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Preconditioning of 3T3 cells by fresh medium together with genistein enhances prostaglandin E_2 release

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Abstract

Bradykinin induced prostaglandin E_2 release from the Swiss 3T3 fibroblasts, preconditioned with fresh culture medium. Although treatment with genistein for the entire period of preconditioning and incubation with bradykinin attenuated prostaglandin E_2 release, treatment with fresh culture medium and genistein for only the preconditioning period further augmented the prostaglandin E_2 release. In the cells preconditioned with fresh culture medium and genistein, bradykinin caused the phosphorylation of protein tyrosine and mitogen-activated protein kinase/extracellular-regulated kinase (MAPK/ERK), followed by arachidonic acid release. Interestingly, preconditioning with genistein alone also caused phosphorylation and arachidonic acid release, probably reflecting rebound activation after the washout of genistein. However, preconditioning with genistein alone induced neither the augmentation of prostaglandin E_2 release nor the expression of cyclooxygenase-2. The further potentiation of bradykinin-induced prostaglandin E_2 release by combined preconditioning with fresh culture medium and genistein may be due to the activation of the MAPK/ERK-c phospholipase A_2 pathway by preconditioning with genistein.

Keywords: Medium change; Genistein; Prostaglandin E_2; Cytosolic phospholipase A_2; Cyclooxygenase-2; MAP (Mitogen-activated protein) kinase

1. Introduction

Phospholipase A_2, which catalyzes the hydrolysis of the sn-2 fatty acyl bond of phospholipids to yield arachidonic acid lysophospholipids, exists in three groups, extracellular secretory phospholipase A_2, intracellular cytosolic phospholipase A_2 and Ca^{2+}-independent phospholipase A_2. Cytosolic phospholipase A_2 is activated under Ca^{2+} concentrations found in the cytosol (Lin et al., 1992) by G-protein-coupled or tyrosine kinase-coupled receptor stimulation (Clark et al., 1991; Sharp et al., 1991; Kim et al., 1991). Recent evidence suggests that mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) phosphorylates and activates cytosolic phospholipase A_2 result in the release of arachidonic acid (Lin et al., 1993; Pumiglia and Decker, 1997).

Prostaglandins, arachidonic acid metabolites of the cyclooxygenase pathway, are major mediators in the regulation of inflammation and immune function (Gyires and Knoll, 1975). Cyclooxygenase exists in two isoforms, the constitutive form, cyclooxygenase-1, and the inducible form, cyclooxygenase-2. Cyclooxygenase-1 is constitutively expressed in a wide range of cells and tissues (Funk et al., 1991) and may undergo slow changes in the levels of expression associated with cellular differentiation (Smith et al., 1993), whereas cyclooxygenase-2 is highly expressed in inflammatory cells stimulated (e.g. macrophages) by a variety of pro-inflammatory agents (O'Sullivan et al., 1992). The characteristics of their expression suggest that cyclooxygenase-1 may be important for the production of prostaglandins mediating homeostatic functions, while cyclooxygenase-2 may contribute mainly to the increased prostaglandin production localized to specific tissues affected by inflammation.

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Tyrosine phosphorylation has been implicated in normal and abnormal cell growth (Hunter and Cooper, 1985; Perlmutter et al., 1988; Yarden and Ullrich, 1988; Ullrich and Schlessinger, 1990). Growth factor receptors are known to be protein tyrosine kinases in themselves (Yarden and Ullrich, 1988), the enzymatic activation of which is central to their signal transduction processes (Perlmutter et al., 1988). Genistein (Akiyama et al., 1987), a putative tyrosine kinase inhibitor, is widely used as a pharmacological tool to inhibit tyrosine kinases in cell signaling studies. It is suggested that tyrosine kinase is important for the activation of cytosolic phospholipase A_2 (Zor et al., 1993). Previously, we showed that preconditioning of cells with fresh culture medium prior to the stimulation accelerated bradykinin-induced prostaglandin E_2 release from Swiss 3T3 fibroblasts (Nakatani et al., 1998). In the present study, we found that the combined preconditioning of cells with fresh medium and genistein potently enhanced bradykinin-induced prostaglandin E_2 release. Then, we attempted to clarify the mechanism of the augmentation of prostaglandin E_2 release by combined preconditioning of the cells with fresh medium and genistein.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) and Eagle's minimum essential medium (EMEM) were purchased from Nissui Pharmaceutical (Tokyo, Japan). Fetal calf serum was obtained from JRH Biosciences (Lanexa, KS, USA). Bovine serum albumin and bradykinin were from Sigma (St. Louis, MO, USA). Prostaglandin E_2 and anti-prostaglandin E_2 antibody were generous gifts from Ono Pharmaceuticals (Osaka, Japan). [^{3}H]prostaglandin E_2 (200 Ci/mmol) and [^{14}C]arachidonic acid (50 mCi/mmol) were from NEN/DuPont (Boston, MA, USA). Anti-phospho-MAPK antibody and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG) were obtained from New England Biolabs (Beverly, MA, USA). Anti-phosphotyrosine antibody was from Upstate Biotechnology (Lake Placid, NY, USA) and alkaline phosphatase-conjugated goat anti-mouse IgG was from Bio-Rad (Hercules, CA, USA). Anti-cyclooxygenase-1 and -2 antibodies were obtained from Santa Cruz (Delaware, CA, USA). Alkaline phosphatase-conjugated affinity-purified anti-goat IgG was from Rockland (Gilbertsville, PA, USA). Genistein and sodium dodecyl sulfate (SDS) were obtained from Wako (Tokyo, Japan). Other chemicals and drugs were of reagent grade or of the highest quality available.

2.2. Cell culture

Swiss 3T3 fibroblasts were cultured in DMEM, supplemented with 10% fetal calf serum, 50 units/ml of penicillin and 50 μg/ml of streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C until preconfluent.

2.3. Assay of prostaglandin E_2

Swiss 3T3 fibroblasts were seeded into 12-well plates at a density of 1.0×10^4 cells/well and were used 2 days after subculture. The old culture medium was replaced by fresh culture medium with or without genistein. In some experiments, genistein was added to the old medium without replacing the medium. Then, the cells were incubated for 1 h (preconditioning). After incubation, the cells were washed twice with EMEM buffered with 20 mM HEPES, pH 7.35 (EMEM–HEPES) and then incubated with bradykinin for 10 min after the preincubation for 10 min. The reaction medium was collected in an ice-cold tube at the end of incubation. Medium was stored at −20 °C until extraction of prostaglandin E_2. Prostaglandin E_2 was extracted twice with ethyl acetate after acidification of the medium to pH 4.0 with 1 N HCl. Ethyl acetate was evaporated under a stream of N_2 gas at 40 °C. Prostaglandin E_2, which was dissolved in 10 mM Tris–HCl (pH 7.6), was determined by radioimmunoassay, as described previously (Nakahata et al., 1996). The sample was incubated overnight at 4 °C with [^{3}H]prostaglandin E_2 (10,000 dpm) and anti-prostaglandin E_2 antibody (450 times dilution) in a final volume of 150 μl containing 0.5% bovine serum albumin in 100 mM Tris–HCl (pH 7.6). The free [^{3}H]prostaglandin E_2 was sedimented by centrifugation after the addition of 0.5 ml of a buffer containing 0.5% charcoal, 0.075% dextran and 0.5% bovine serum albumin in 100 mM Tris–HCl (pH 7.6). The [^{3}H]prostaglandin E_2 bound to antibody was counted by liquid scintillation counting.

2.4. Immunoblotting

Swiss 3T3 fibroblasts were seeded into six-well plates at a density of 1.0×10^5 cells/well and were used 2 days after subculture. The old culture medium was replaced by fresh culture medium with or without genistein. In some experiments, genistein was added to the old medium without replacing the medium. Then, the cells were incubated for 1 h (preconditioning). After incubation, the reaction was terminated by the addition of Laemmli sample buffer (final concentration, 187.5 mM Tris–HCl, 6% SDS, 30% glycerol, 15% 2-mercaptoethanol, pH 6.8) after aspiration of the medium for the analysis of cyclooxygenase-1 or -2. For the analysis of MAPK or tyrosine kinase, the cells were washed twice with EMEM–HEPES and preincubated for 10 min at 37 °C. The cells were incubated with bradykinin for an additional 2 min, and the reaction was terminated by the addition of Laemmli sample buffer after aspiration of the medium (Laemmli,

1970). The sample was boiled at 95 °C for 5 min and separated on 8–11% SDS-polyacrylamide gels. Proteins were electrically transferred from the gel onto Immobilon polyvinylidene difluoride membranes (Millipore) by a semi-dry blotting method (Ohkubo et al., 1996). The blots, blocked with 2% bovine serum albumin in Tris-buffered saline (10 mM Tris–HCl, 100 mM NaCl, pH 7.5) containing 0.05% Tween 20 (TBST) at 25 °C for 2 h, were incubated with anti-phospho-MAPK antibody (rabbit) at 1 μg/ml (Hunter, 1995; Pang et al., 1995; Cowley et al., 1994), anti-phosphotyrosine antibody (mouse) at 1 μg/ml, anti-cyclooxygenase-1 antibody (goat) at 0.1 μg/ml or anti-cyclooxygenase-2 antibody (goat) at 0.1 μg/ml (Adams et al., 1996; Morham et al., 1995; O'Neill et al., 1994) at 25 °C for 2 h. After it has been washed with TBST for several times, the membranes were incubated with a 1:2000–4000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG, alkaline phosphatase-conjugated affinity-purified anti-mouse IgG or alkaline phosphatase-conjugated affinity-purified anti-goat IgG in TBST containing 2% bovine serum albumin overnight at 25 °C for 2 h. Immunoreactivity was determined by a chemiluminescence assay kit (Bio-Rad) and visualized by exposing the chemiluminescence from the membrane to Hyper-film ECL (Amersham).

2.5. Analysis of arachidonic acid

Swiss 3T3 fibroblasts were seeded into 12-well plates at a density of 1.0×10^4 cells/well. Two days after seeding, the cells were cultivated in DMEM containing 0.3 μCi/ml of [^{14}C]arachidonic acid for 18 h. Prior to assay, the cells were incubated with fresh or old culture medium containing 0.3 μCi/ml of [^{14}C]arachidonic acid in the presence or absence of genistein for 1 h. The cells were washed twice with EMEM–HEPES–albumin solution (pH 7.35), preincubated for 10 min, and then incubated with bradykinin for 7 min at 37 °C. The medium (0.6 ml) was transferred to tubes, and 0.6 ml of 1 N HCl–methanol solution was added (Rho et al., 1997). After further addition of chloroform (1.2 ml) and water (0.6 ml), the lower phase was dried under a stream of nitrogen gas and applied to a thin-layer chromatography (TLC) plate (LK5D, Whatman, Clifton, NJ). The developer used was the upper phase of benzene–isooctane–acetic acid (60:30:3, v/v) (Rho et al., 1997). [^{14}C]Arachidonic acid metabolites were visualized in the radioluminogram with a molecular imager (GS363, Bio-Rad).

2.6. Statistical analysis of the data

The data are expressed as means ± S.E.M., and a significant difference ($P < 0.05$) was analyzed by means of a two-way analysis of variance (two-way ANOVA).

3. Results

3.1. Effect of preconditioning of Swiss 3T3 fibroblasts with fresh medium and genistein on bradykinin-induced prostaglandin E_2 release

While bradykinin did not cause prostaglandin E_2 release in Swiss 3T3 fibroblasts under normal culture conditions, preconditioning of the cells with fresh DMEM containing 10% fetal calf serum (fresh medium) prior to stimulation resulted in potent prostaglandin E_2 release induced by bradykinin (Fig. 1). Treatment of the cells with genistein in fresh medium for 1 h resulted in a further enhancement of bradykinin-induced prostaglandin E_2 release. The potentiation by genistein was concentration dependent (Fig. 2). However, treatment of the cells with 50 μM genistein alone in old culture medium for 1 h prior to the assay did not cause bradykinin-induced prostaglandin E_2 release (Fig. 1). Genistein was tested in a concentration range of 10–100 μM (data not shown). These results indicate that genistein augments the preconditioning effect of fresh medium on bradykinin-induced PGE_2 release.

3.2. Effect of preconditioning of cells with fresh medium and genistein on protein tyrosine phosphorylation and MAPK phosphorylation

Genistein caused an augmentation of the preconditioning effect of fresh medium on bradykinin-induced prostaglandin

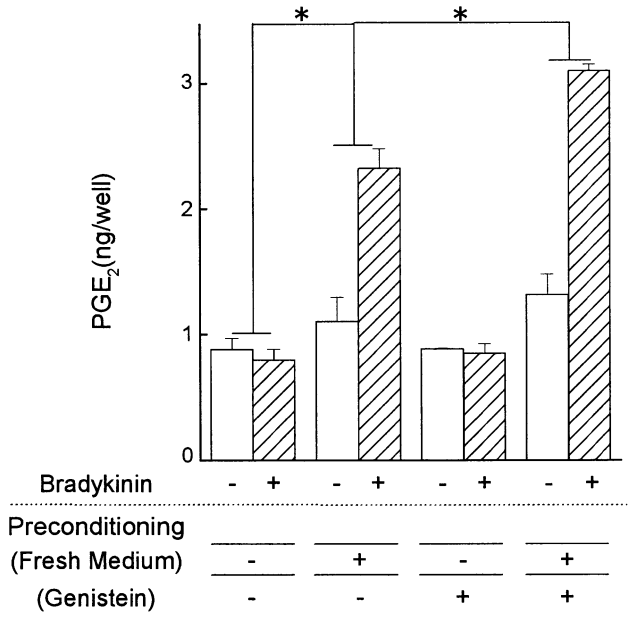

Fig. 1. Effect of preconditioning of cells with fresh medium and genistein (50 μM) on bradykinin-induced prostaglandin E_2 release. The cells were incubated with (hatched column) or without (open column) bradykinin (1 μM). Values are means ± S.E.M. ($n = 3$). Statistical significance is indicated: * $P < 0.05$, different from control by two-way ANOVA.

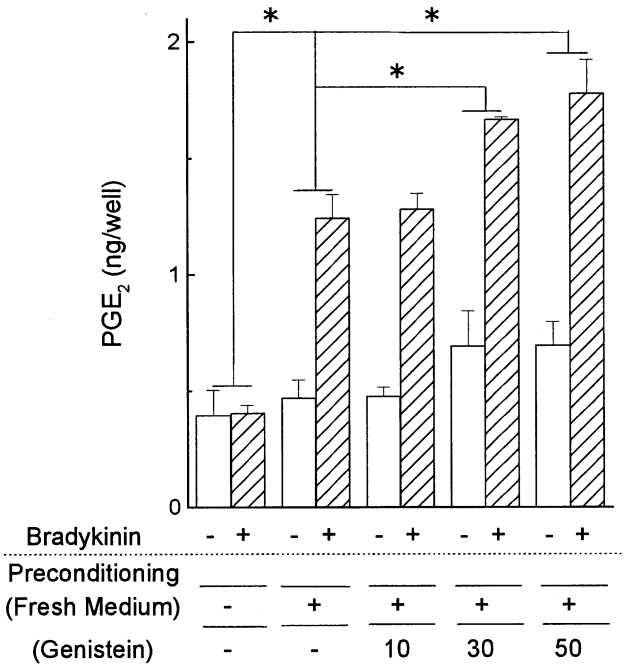

Fig. 2. Concentration-dependent augmentation by genistein preconditioning of bradykinin-induced prostaglandin E_2 release in cells preconditioned with fresh medium. The cells were incubated with (hatched column) or without (open column) bradykinin (1 μM). Values are means ± S.E.M. ($n=3$). Statistical significance is indicated: * $P<0.05$, different from control by two-way ANOVA.

E_2 release, although it is known as an inhibitor of protein tyrosine kinase (Fig. 3). Therefore, we examined the effect of genistein on protein tyrosine phosphorylation. The p150 and p55 proteins were used as typical proteins for quantitative analysis of tyrosine phosphorylation (Fig. 3B). Treatment of the cells with genistein for the entire period of preconditioning and incubation with bradykinin resulted in an inhibition of bradykinin-induced protein tyrosine phosphorylation (Fig. 3, lane 6). However, treatment of the cells with genistein alone in old medium prior to the assay resulted in a marked activation of protein tyrosine phosphorylation (Fig. 3, lane 3).

The MAPK family is involved in a wide range of cellular functions, including cytosolic phospholipase A_2 activation (Pumiglia and Decker, 1997). Then, we examined the protein phosphorylation of MAPK/ERK, determined by immunoblotting using an anti-phospho-MAPK/ERK antibody, in order to clarify the involvement of MAPK/ERK in the augmentation of bradykinin-induced prostaglandin E_2 release by combined preconditioning with fresh medium and genistein (Fig. 4). When the cells were preconditioned with fresh medium for 1 h, the phosphorylation of MAPK/ERK was markedly activated by bradykinin. Preconditioning of the cells with fresh medium and genistein for 1 h further enhanced the bradykinin-induced phosphorylation of MAPK/ERK. Interestingly, preconditioning of the cells with genistein alone in old medium prior to the assay resulted in a marked activation of the bradykinin-induced phosphorylation of MAPK/ERK (Fig. 4), although it did not cause bradykinin-induced prostaglandin E_2 release (Fig. 1).

3.3. Effect of preconditioning of cells with fresh medium and genistein on arachidonic acid liberation

Since preconditioning with genistein alone caused the augmentation of bradykinin-induced phosphorylation of

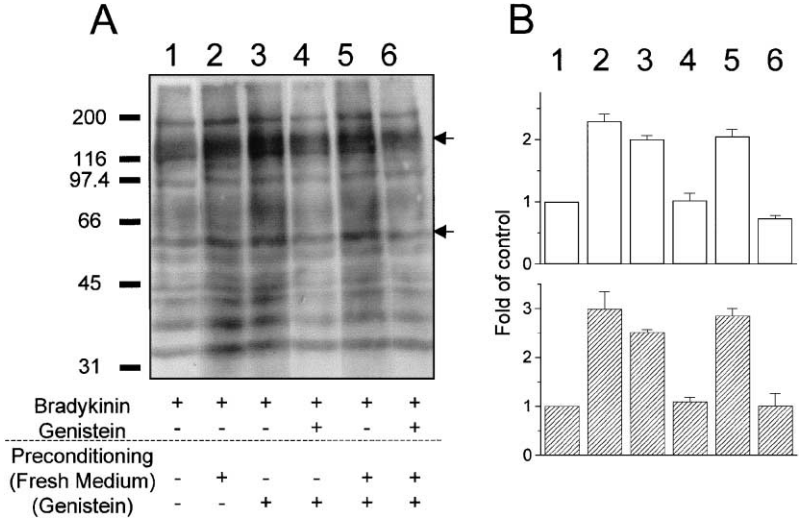

Fig. 3. Effect of preconditioning of cells with fresh medium and genistein on tyrosine phosphorylation. (A) Immunoblotting. Prior to assay, the old culture medium was replaced by fresh culture medium in the presence (lanes 5, 6) or absence (lane 2) of 50 μM genistein, or 50 μM genistein was added to the old culture medium (lanes 3, 4). The cells were further incubated with bradykinin (1 μM) in the presence (lanes 4, 6) or absence (lanes 1–3, 5) of genistein (50 μM). The same amount of protein was loaded in each lane, determined by Coomassie staining of the gel after electrophoresis. (B) Densitometric analysis of typical proteins. Upper figure, the phosphorylation of p150 protein indicated as upper arrow in (A). Lower figure, the phosphorylation of p55 protein indicated as lower arrow in (A). The results are shown as the fold increase of control (without fresh medium or genistein). Values are means ± S.E.M. ($n=3$).

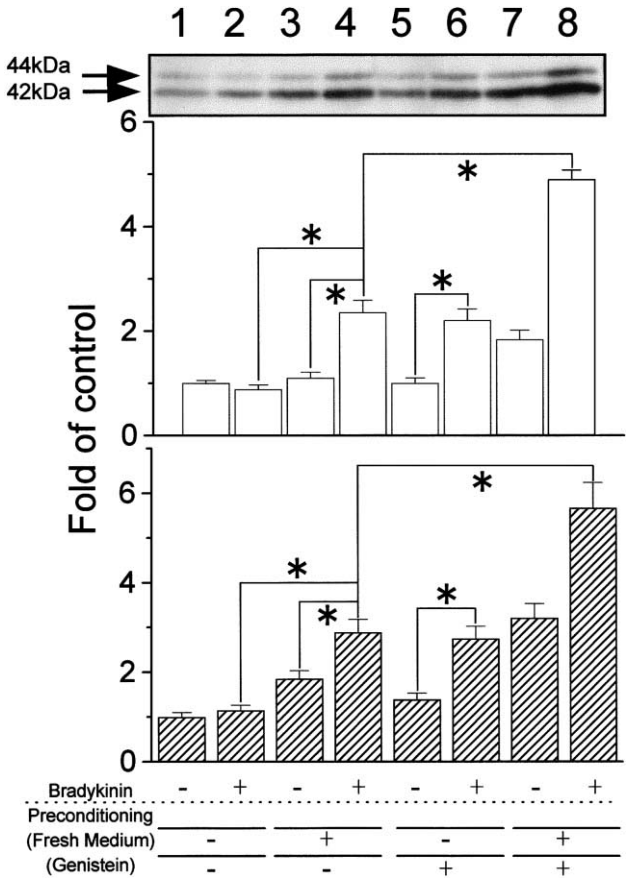

Fig. 4. Effect of preconditioning of the cells with fresh medium and genistein on MAPK/ERK phosphorylation. Prior to assay, the old culture medium was replaced by fresh culture medium in the presence (lanes 7, 8) or absence (lanes 3, 4) of genistein (50 μM), or genistein was added to the old culture medium (lanes 5, 6). After the cells were preconditioned for 1 h and washed, they were further incubated in the presence (lanes 2, 4, 6, 8) or absence (lanes 1, 3, 5, 7) of bradykinin (1 μM) for 2 min. The same amount of protein was loaded in each lane, determined by Coomassie staining of the gel after electrophoresis. Immunoblotting (upper figure) and densitometric analysis results (middle and lower figures) are shown. Open column in the middle figure: the phosphorylation of p44 MAPK; hatched column in the lower figure: the phosphorylation of p42 MAPK. The results are shown as the fold increase of control (without fresh medium or bradykinin). Values are means ± S.E.M. ($n=3$). Statistical significance is indicated in the figure: * $P<0.05$, by one-way ANOVA.

MAPK/ERK (Fig. 4) in spite of a lack of prostaglandin E_2 release (Fig. 1), we examined the arachidonic acid liberation in [^{14}C]arachidonic acid-labelled cells (Fig. 5). When the cells were preconditioned with fresh medium for 1 h, bradykinin markedly liberated arachidonic acid. The preconditioning of the cells with fresh medium and genistein for 1 h prior to stimulation resulted in a further enhancement of the bradykinin-induced liberation of arachidonic acid. These observations were consistent with the results of the augmented bradykinin-induced prostaglandin E_2 release seen in the cells preconditioned with fresh medium and genistein (Figs. 1 and 2). Interestingly, preconditioning with genistein alone in old medium caused the augmentation of bradykinin-induced arachidonic acid liberation. The results were consistent with the phosphorylation of MAPK/ERK (Fig. 4), but not with prostaglandin E_2 release (Fig. 1).

3.4. Effect of preconditioning of cells with fresh medium and genistein on the expression of cyclooxygenase-1 or -2

Cyclooxygenase is the rate-limiting enzyme in the conversion of arachidonic acid to prostaglandins (Rosen et al., 1989). Since preconditioning with genistein alone in old medium resulted in the augmentation of bradykinin-induced arachidonic acid liberation, we next examined the

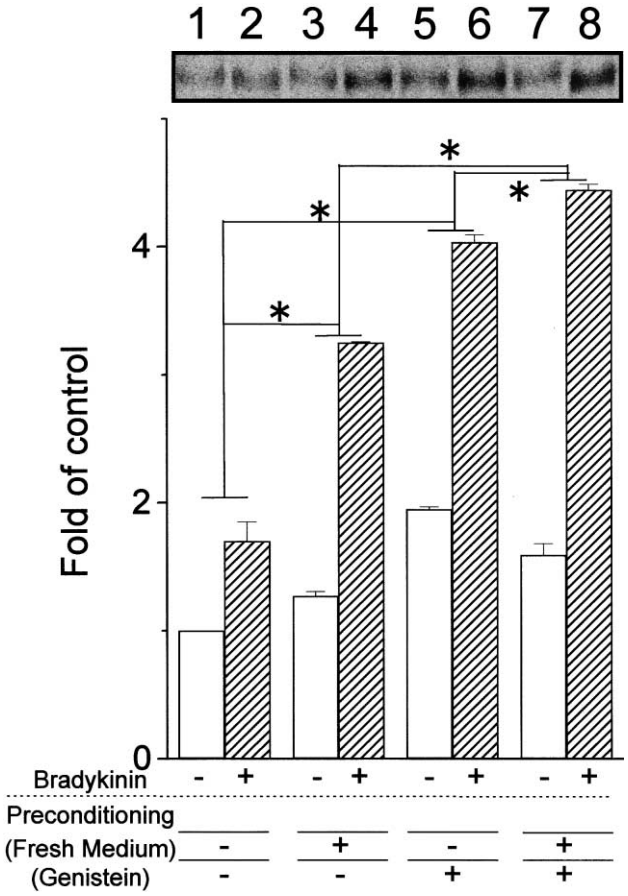

Fig. 5. Effect of preconditioning of cells with fresh medium and genistein on the liberation of arachidonic acid. Prior to assay, the cells were preconditioned with fresh culture medium containing 0.3 μCi/ml of [^{14}C]arachidonic acid in the presence (lanes 7, 8) or absence (lanes 3, 4) of genistein (50 μM), or with old medium in the presence (lanes 5, 6) or absence (lanes 1, 2) of genistein for 1 h. After being washed, the cells were incubated in the presence (lanes 2, 4, 6, 8) or absence (lanes 1, 3, 5, 7) of 1 μM bradykinin for 10 min. [^{14}C]arachidonic acid separated by thin-layer chromatography was analyzed by radioluminography (upper figure). Lower figure shows densitometric analysis of the radioluminogram. Open column: basal liberation, hatched column: bradykinin-induced liberation. The results are shown as the fold increase of control (without fresh medium or bradykinin). Values are means ± S.E.M. ($n=3$). Statistical significance is indicated: * $P<0.05$, two-way ANOVA.

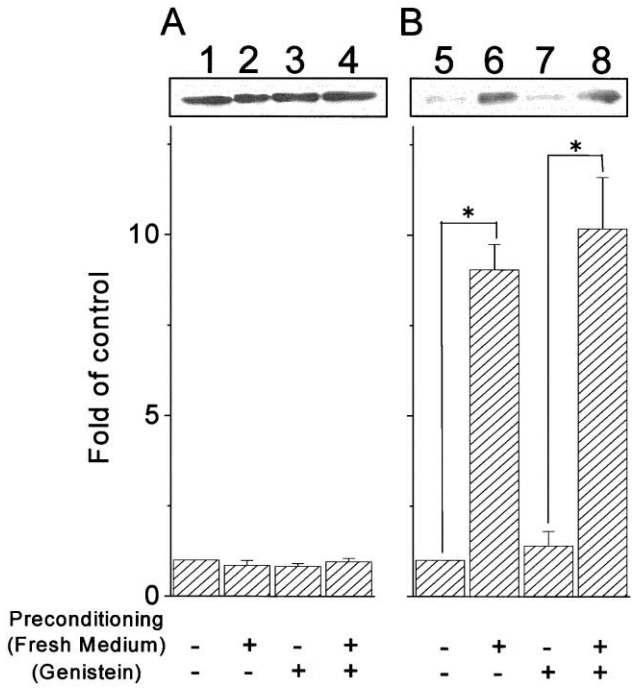

Fig. 6. Effect of preconditioning of cells with fresh medium and genistein on the expression of cyclooxygenase-1 (A) or cyclooxygenase-2 (B). The old culture medium was replaced by fresh culture medium with (lanes 4, 8) or without (lanes 2, 6) genistein (50 μm), or genistein was added to the old culture medium (lanes 3, 7). Immunoblotting (upper figure) and densitometric analysis results (lower figure) are shown. The densitometric data are calculated as the fold increase of control (without medium change or genistein). Values are means ± S.E.M. ($n = 3$). Statistical significance is indicated: * $P < 0.05$, by one-way ANOVA.

expression of cyclooxygenase-1 or cyclooxygenase-2 protein by immunoblotting using anti-cyclooxygenase-1 or anti-cyclooxygenase-2 antibody. The expression of cyclooxygenase-1 was not changed by the preconditioning of the cells for 1 h with either fresh medium, genistein alone or fresh medium containing genistein (Fig. 6A). In contrast, the expression of cyclooxygenase-2 was enhanced by the preconditioning of the cells with fresh medium for 1 h (Fig. 6B). Preconditioning with fresh medium containing genistein enhanced the expression of cyclooxygenase-2 to a similar extent to that was seen with fresh medium alone. However, preconditioning with genistein alone in old medium did not enhance cyclooxygenase-2 expression (Fig. 6B).

4. Discussion

Under normal culture conditions (DMEM-10% fetal calf serum), Swiss 3T3 fibroblasts produce little prostaglandin E_2 in response to bradykinin. Previously, we have shown that preconditioning of the cells with fresh medium prior to stimulation resulted in the augmentation of bradykinin-induced prostaglandin E_2 release (Nakatani et al., 1998). The present study demonstrated that preconditioning of the cells with genistein, a tyrosine kinase inhibitor, together with fresh medium further enhanced bradykinin-induced prostaglandin E_2 release.

Tyrosine-specific protein kinase activity is associated with a variety of cellular responses (Jerome et al., 1991), including the activation of cytosolic phospholipase A_2 (Goldman et al., 1994). Purified cytosolic phospholipase A_2 can be stoichiometrically phosphorylated and activated by MAPK/ERK (Lin et al., 1993; Sa et al., 1995), and phosphorylation of tyrosine and threorine residues is required for the activation of MAPK/ERK (Nishida and Gotoh, 1993; Davis, 1994). Although the treatment of the cells with genistein for the entire period of preconditioning and incubation with bradykinin resulted in a reduction of bradykinin-induced prostaglandin E_2 release (Nakatani et al., 1998), preconditioning of the cells with fresh medium and genistein resulted in a potent enhancement of a bradykinin-induced prostaglandin E_2 release after the removal of genistein from the medium. This enhancement was accompanied by an augmentation of the phosphorylation of protein tyrosine and MAPK/ERK, arachidonic acid liberation and cyclooxygenase-2 expression.

In contrast to the effect of the combined preconditioning with fresh medium and genistein, treatment of the cells with genistein alone in old medium for 1 h prior to the assay did not cause bradykinin-induced prostaglandin E_2 release. However, preconditioning with genistein alone augmented the bradykinin-induced phosphorylation of protein and MAPK/ERK, and arachidonic acid liberation. Cyclooxygenase is the rate-limiting enzyme in the conversion of arachidonic acid to prostaglandins (Rosen et al., 1989). While the expression of cyclooxygenase-1 was not augmented by preconditioning of the cells with fresh medium or fresh medium plus genistein, the expression of cyclooxygenase-2 was enhanced by preconditioning. Thus, the bradykinin-induced prostaglandin E_2 release from cells preconditioned with fresh medium is due not only to the activation of MAPK/ERK and cytosolic phospholipase A_2, but also to the enhanced expression of cyclooxygenase-2. However, preconditioning with genistein alone induced the expression of neither cyclooxynase-1 nor -2. Since, preconditioning with genistein alone stimulated arachidonic acid liberation, it is assumed that the expression of cyclooxygenase-2 is critical for the production of prostaglandin E_2 in Swiss 3T3 cells. Cyclooxygenase-1 may not be responsible for its synthesis. It is interesting to know why preconditioning with genistein activated the MAPK/ERK-cytosolic phospholipase A_2 pathway. One possible explanation of the results is that the removal of genistein after its preconditioning may change the regulatory state of some tyrosine kinases in the cells, resulting in the phosphorylation of MAPK/ERK via activation of tyrosine kinases. Similar phenomena are often called rebound activation. Recently, it was shown that genistein in a higher concentration range stimulated human platelet MAPK activity in a concentration- and time-dependent manner

(Kansra et al., 1999), even though platelets were treated with genistein for the entire study period. Further study is necessary to clarify the detailed mechanism of genistein action.

In conclusion, preconditioning of Swiss 3T3 fibroblasts with fresh medium prior to stimulation results in the augmentation of bradykinin-induced prostaglandin E_2 release through the activation of the MAPK/ERK-cytosolic phospholipase A_2 pathway with an increased expression of cyclooxygenase-2. Preconditioning with genistein alone results in the activation of the MAPK/ERK-cytosolic phospholipase A_2 pathway without cyclooxygenase-2 expression. The further potentiation of bradykinin-induced prostaglandin E_2 release by combined preconditioning with fresh medium and genistein may be because preconditioning with genistein causes the activation of the MAPK/ERK-cytosolic phospholipase A_2 pathway. Further detailed analysis of the augmentation will be helpful for understanding the molecular regulatory mechanism of the receptor-mediated activation of the arachidonic acid cascade.

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References

Adams, J., Collaco-Moraes, Y., de Belleroche, J., 1996. Cyclooxygenase-2 induction in cerebral cortex: an intracellur response to synaptic excitation. J. Neurochem. 66, 6–13.

Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S., Itoh, N., Shibuya, M., Fukami, Y., 1987. Genistein: a specific inhibitor of tyrosine specific protein kinases. J. Biol. Chem. 262, 5592–5595.

Clark, J.D., Lin, L.L., Kriz, R.W., Ramesha, C.S., Sultzman, L.A., Lin, A.Y., Milota, N., Knopf, J.K., 1991. A novel arachidonic acid-selective cytosolic PLA_2 contains a Ca^{2+}-dependent translocation domain with homology to PKC and GAP. Cell 65, 1043–1051.

Cowley, S., Paterson, H., Kemp, P., Marshall, C.J., 1994. Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. Cell 77, 841–852.

Davis, R.J., 1994. MAPKs: New JNK expands the group. Trends Biochem. Sci. 19, 470–473.

Funk, C.D., Funk, L.B., Kennedy, M.E., Pong, A.S., Fitzgerald, G.A., 1991. Human platelet/erythroleukemia cell prostaglandin G/H synthase: cDNA cloning, expression, and chromosomal assignment. FASEB J. 5, 2304–2312.

Goldman, R., Ferber, E., Meller, R., Zor, U., 1994. A role for reactive oxygen species in zymosan and beta-glucan induced protein tyrosine phosphorylation and phospholipase A_2 activation in murine macrophages. Biochim. Biophys. Acta 1222, 265–276.

Gyires, K., Knoll, J., 1975. Inflammation and writhing syndrome inducing effect of PGE_1, PGE_2 and the inhibition of these actions. Pol. J. Pharmacol. Pharm. 27, 257–264.

Hunter, T., 1995. Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. Cell 80, 225–236.

Hunter, T., Cooper, J.A., 1985. Protein-tyrosine kinases. Annu. Rev. Biochem. 54, 897–930.

Jerome, V., Leger, J., Devin, J., Baulieu, E.E., Catelli, M.G., 1991. Growth factors acting via tyrosine kinase receptors induce HSP90 alpha gene expression. Growth Factors 4, 317–327.

Kansra, S.V., Reddy, M.A., Weng, Y.I., Shukla, S.D., 1999. Activation of mitogen activated protein kinase in human platelets by genistein. Pharmacol. Res. 39, 21–31.

Kim, D.K., Kudo, I., Inoue, K., 1991. Purification and characterization of rabbit platelet cytosolic phospholipase A_2. Biochim. Biophys. Acta 1083, 80–88.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lin, L.L., Lin, A.Y., Dewitt, D.L., 1992. Interleukin-1 alpha induces the accumulation of cytosolic phospholipase A_2 and the release of prostaglandin E_2 in human fibroblasts. J. Biol. Chem. 267, 23451–23454.

Lin, L.L., Wartmann, M., Lin, A.Y., Knopf, J.V., Hyslop, P.A., Jakubowski, J.A., 1993. $cPLA_2$ is phosphorylated and activated by MAP kinase. Cell 72, 269–278.

Morham, S.G., Langenbach, R., Loftin, C.D., Tiano, H.F., Vouloumanos, N., Jennett, J.C., Mahler, J.F., Kluckman, K.D., Ledford, A., Lee, C.A., Smithies, O., 1995. Prostaglandin synthase 2 gene disruption causes severe renal pathology in the mouse. Cell 83, 473–482.

Nakahata, N., Imata, K., Okawa, T., Watanabe, Y., Ishimoto, H., Ono, T., Ohizumi, Y., Nakanishi, H., 1996. Mastoparan elicits prostaglandin E_2 generation and inhibited inositol phosphate accumulation via different mechanisms in rabbit astrocytes. Biochim. Biophys. Acta 1310, 60–66.

Nakatani, K., Nakahata, N., Hamada, Y., Tsurufuji, S., Ohizumi, Y., 1998. Medium change amplifies mitogen-activated protein kinase-mediated PGE_2 synthesis in Swiss 3T3 fibroblasts. Eur. J. Pharmacol. 356, 91–100.

Nishida, E., Gotoh, Y., 1993. The MAP kinase cascade is essential for diverse signal transduction pathways. Trends Biochem. Sci. 18, 128–131.

Ohkubo, S., Nakahata, N., Ohizumi, Y., 1996. Thromboxane A_2 stimulates mitogen-activated protein kinase and arachidonic acid liberation in rabbit platelets. Prostaglandins 52, 403–413.

O'Neill, G.P., Mancini, J.A., Kargman, S., Yergey, J., Kwan, M.Y., Falgueyret, J.P., Abromozitz, M., Kennedy, B.P., Ouellet, M., Cromlish, W., Culp, S., Evans, J.F., Ford-Hutchinson, A.W., Vickers, P.J., 1994. Overexpression of human prostaglandin G/H synthase-1 and -2 by recombinant vaccinia virus: inhibition by nonsteroidal anti-inflammatory drugs and boisynthesis of 15-hydroeicosatetraenoic acid. Mol. Pharmacol. 45, 245–254.

O'Sullivan, M.G., Chilton, F.H., Huggins, E.M., McCall, C.E., 1992. Lipopolysaccharide priming of alveolar macrophages for enhanced synthesis of prostanoids involves induction of a novel prostaglandin H synthase. J. Biol. Chem. 267, 14547–14550.

Pang, L., Sawada, T., Decker, S.J., Saltiel, A.R., 1995. Inhibition of MAP kinase kinase blocks the differentiation of PC-12 cells induced by nerve growth factor. J. Biol. Chem. 270, 13585–13588.

Perlmutter, R.M., Marth, J.D., Ziegler, S.F., Carvin, A.M., Pawar, S., Cooke, M.P., Abraham, K.M., 1988. Specialized protein tyrosine kinase protooncogenes in hematopoietic cells. Biochem. Biopshys. Acta 948, 245–262.

Pumiglia, K.M., Decker, S.J., 1997. Cell cycle arrest mediated by the MEK/mitogen-activated protein kinase pathway. Proc. Natl. Acad. Sci. U. S. A. 94, 448–452.

Rho, M.C., Nakahata, N., Nakamura, H., Murai, A., Ohizumi, Y., 1997. Involvement of phospholipase C-γ2 in activation of mitogen-activated protein kinase and phospholipase A_2 by zooxanthellatoxin-A in rabbit platelets. J. Pharmacol. Exp. Ther. 282, 496–504.

Rosen, G.D., Birkenmeier, T.M., Raz, A., Holtzman, M.J., 1989. Identifi-

cation of a cyclooxygenase-related gene and its potential role in prostaglandin formation. Biochem. Biophys. Res. Commun. 164, 1358–1365.

Sa, G., Murugesan, G., Jaye, M., Ivashchenko, Y., Fox, P.L., 1995. Activation of cytosolic phospholipase A_2 by basic fibroblast growth factor via a p42 mitogen-activated protein kinase-dependent phosphorylation pathway in endothelial cells. J. Biol. Chem. 270, 2360–2366.

Sharp, J.D., White, D.L., Chiou, X.G., Goodson, T., Gamboa, G.C., McClure, D., Burgett, S., Hoskins, J., Skatrud, P.L., Sportsman, J.R., Becker, G.W., Kang, L.H., Roberts, E.F., Kramer, R.M., 1991. Molecular cloning and expression of human Ca^{2+}-sensitive cytosolic phospholipase A_2. J. Biol. Chem. 266, 14850–14853.

Smith, C.J., Morrow, J.D., Roberts, L.J., Marnett, L.J., 1993. Differentiation of monocytoid THP-1 cells with phorbol ester induces expression of prostaglandin endoperoxide synthase-1 (cyclooxygenase-1). Biochem. Biophys. Res. Commun. 192, 787–793.

Ullrich, A., Schlessinger, J., 1990. Signal transduction by receptors with tyrosine kinases activity (review). Cell 61, 203–212.

Yarden, Y., Ullrich, A., 1988. Growth factor receptor tyrosine kinases. Annu. Rev. Biochem. 57, 443–478.

Zor, U., Ferber, E., Gergely, P., Szucs, K., Dombradi, V., Goldman, R., 1993. Reactive oxygen species mediate phorbol ester-regulated tyrosine phosphorylation and phospholipase A_2 activation: potentiation by vanadate. Biochem. J. 295, 879–888.