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Short communication

Tyrphostin 23 blocks phosphorylation of p42 but not p38 mitogen-activated protein kinase by zooxanthellatoxin-A

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Abstract

Zooxanthellatoxin-A isolated from a symbiotic dinoflagellate, caused aggregation in rabbit platelets that was inhibited by genistein (50 μM) and tyrphostin 23 (500 μM). Zooxanthellatoxin-A increased tyrosine phosphorylation of 42-kDa proteins which were identified as p42 and p38 mitogen-activated protein kinase (MAPK) by immunoprecipitation. Tyrphostin 23 inhibited the tyrosine phosphorylation of p42 MAPK but not p38 MAPK. In contrast, genistein abolished zooxanthellatoxin-A-induced tyrosine phosphorylation of both p42 and p38 MAPK. The results suggest that tyrphostin 23 selectively inhibits tyrosine phosphorylation of p42 MAPK. The p38 MAPK tyrosine phosphorylation is not involved in zooxanthellatoxin-A-induced platelet aggregation.

Keywords: Zooxanthellatoxin-A; Tyrphostin 23; Mitogen-activated protein kinase; Platelet aggregation

1. Introduction

Mitogen-activated protein kinase (MAPK) has been shown to function in a wide variety of biological processes (Nishida and Gotoh, 1993; Marshall, 1995). p42 MAPK and p44 MAPK phosphorylate a number of intracellular proteins including several kinases, transcription factors, and cytoplasmic and cytoskeletal proteins. They are important for proliferative and differentiative responses (Marshall, 1995). On the other hand, p38 MAPK is the mammalian homologue of the yeast high osmolarity glycerol response kinase and participates in a cascade controlling cellular response to a variety of cellular stresses including osmotic shock, lipopolysaccharides and growth factors (Saklatvala et al., 1996).

It has been reported that tyrphostin 23 inhibited tyrosine phosphorylation of p42 MAPK and p44 MAPK in a small intestinal crypt cell line (IEC-6) (Oliver et al., 1994) and adherent neutrophils (Johnson and Gomez-Cambronero, 1995). It has been also reported that preincubation of platelets with the tyrosine kinase inhibitors, genistein and tyrphostin 23, completely inhibited platelet shape change and protein tyrosine phosphorylation induced by a partial peptide of thrombin receptor YFLLRNP (Negrescu et al., 1995).

In the present study, we examined the effects of genistein and tyrphostin 23 on tyrosine phosphorylation of p42 MAPK and p38 MAPK induced by zooxanthellatoxin-A in rabbit washed platelets.

2. Materials and methods

2.1. Materials

Zooxanthellatoxin-A was isolated as described previously (Rho et al., 1995). Indomethacin was obtained from Merck (Rahway, NJ, USA). Genistein and tyrphostin 23 were obtained from Wako (Osaka, Japan). Bovine serum albumin was from Sigma (St. Louis, MO, USA). Anti-phosphotyrosine antibody (4G10) was obtained from Upstate Biotechnology (New York, NY, USA). Anti-extracellular signal-regulated kinase 2 (ERK2, C-14) antibody was obtained from Santa Cruz Biotechnology (Santa

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Cruz, CA, USA). Anti-p38 MAPK antibody was obtained from New England Biolabs (Beverly, MA, USA). Protein A-Sepharose was obtained from Zymed Laboratories (South San Francisco, CA, USA). All other chemicals were of analytical grade.

2.2. Preparation of washed platelets and determination of platelet aggregation

Fresh blood was obtained from male rabbits (Japanese white rabbits weighing about 2–3 kg) and washed platelets were prepared as described previously (Rho et al., 1995). Platelet aggregation was determined by a standard turbidimetric method (Rho et al., 1995).

2.3. Immunoblotting

For analysis of total platelet proteins, the reaction was terminated by the addition of Laemmli sample buffer, and the mixture was then boiled for 5 min, and analyzed on an 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblot assays were performed as described by Papkoff et al. (1994), with slight modifications. Proteins were electrically transferred to the polyvinylidene difluoride membrane for 80 min at 120 mA. Blots were incubated for 2 h with 1% (w/v) bovine serum albumin in Tris-buffered saline (TBS) to block residual protein binding sites. Immunodetection of tyrosine phosphorylation was achieved by using a specific antiphos-

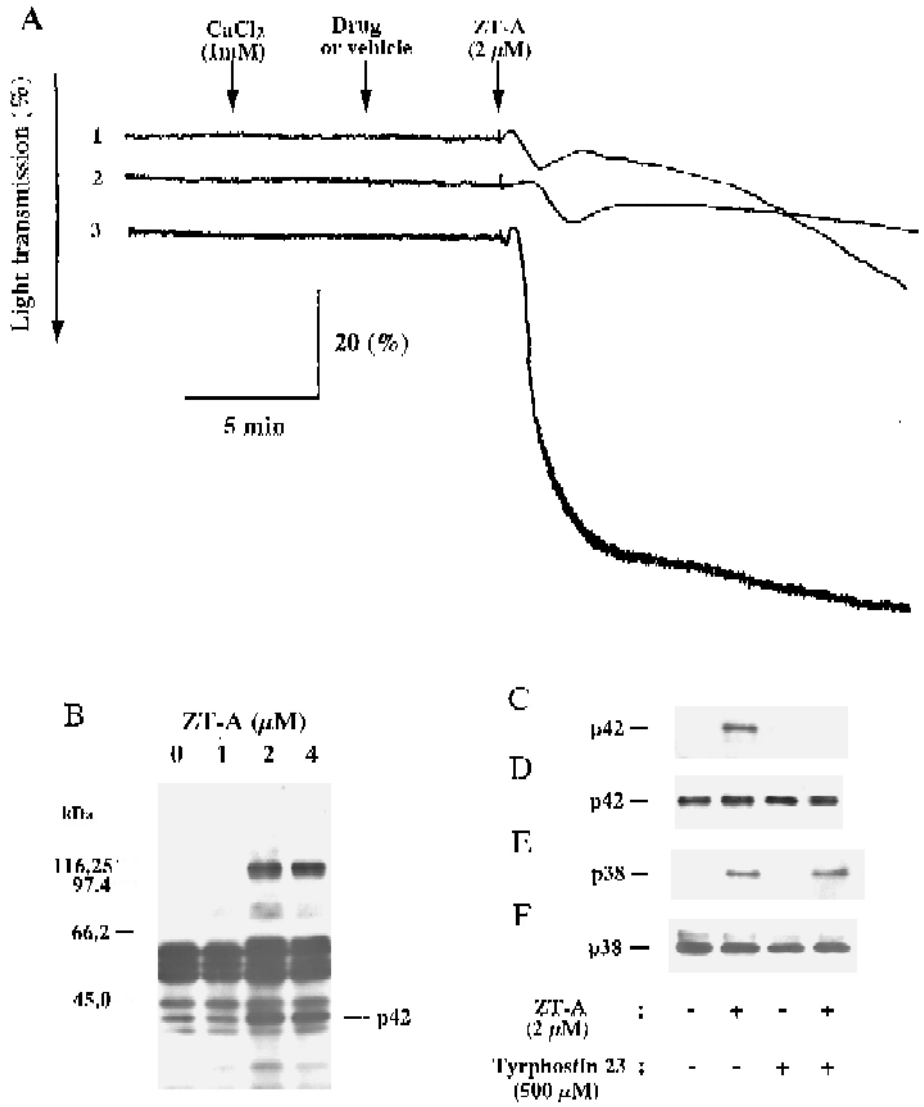

Fig. 1. Effect of genistein and tyrphostin 23 on zooxanthellatoxin-A (ZT-A)-induced platelet activation. (A) ZT-A-induced platelet aggregation. Drugs were added 5 min before the addition of ZT-A (2 μM) in the presence of 1 mM $CaCl_2$: 1, genistein (50 μM); 2, tyrphostin 23 (500 μM); 3, vehicle (dimethyl sulfoxide, final 0.05%). A typical experiment representative of three is shown. (B–F) Protein tyrosine phosphorylation upon ZT-A stimulation. Platelets were incubated with ZT-A (2 μM) for 5 min in the presence of 1 mM $CaCl_2$. Platelet lysates were fractionated on a 8% SDS-PAGE followed immunoblotting with the anti-phosphotyrosine antibody (B), and immunoprecipitated with anti-p42 MAPK antibody (C, D) or anti-p38 MAPK antibody (E, F). Immunoprecipitates were fractionated on a 10% SDS-PAGE following by immunoblotting with anti-phosphotyrosine antibody (C, E), anti-p42 MAPK antibody (D) or anti-p38 MAPK antibody (F) as described in Section 2. The results are representative of three similar experiments.

photyrosine monoclonal murine antibody (4G10, 2 μg/ml) in TBS containing 1% bovine serum albumin for 2 h. p42 MAPK and p38 MAPK were detected by incubation of rabbit anti-ERK2 (C-14) antibody (1 μg/ml) and rabbit anti-p38 antibody (1 μg/ml) for 2 h, respectively. To detect the primary antibody, blots were incubated with alkaline phosphatase-conjugated anti-mouse or anti-rabbit antibody (Bio-Rad) diluted to 1:3000 in TBS containing 1% bovine serum albumin for 2 h. After the blots were exposed to enhanced chemiluminescence reagents (Bio-Rad) for 30 min, they were then exposed to Hyper film-enhanced chemiluminescence (Amersham) for 10–30 min.

2.4. Immunoprecipitation

For immunoprecipitation of p42 MAPK or p38 MAPK, platelet suspensions (0.3 ml) were harvested by the addition of 20 μl denaturing buffer (10% SDS, 10 mM dithiothreitol, 20 mM HEPES, pH 7.4) (Papkoff et al., 1994). The sample was heated at 95°C for 5 min and diluted with 0.8 ml of immunoprecipitation buffer (150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 2 mM EGTA, 10 μg/ml leupeptin, 10 μg/ml phenylmethanesulfonyl fluoride, 0.5 mM sodium orthovanadate, and 50 mM HEPES, pH 7.4). The samples were centrifuged at 15 000 $\times g$ for 20 min. The supernatants (0.8 ml) were then incubated for 3 h at 4°C with 1 μg/ml of the anti-ERK2 (C-14) antibody or the anti-p38 MAPK antibody, and were further incubated for 1 h at 4°C after the addition of protein A-Sepharose (20 μg). Immune complexes were collected by centrifugation in a microcentrifuge and then washed three times with washing buffer (150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 1% aprotinin, 10 μg/ml leupeptin, 10 μg/ml phenylmethanesulfonyl fluoride, 0.5 mM sodium orthovanadate, and 50 mM HEPES, pH 7.4). The precipitate was solublized by Laemmli sample buffer, and the mixture was then boiled for 10 min, and applied to 10% SDS-PAGE and immunoblotting described above.

3. Results

Zooxanthellatoxin-A caused aggregation in the presence of external Ca^{2+} (Fig. 1A). Zooxanthellatoxin-A (2 μM)-induced platelet aggregation was inhibited by genistein (50 μM) and tyrphostin 23 (500 μM), suggesting the involvement of tyrosine phosphorylation in zooxanthellatoxin-A-induced aggregation.

The ability of zooxanthellatoxin-A to induce protein tyrosine phosphorylation in rabbit washed platelets was assessed by using antibody to phosphotyrosine and immunoblotting. Zooxanthellatoxin-A caused an increase in tyrosine phosphorylation of 105, 100, 60 and 42-kDa proteins in a concentration-dependent manner (Fig. 1B). In order to identify the 42-kDa protein, platelet lysates were immunoprecipitated with anti-ERK2 (C-14) antibody or anti-p38 MAPK antibody. Zooxanthellatoxin-A (2 μM)

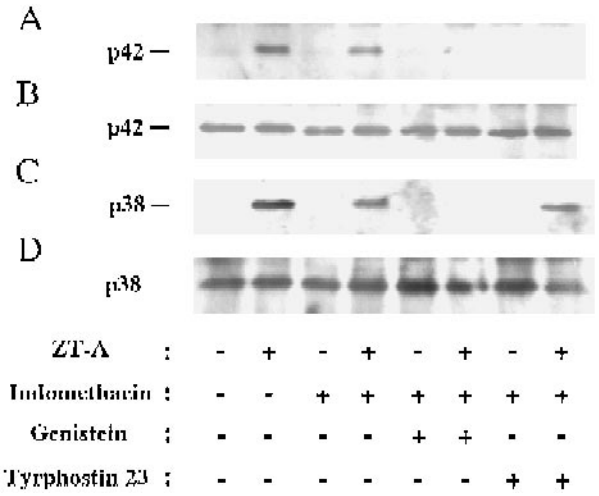

Fig. 2. Effects of indomethacin, genistein and tyrphostin 23 on zooxanthellatoxin-A (ZT-A)-induced tyrosine phosphorylation of p42 or p38 MAPK. Platelets were preincubated with indomethacin (10 μM) for 5 min and were then incubated with ZT-A (2 μM) for 5 min. Platelets were preincubated with genistein (50 μM) or tyrphostin 23 (500 μM) for 5 min in the presence of indomethacin (10 μM) and were then incubated with ZT-A (2 μM) for 5 min. The lysates were immunoprecipitated with anti-p42 MAPK antibody (A, B) or anti-p38 MAPK antibody (C, D). Immunoprecipitates were fractionated on a 10% SDS-PAGE followed immunoblotting with anti-phosphotyrosine antibody (A, C), anti-p42 MAPK antibody (B) or anti-p38 MAPK antibody (D) as described in Section 2. The results are representative of three similar experiments.

increased tyrosine phosphorylation of both p42 MAPK and p38 MAPK (Fig. 1C and E). Although tyrosine phosphorylation of p42 MAPK induced by zooxanthellatoxin-A (2 μM) was inhibited by tyrphostin 23 (500 μM), the phosphorylation of p38 MAPK was resistant to tyrphostin 23 (Fig. 1C and E).

Zooxanthellatoxin-A caused thromboxane A_2 release in rabbit washed platelets (Rho et al., 1995) and thromboxane A_2 activated p38 MAPK in human platelets (Saklatvala et al., 1996). Indomethacin (10 μM), a cyclooxygenase inhibitor, weakly inhibited tyrosine phosphorylation of p42 MAPK and p38 MAPK in response to zooxanthellatoxin-A (2 μM), suggesting that thromboxane A_2 released by zooxanthellatoxin-A was responsible for the phosphorylation of p42 MAPK and p38 MAPK. In the presence of indomethacin (10 μM), genistein also inhibited zooxanthellatoxin-A-induced phosphorylation of p42 MAPK and p38 MAPK. However, tyrphostin 23 inhibited the phosphorylation of p42 MAPK, but not that of p38 MAPK (Fig. 2).

4. Discussion

Several subgroups of MAPK exist in mammalian cells, including p42 MAPK and p44 MAPK, c-Jun N-terminal kinase, and p38 MAPK (Davis, 1994). These MAPK isoforms are activated by dual phosphorylation on their threonine and tyrosine residues. Recently, it has been

reported that p42 MAPK and p44 MAPK, and p38 MAPK were regulated by different signal transduction pathways (Derijard et al., 1995). It has been shown that p42 MAPK and p44 MAPK are identified in human platelets, and thrombin stimulates the activation of p42 MAPK but not p44 MAPK (Papkoff et al., 1994). In the present study, we also observed that zooxanthellatoxin-A increased tyrosine phosphorylation of p42 MAPK but not p44 MAPK in rabbit platelets. On the other hand, p38 MAPK was identified in several cell lines on the basis of its immunological reactivity and substrate specificity (Rouse et al., 1994; Freshney et al., 1994). It has been also reported that p38 MAPK is activated in human platelets by collagen fibers, a collagen-related cross-linked peptide, thrombin, or the thromboxane analogue 9,11-dideoxy-9α,11α-epoxymethanoprostaglandin $F_{2\alpha}$ (U46619) (Saklatvala et al., 1996). In agreement with the above reports, zooxanthellatoxin-A also increased tyrosine phosphorylation of p38 MAPK.

Protein tyrosine kinase inhibitors would be valuable reagents for studying the role of tyrosine kinases in signal transduction. Two structurally different tyrosine kinase inhibitors, genistein and tyrphostins, were commonly used for investigating the physiological role of tyrosine phosphorylation (Negrescu et al., 1995; Ramdas et al., 1994; Martinson et al., 1994). In our study, genistein inhibited tyrosine phosphorylation of both p42 MAPK and p38 MAPK in response to zooxanthellatoxin-A, accompanied with inhibition of platelet aggregation. However, tyrphostin 23 inhibited the phosphorylation of p42 MAPK induced by zooxanthellatoxin-A, but not that of p38 MAPK. Since zooxanthellatoxin-A-induced aggregation is inhibited by tyrphostin 23, zooxanthellatoxin-A causes aggregation not mediated via phosphorylation of p38 MAPK. Tyrphostin 23 seems to have a selective inhibitory effect on phosphorylation of p42 MAPK but not on that of p38 MAPK.

Zooxanthellatoxin-A-induced aggregation was inhibited by indomethacin, a cyclooxygenase inhibitor, and zooxanthellatoxin-A caused the release of thromboxane A_2 in rabbit platelets (Rho et al., 1995). Since zooxanthellatoxin-A-induced tyrosine phosphorylation of p42 MAPK and p38 MAPK was weakly inhibited by indomethacin, thromboxane A_2 released by zooxanthellatoxin-A may cause tyrosine phosphorylation of p42 MAPK and p38 MAPK. The results were consistent with the observation that p38 MAPK (Saklatvala et al., 1996) and p42 MAPK (Ohkubo et al., 1996) were activated by U46619, a thromboxane A_2 analogue. The remaining zooxanthellatoxin-A-induced tyrosine phosphorylation of p42 MAPK and p38 MAPK in the presence of indomethacin is due to zooxanthellatoxin-A by itself.

In conclusion, zooxanthellatoxin-A increases tyrosine phosphorylation of p42 MAPK and p38 MAPK, and tyrphostin 23 inhibits the tyrosine phosphorylation of p42 MAPK but not that of p38 MAPK. The p38 MAPK tyrosine phosphorylation may not be responsible for platelet aggregation induced by zooxanthellatoxin-A.

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