mediated pathway is regulated by ERK1/2 activation. During the regulation, even though the extent of ERK1/2 activation was very low and barely detectable,  $G\alpha_{13}$ -dependent activation of a phosphatase was responsible for these different levels of ERK regulation (Mariggio et al., 2006). Therefore, even though the phosphorylation level of ERK1/2 in 0.3 mM ampicillin was not high enough, researchers need to consider the effect of ampicillin on MAPK signal transduction.

Interestingly, ERK participates in some immune response mechanisms; ERK activates expression of cytokines such as IL-10 (Lucas et al., 2005) and blocks

T-cell apoptosis (Gu et al., 2013), and fungal infection blocks the expression of proinflammatory transcripts and interleukin proteins through ERK1/2 (Zanello et al., 2011). This suggests that ampicillin-induced activation of ERK phosphorylation is related to activation or induced expression of cytokines within macrophages or other immune response cells. Our future goal is to identify whether ampicillin can activate immune response cells and induce cytokine expression.

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