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Short communications

Inhibitory effect of tyrphostin 47 on Shiga toxin-induced cell death

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(Abstract - 99 words)
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Abstract

The inhibitory effects of tyrosine kinase inhibitors including tyrphostin 25, 47 and 51 on Shiga toxin 1-induced cell death and p38 mitogen-activated protein kinase (p38 MAPK) phosphorylation were examined in Vero cells. Tyrphostin 47 significantly inhibited Shiga toxin 1-induced cell death and p38 MAPK phosphorylation. In contrast, tyrphostin 25 and 51 had no significant effect on the Shiga toxin 1-induced responses. These data indicate that Shiga toxin 1-induced cell injury occurs through a pathway sensitive to tyrphostin 47, and the target molecule for tyrphostin 47 opens up new opportunities for pharmacological intervention against Shiga toxin-producing *Escherichia coli* infectious diseases. (99 words)

Key words: Shiga toxin, p38 MAPK, tyrosine kinase, tyrosine kinase inhibitor, tyrphostin 47

1. Introduction

Shiga toxins (Shiga toxins) are a group of bacterial protein toxins produced by *Shigella dysenteriae* type 1, Shiga toxin-producing strains of *Escherichia coli* as well as other bacteria. Two groups of Shiga toxins are found: the Shiga toxin 1 family and Shiga toxin 2 family. The amino acid sequence of Shiga toxin 1 has about 60% homology with that of Shiga toxin 2. Shiga toxins are composed of an enzymatically active A subunit that is a translational inhibitor, thereby inhibiting protein synthesis, and five B subunits that confer the binding of holotoxin to globotriaosyl ceramide. There are currently no specific therapeutic approaches to prevent or block the progression of Shiga toxin-producing bacterial infectious diseases except for supportive care. Infections with Shiga toxin-producing bacteria are therefore responsible for wide spread disease and for the death of large number of people (Takeda et al., 1993; Sandvig, 2001).

p38 mitogen-activated protein kinases (p38 MAPKs) are members of the MAPK superfamily that plays an important role in widespread biological responses including mitosis, apoptosis and inflammation. p38 MAPKs are activated in response to a variety of extracellular stimuli such as ultraviolet (UV) radiation, oxidative stress, hyperosmotic shock, proinflammatory cytokines or withdrawal of a trophic factor (Garrington and Johnson, 1999; Kyriakis and Avruch, 2001). The activation of p38 MAPKs by the extracellular stimuli is known to be mediated by tyrosine kinases, p21-activated kinases and small G proteins (Yamauchi et al., 1997; Garrington and Johnson, 1999; Ohanian et al., 2001).

Several groups have shown that Shiga toxins regulate cytokine expression through a p38 MAPK signaling pathway (Thorpe et al., 1999; Foster and Tesh, 2002; Cameron et al., 2003). In addition, Foster et al. (2000) have reported that Shiga toxin-induced cytokine production is mediated by tyrosine kinases. These data strongly suggest that the signal transduction pathway stimulated by Shiga toxins includes both p38 MAPKs and tyrosine kinases.

We showed that both Shiga toxin 1 and Shiga toxin 2 activated p38 MAPK and this activation was involved in Shiga toxin-induced cell death (Ikeda et al., 2000). Furthermore it has been reported that Shiga toxin 1 induces apoptosis (Inward et al., 1995) and this apoptosis is associated with p38 MAPK activation (Smith et al., 2003). However, the roles of tyrosine kinases in Shiga toxin-induced cell death have yet to be elucidated. In this study, we examined the effects of tyrosine kinase inhibitors on the Shiga toxin 1-induced cell death and p38 MAPK phosphorylation. Interestingly, only tyrphostin 47 significantly exerted inhibitory effects of Shiga toxin 1-induced p38 MAPK phosphorylation and cell death, and none of the other tyrosine kinase inhibitors, including tyrphostin 25 and 51, had any significant effect on the Shiga toxin 1-induced responses. This work provides evidence that Shiga toxin 1-induced cell injury is mediated by tyrphostin 47-sensitive molecule and this molecule may prove to be an important new target for the therapy of Shiga toxin-producing bacterial infectious diseases.

2. Materials and methods

2.1. Cell culture

Vero cells were grown and maintained in Eagle minimum essential medium (MEM; Gibco-BRL, USA) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), tylosin (8 µg/ml) and 10% fetal bovine serum (FBS). All experiments were performed under conditions of serum starvation. Vero cells were grown to 80% confluence in MEM containing 10% FBS and transferred to MEM containing 0.5% FBS for 48-72 h.

2.2. Measurement of cell survival

Cell survival after the stimulation with Shiga toxin 1 was determined by the crystal violet assay. Quiescent Vero cells were incubated with tyrphostins at the indicated concentration or 1% dimethyl sulfoxide (DMSO) for 1 h. Cells were then treated with or without 20 pg/ml Shiga toxin 1 for 48 h in the presence of a drug. Thereafter cells were fixed in 0.25% gluteraldehyde solution, washed with tap water and incubated with 0.3% crystal violet. Finally cells were washed with tap water and solubilized with 1% SDS. The absorbance was measured at 590 nm. Data are expressed as % of the mean value obtained from the difference between the absorbance values in cells untreated (without both Shiga toxin 1 and DMSO) and in those treated with both Shiga toxin 1 and DMSO.

In our previous study, we observed that the 50% cytotoxic concentration for 48 h of the treatment with Shiga toxin 1 was 11 pg/ml by the 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate assay in quiescent Vero cells (Ikeda et al., 2000). Based on this data, we tested 20 pg/ml of Shiga toxin 1 on the quiescent Vero cell survival by the crystal violet assay. After the cells were incubated with 20 pg/ml Shiga toxin 1 for 48 h, 50.6 ± 7.7% (n = 4) of the cells survived. Therefore we

used this concentration of Shiga toxin 1 in this study. Furthermore we preliminarily examined whether 1% DMSO which was used as a solvent for the tyrphostins affects the Shiga toxin 1-induced cell injury or not. Quiescent Vero cells were incubated with 1% DMSO for 1 h and were then treated with 20 pg/ml Shiga toxin 1 for 48 h. After the incubation, the extent of cell survival judged by the crystal violet assay was 51.6 ± 8.1% (n = 4). As this value did not significantly differ from the above-mentioned value without DMSO, it was thought that 1% DMSO can be used as a solvent for tyrphostins.

2.3. Evaluation of phosphorylation of p38 MAPK

Activation of p38 MAPK was determined by Western blotting with anti-phospho-p38 MAPK antibody as described previously (Ikeda et al., 2000). Quiescent Vero cells were incubated with each drug (100 μM each) or 1% DMSO for 1 h, and were then stimulated with 20 pg/ml Shiga toxin 1 for 2 h in the presence of drug. After the treatment, the cells were washed quickly with ice-cold Tris-buffered saline (TBS; 25 mM Tris, pH 7.4, 150 mM NaCl) containing 1 mM Na_3VO_4, and then lysed in lysis buffer (50 mM Tris, pH 7.4, 50 mM NaCl, 50 mM NaF, 5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM Na_3VO_4, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml antipain, 100 μg/ml benzamidine, 10 μg/ml aprotinin, 100 μg/ml soybean trypsin inhibitor, 1% glycerol) for 20 min at 4°C. The cells were then scraped off the dish, the cell lysate was centrifuged at 12,000g for 10 min, and the protein concentration in the supernatants was estimated by the DC protein assay (Nippon Bio-Rad, Japan). The remaining supernatants were combined with 4 x SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (0.5 M Tris, pH 6.8, 50% glycerol, 8% SDS, 0.4 M dithiothreitol, 0.05% bromophenolblue) and heated at 95°C for 3 min. The samples were then frozen at -80°C until use.

After separation by SDS-PAGE, the protein was transferred on to a nitrocellulose or a polyvinylidene difluoride membrane and analyzed by immunoblotting. The membrane was incubated in blocking solution and then with the primary antibody for 1.5 h at 37°C. Excess primary antibody was removed by washing the membrane in TBS containing 0.05% Tween 20. The blots were then incubated with an anti-rabbit secondary antibody for 1 h at 37°C, and the membrane was washed and proteins associated with antibodies were detected by the enhanced (Amersham Pharmacia Biotech, Japan) or Super Signal^{R} chemiluminescence detection system (Pierce Biotechnology, Inc., USA).

2.4. Chemicals and Reagents

Shiga toxin 1 was purified as described previously (Noda et al., 1987). Rabbit polyclonal anti-phospho-p38 MAPK antibody specific for dual-phosphorylated ^{180}Thr and ^{182}Tyr of p38 MAPK was from New England Biolabs Inc. (USA) or CHEMICON Int., Inc. (USA) and rabbit polyclonal anti-p38 MAPK antibody, which detects phosphorylation-state independent p38 MAPK, was from Santa Cruz Biotechnology Inc. (USA). Horseradish peroxidase-conjugated donkey or goat anti-rabbit secondary antibody was from Amersham Pharmacia Biotech. (USA) or Cell Signaling Tec., Inc (USA), respectively. All other chemicals and reagents were from Sigma Chemical Co. (USA) or Wako Pure Chemicals (Japan).

3. Results

3.1. Effects of tyrosine kinase inhibitors on Shiga toxin-induced cell death

The effects of tyrosine kinase inhibitors on Shiga toxin 1-induced cell death were examined by crystal violet assay. As shown in Fig. 1, 100 μM tyrphostin 47 significantly reduced Shiga toxin 1-induced cell death. In contrast to tyrphostin 47, both tyrphostins 25 and

51 tended to worsen the cytotoxicity caused by Shiga toxin 1, although the effects did not achieve significance.

3.2. Effect of tyrosine kinase inhibitors on Shiga toxin -induced p38 MAPK phosphorylation

In the previous reports, we observed that Shiga toxin 1 caused a p38 MAPK phosphorylation and this phosphorylation was in part related to the Shiga toxin 1-induced cell death (Ikeda et al., 2000). Therefore, we next examined the effect of tyrosine kinase inhibitors on Shiga toxin 1-induced phosphorylation of p38 MAPK.

As shown in Fig. 2 A and B, treatment of the cells with tyrphostin 47 significantly reduced a Shiga toxin 1-induced phosphorylation of p38 MAPK, while the protein level of p38 MAPK was not significantly affected by tyrphostin 47 treatment. In contrast, both tyrphostin 25 and 51 did not inhibit the Shiga toxin 1-induced phosphorylations of p38 MAPK. On the contrary, they tended to enhance the Shiga toxin 1-induced phosphorylation of p38 MAPK.

4. Discussion

In this study, we clearly showed that tyrphostin 47 but neither tyrphostin 25 nor tyrphostin 51 reduced Shiga toxin 1-induced cytotoxicity. Consonant with this reduction, only tyrphostin 47 inhibited Shiga toxin 1-induced p38 MAPK phosphorylation. Since inhibitors for p38 MAPK partially but significantly inhibited Shiga toxin 1-induced cell death in our previous study (Ikeda et al., 2000), tyrphostin 47 is likely to protect against Shiga toxin 1-induced cytotoxicity in part via the inhibition of a signaling pathway related p38 MAPK.

Tyrphostins 25, 47, and 51 were originally thought to be broad spectrum inhibitors for tyrosine kinase and inhibited the epidermal growth factor receptor kinase activity with similar IC50 values of 3 μM for tyrphostin 25, 2.4 μM for tyrphostin 47, and 0.8 μM for tyrphostin 51 (Miranda et al., 2004). In the present study, tyrphostins 25 and 51 tended to

enhance the Shiga toxin 1-induced cell injury and p38 MAPK activation, suggesting that the tyrphostins 25 and 51 could be active under the present experimental condition. In addition to this observation, in the preliminary experiment, when given to the cultured cells each tyrphostin inhibited cell proliferation over a similar concentration range (IC50 values of 6 μM for tyrphostin 25, 23 μM for tyrphostin 47, and 7 μM for tyrphostin 51). Although we observed a selective action of tyrphostin 47, each tyrosine kinase inhibitor therefore might have efficiently inhibited the tyrosine kinase in the present experimental condition and it is conceivable that the selective action of tyrphostin 47 is mediated by molecules that are insensitive to tyrphostins 25 and 51.

It is known that p38 MAPKs are activated by concomitant Tyr and Thr phosphorylation within a conserved Thr-X-Tyr motif in the activation loop of the kinase domain subdomain VIII (Kyriakis and Avruch, 2001). Therefore, one possible explanation for the selective action of tyrphostin 47 is that tyrphostin 47 but neither tyrphostin 25 nor 51 has an ability to directly inhibit the Tyr-phosphorylation of the activation loop of the p38 MAPKs. Alternatively, the selective action of tyrphostin 47 might be explained by the presence of target molecules for tyrphostin 47 other than tyrosine kinase. For example, tyrphostins 25 and 47 but not the other 13 tyrphostins tested have been shown to selectively stimulate the methylation of kidney cytosolic protein in the presence of vanadate (Miranda et al., 2004), even though the authors used 100 μM of tyrphostins which is a higher concentration than the concentration required for 50% inhibition of the epidermal growth factor receptor kinase activity. Furthermore, a certain type of tyrphostins is known to inhibit GTP-utilizing enzymes including guanylyl cyclase and fructose-6-phosphate kinase (Jaleel et al., 2004). Although further studies are needed to explore a target molecule for tyrphostin 47, tyrphostin 47-sensitive molecule may be different from tyrosine kinases.

In conclusion, we provided evidence that tyrphostin 47 selectively exerted inhibitory effects on the Shiga toxin 1-induced p38 MAPK activation and cell death, and the tyrphostin

47-sensitive molecule may prove to be an important new target for the therapy of Shiga toxin -producing bacterial infectious diseases.

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Figure legends

Fig. 1. Effect of tyrphostins 25, 47 and 51 on Shiga toxin 1-induced cell injury.
Effect of tyrphostins 25 (TP25), 47 (TP47) and 51 (TP51) on Shiga toxin 1-induced cell injury assessed by crystal violet assay. Data are expressed as % of the mean value obtained from the difference between the absorbance values from cells untreated (without both Shiga toxin 1 and DMSO) and those treated with both Shiga toxin 1 and DMSO. Zero % indicates the level of cell death when the cells were treated with both Shiga toxin 1 and DMSO. Values are presented as mean ± SE from two to three independent experiments performed in

duplicate. * represents the significance level of $P < 0.05$, tested for the difference between DMSO and test agents (Dunnett's method).

Fig. 2. Effect of tyrphostins 25, 47 and 51 on Shiga toxin 1-induced p38 MAPK phosphorylation.

(**A**) Typical examples of Shiga toxin 1-induced p38MAPK phosphorylation in the presence or absence of TP 25, 47, and 51 (100 μM each). An equal amount of protein was immunoblotted with anti-phospho-p38 MAPK antibody (p-p38) or with anti-p38 MAPK antibody (p38).

(**B**) Densitometric analysis of the phosphorylated p38 MAPK protein (p-p38 MAPK) is summarized. Data are expressed as % of the control value obtained from the cells treated with both Shiga toxin 1 and DMSO. Values are presented as mean ± SE from three to four independent experiments. * represents the significance level of $P < 0.05$, tested for the difference between DMSO and test groups (Dunnett's method).

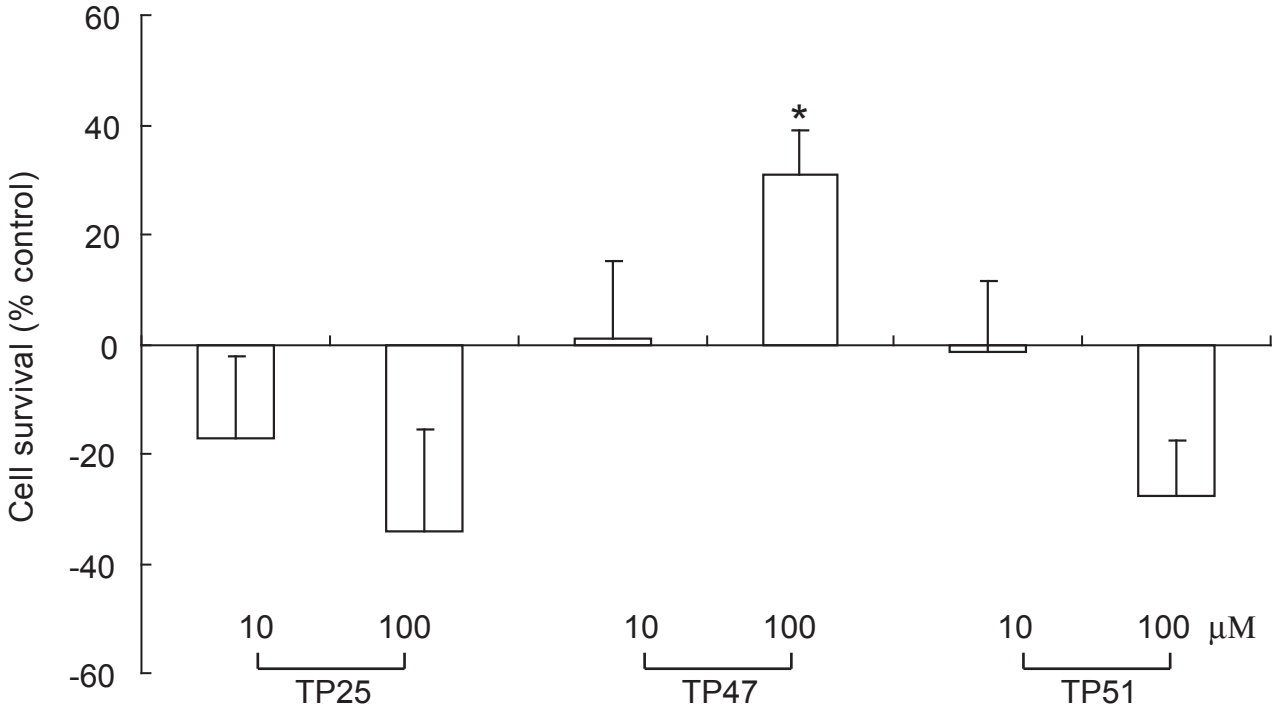

Fig. 1. Effect of tyrphostins 25, 47 and 51 on Stx1-induced cell injury.

Effect of tyrphostins 25 (TP25), 47 (TP47) and 51 (TP51) on Shiga toxin 1-induced cell injury assessed by crystal violet assay. Data are expressed as % of the mean value obtained from the difference between the absorbance values from cells untreated (without both Shiga toxin 1 and DMSO) and those treated with both Shiga toxin 1 and DMSO. Zero % indicates the level of cell death when the cells were treated with both Shiga toxin 1 and DMSO.

Values are presented as mean ± SE from two to three independent experiments performed in duplicate.

* represents the significance level of $P < 0.05$, tested for the difference between DMSO and test agents (Dunnett's method).

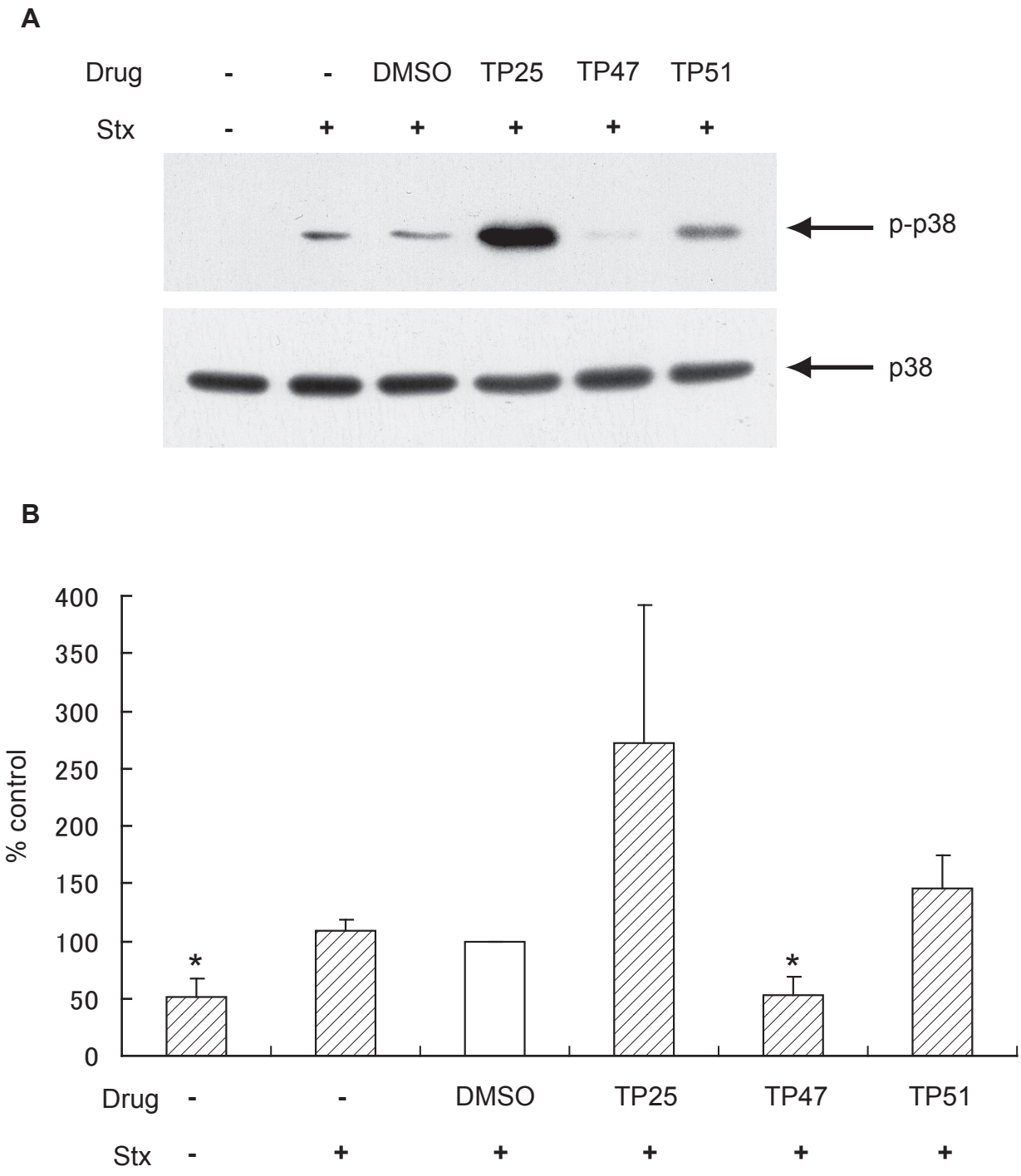

Fig. 2. Effect of tyrphostins 25, 47 and 51 on Stx1-induced p38 MAPK phosphorylation.
(**A**) Typical examples of Shiga toxin 1-induced p38MAPK phosphorylation in the presence or absence of TP 25, 47, and 51 (100 μM each). An equal amount of protein was immunoblotted with anti-phospho-p38 MAPK antibody (p-p38) or with anti-p38 MAPK antibody (p38).
(**B**) Densitometric analysis of the phosphorylated p38 MAPK protein (p-p38 MAPK) is summarized. Data are expressed as % of the control value obtained from the cells treated with both Shiga toxin 1 and DMSO. Values are presented as mean ± SE from three to four independent experiments.
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