(−)-Epigallocatechin gallate inhibits Mos activation-mediated *Xenopus* oocyte maturation induced by progesterone

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Abstract (−)-Epigallocatechin gallate (EGCG), one of the constituents of green tea known to have a tumor preventing effect, inhibited maturation of *Xenopus laevis* oocytes induced by progesterone when this polyphenol was microinjected into oocytes at a final concentration of about 1 mM. Western blot and activity measurement analyses showed that Mos translation and the subsequent activations of mitogen-activated protein kinase and p90^{rsk}, probably by protein phosphorylation, seemed to have been inhibited by the microinjection of EGCG. These results suggest that EGCG may have the ability to control *Xenopus* oocyte maturation at least during the stage of Mos activation.

Key words: (−)-Epigallocatechin gallate; Oocyte maturation; Mos; Mitogen-activated protein kinase; Progesterone

1. Introduction

It has been shown that tea polyphenols such as (−)-epigallocatechin gallate (EGCG) have tumor preventing effects caused by antioxidative and antiproliferative activity [1]. Consistent with this report, EGCG has been shown to inhibit tumor cell growth by interfering with the binding of various growth factors to their receptors and subsequent activation of receptor tyrosine kinases [2] or tumor promotion induced by the 12-*O*-tetradecanoyl-phorbol 13-acetate-type promoter, teleocidin, in a two-step carcinogenesis system using mouse skin [3]. Direct inhibition by EGCG of teleocidin-activated protein kinase C (PKC) or protein-tyrosine kinase activity has also been demonstrated in purified assay systems [2,3]. Another report also indicated that EGCG had the ability to arrest cell cycle transfer at the G_2-M stage [4]. This evidence suggests that EGCG may have some pleiotropic effects in the control of cell proliferation.

Recently, the decreased activity or the decreased phosphorylation level of the three types of mitogen-activated protein (MAP) kinase was demonstrated using various stimulated or transformed cells after treatment with EGCG [5–7]. A previous report from our laboratory indicated that EGCG showed an inhibitory effect on extracellular signal-regulated-type MAP kinase activity in vitro [8]. In an attempt to clarify the effect of EGCG on MAP kinase in a cellular system, the *Xenopus laevis* oocyte was selected because MAP kinase has been shown to play a pivotal role in oocyte maturation induced by progesterone [9]. It has also been established that the protein serine/threonine kinase cascade exists as the signal transducing mechanism initiated by this hormone [10–12]. Among the protein kinases, Mos seemed to be the first one whose translation was initially stimulated and then activated by progesterone [13–18]. The present results suggest that EGCG might seriously affect oocyte maturation at a point(s) upstream from MAP kinase.

2. Materials and methods

2.1. Materials and chemicals

X. laevis females were purchased from Johoku Seibutsu Kyozai (Shizuoka) and maintained in aquariums at 25°C. Collagenase (type 1A), progesterone and myelin basic protein (MBP) were obtained from Sigma. EGCG and [γ-^{32}P]ATP were purchased from Kurita Kogyo (Tokyo, Japan) and Amersham, respectively. Polyclonal antibodies against Mos and p90^{rsk} and agarose-conjugated p90^{rsk} antibody were obtained from Santa Cruz Biotechnology. Monoclonal antibody against phospho(threonine-202/tyrosine-204) extracellular signal-regulated-type MAP kinase and its agarose conjugate were purchased from New England Biolabs. protein kinase A (PKA) and PKC inhibitor peptides were prepared as indicated earlier [19]. The 40S ribosomal subunits, prepared as indicated previously [20], were a kind gift of Dr. K. Mizuta (Hiroshima University).

2.2. Culture of oocytes and microinjection

Xenopus ovaries were isolated as indicated earlier [19] except that modified Barth solution medium which contained 10 mM HEPES at pH 7.5, 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$ and 0.41 mM $CaCl_2$ was employed. After separation into small pieces, collagenase digestion was performed at a concentration of 2 mg/ml in the same medium (Ca^{2+}-free) at room temperature with gentle rotation. Isolated oocytes were washed thoroughly with the Ca^{2+}-containing medium and those at stage VI were selected. EGCG was injected into about 30 oocytes in a volume of 50 nl at a final concentration of about 1 mM using a Dramond 203-XV autoinjector pipette. For the control, distilled water was injected into about 30 oocytes. These oocytes were cultured in the presence of 2 μg/ml progesterone or the same concentration of ethanol at 20°C. Germinal vesicle break-down (GVBD) was observed as the appearance of a white spot on the animal pole.

2.3. Preparation of enzymes

After incubation, oocytes were homogenized in a solution (22 μl per

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Abbreviations: EGCG, (−)-epigallocatechin gallate; PKA, protein kinase A; PKC, protein kinase C; MBP, myelin basic protein; rsk, ribosomal protein S6 kinase; GVBD, germinal vesicle break-down

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oocyte) which contained 50 mM β-glycerophosphate at pH 7.3, 7 mM NaF, 0.3 mM EDTA, 15 mM $MgCl^2$, 2 mM dithiothreitol using a pellet pestle mixer (Kontes) by 10 strokes as indicated earlier [21]. The homogenate was ultracentrifuged at 100 000×*g* for 20 min at 5°C using a Hitachi CS-120 ultracentrifuge and the resultant supernatant was used as an enzyme source. The solution was divided into small portions and stored at −80°C until use.

2.4. Immunoprecipitation and detection of protein kinase activities

To each enzyme solution (87 μg protein) obtained above, EGTA, phenylmethylsulfonyl fluoride and leupeptin were supplemented at final concentrations of 1 mM (for MAP kinase) or 20 mM (for $p90^{rsk}$), 1 mM and 10 μg/ml, respectively. An aliquot of the original agarose-conjugated antibody (15 μl for each enzyme) was added to the enzyme solution and this mixture was rotated overnight (for MAP kinase) for 2 h (for $p90^{rsk}$) at 4°C. For kinase activity measurement, the recovered agarose gel was washed twice each with the homogenizing buffer containing EGTA and protease inhibitors and the kinase buffer (omitting substrate and ATP) indicated below. The agarose gel was finally suspended in 50 μl of the kinase buffer.

Both enzyme activities were measured based on the method described previously [21]. The reaction mixture (40 μl) contained 25 mM MOPS at pH 7.2, 60 mM β-glycerophosphate, 30 mM *p*-nitrophenyl phosphate, 15 mM EGTA, 15 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM Na_3VO_4, 10 μM PKA inhibitor peptide, 10 μM PKC inhibitor peptide, substrate protein (1 mg/ml MBP or 0.5 mg/ml 40S ribosomal subunits) and the enzyme preparation as indicated. The reactions were started with the addition of 50 μM [γ-^{32}P]ATP (1200 cpm/pmol). Incubation was performed at 30°C for 30 min and the reaction was stopped by the addition of the sodium dodecyl sulfate (SDS) sample buffer, which was concentrated five times. After SDS-polyacrylamide gel electrophoresis (PAGE), the radioactive proteins were visualized by autoradiography with intensifying screen for 1–2 days at −80°C.

2.5. Electrophoresis and Western blotting

SDS-PAGE was performed as described by Laemmli [22] utilizing 7.5% (for $p90^{rsk}$), 12.5% (for Mos and MAP kinase and for detection of the $p90^{rsk}$ activity) and 15% (for detection of the MAP kinase activity) acrylamide separating gels and 4.5% stacking gel. Proteins on the gel were transferred to PVDF membrane using a Trans-blot SD Semi-dry Transfer Cell (Bio-Rad) at 12 V for 40 min according to the manual published from the company. The procedures for blocking with 5% skim milk solution and the subsequent reactions with antibodies were essentially the same as those indicated earlier [23].

2.6. Determinations

Protein was determined with bovine serum albumin as a standard according to the method of Bradford [24]. Concentration of the 40S ribosomal subunit was determined as described previously [25]. Radioactivity was determined with a Beckman LS-5801 liquid scintillation counter with Cerenkov radiation.

3. Results

3.1. Effect of microinjection of EGCG on oocyte maturation and Mos activation

Xenopus oocytes were microinjected with EGCG or distilled water and stimulated with progesterone. As shown in Fig. 1A, GVBD was observed in about 80% of water-injected oocytes at 7 h incubation with the hormone. However, only 20% of EGCG-injected oocytes exhibited GVBD at the same time. This result indicated that EGCG had some inhibitory effect on oocyte maturation by progesterone.

It has been well known that Mos translation is stimulated by progesterone and this process is necessary for activation of the MAP kinase cascade and oocyte maturation [9–11,13–18]. In the next experiment, the enzyme extracts were examined for Mos production by an immunoblot procedure. As indicated in Fig. 1B, Mos protein was detected in the sample prepared from progesterone-treated oocytes in the absence of EGCG.

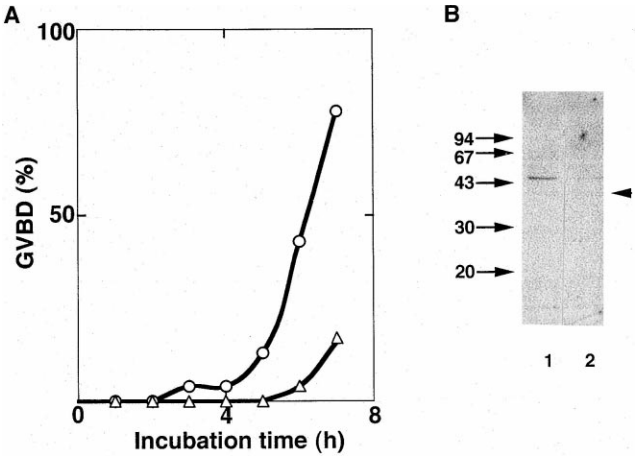

Fig. 1. Inhibition of oocyte maturation (A) and Mos production (B) by microinjection of EGCG. About 30 oocytes each were microinjected with distilled water (○) and EGCG (△), respectively, and stimulated with progesterone; at the time indicated, GVBD was observed as indicated under Section 2. The percentages of oocytes showing GVBD are presented. After incubation for 7 h, each group of oocytes was homogenized and a crude enzyme solution was prepared; an aliquot of each preparation (10 μg of protein) was subjected to Western blot analyses using Mos antibody. Lanes 1 and 2 correspond to the samples prepared from water-injected and hormone-treated, and EGCG-injected and hormone-treated oocytes, respectively. The arrowhead indicates the position of Mos. Numbers on the left side show the positions of molecular mass markers in kDa.

On the other hand, the trace of the protein band was detected in the sample prepared from EGCG-microinjected oocytes.

3.2. Effect of microinjection of EGCG on oocyte MAP kinase and $p90^{rsk}$ activation

Based on the previous reports, it has been established that MAP kinase and $p90^{rsk}$ are protein kinases existing downstream from Mos [9–12,16–18,26]. In the next experiments, the effect of microinjected EGCG was examined on both kinase activations using the immunoblot procedure and activity measurement. The immunoblot analysis of MAP kinase was examined using an antibody which can recognize activated extracellular signal-regulated-type MAP kinase by phosphorylation of both the threonine and tyrosine residues [27]. A more dense protein band was detected using the sample prepared from the hormone-treated and non-EGCG-injected oocytes (Fig. 2A, above). Consistent with this result, several-fold higher MAP kinase activity was detected using the immunoprecipitated enzyme prepared from the same oocyte extract compared with that from EGCG-injected oocytes (Fig. 2A, below).

As it has been well known that $p90^{rsk}$ is one of the substrate proteins of MAP kinase [12,18,26], the same set of experiments were performed on this protein kinase. The results obtained were quite similar to those of MAP kinase. In immunoblot analysis, the slower-migrated protein band, probably due to protein phosphorylation, was observed using the enzyme preparation from hormone-treated and non-EGCG-injected oocytes (Fig. 2B, above). In contrast, the trace of the mobility shift of $p90^{rsk}$ was detected using the sample prepared from EGCG-injected oocytes. Consistent with this result, the higher S6 protein kinase activity was detected using the enzyme sample from hormone-treated and non-EGCG-

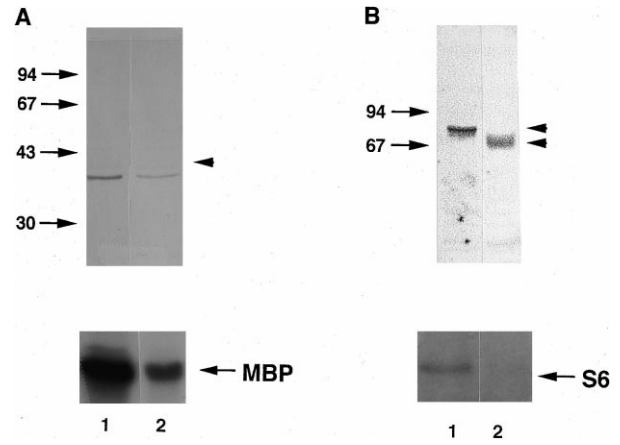

Fig. 2. Effects of microinjection of EGCG on the activation of MAP kinase (A) and p90^{rsk} (B) in oocytes. (above) An aliquot of each preparation (10 μg of protein) obtained as indicated in the legend to Fig. 1 was subjected to Western blot analyses using antibodies of MAP kinase and p90^{rsk}, respectively. Arrowheads in A and B indicate the positions of MAP kinase and p90^{rsk}, respectively. The uppermost arrowhead in the graph seems to correspond to the position of the phosphorylated form of p90^{rsk}. Numbers on the left side showed the positions of molecular mass markers in kDa. (below) An aliquot of each preparation obtained as indicated above was subjected to immunoprecipitation using an agarose-conjugated antibody of MAP kinase or p90^{rsk}. After phosphorylation using 10 μl of the agarose gel containing each enzyme, the radioactive proteins were separated by SDS-PAGE and autoradiography was performed. The positions of radioactive protein bands of MBP and S6 protein are indicated by arrows, respectively. Lanes 1 and 2 correspond to the samples prepared from water-injected and hormone-treated, and EGCG-injected and hormone-treated oocytes, respectively.

injected oocytes compared with that of EGCG-injected oocytes (Fig. 2B, below).

4. Discussion

The results depicted in Fig. 1A show the inhibition of GVBD by the microinjection of EGCG at a final concentration of 1 mM. When EGCG was injected into oocytes at a final concentration of 0.5 mM, inhibition of GVBD was not clear (data not shown). The reason why a relatively higher concentration of EGCG was needed may be due to the nature of this compound having the ability to bind easily to various intracellular constituents [1,2].

At first, we initiated this experiment to examine whether EGCG might show an inhibitory effect on extracellular signal-regulated-type MAP kinase in progesterone-treated oocytes as predicted from the previous analysis performed in vitro [8]. The present results unexpectedly showed that the primary target of EGCG might exist during the process of Mos activation. Previous results indicated that the translation of Mos was stimulated in progesterone-stimulated oocytes [13,14] and this protein plays important roles in both the initiation of oocyte maturation [9–11,13–15] and subsequent metaphase arrest as the cytostatic factor [28,29]. For the oocyte maturation, the activation of Cdc2 kinase by progesterone is essential [10–14]. The signal transduction pathway between Mos and Cdc2 kinase has been extensively studied by many investigators in both in vitro and cellular systems [10–12,16–18]. At present, MAP kinase kinase, MAP kinase, p90^{rsk} and Myt1 have been identified as constituents of this protein kinase cascade [9–12,16–18]. It has been shown that Cdc2 kinase is usually inactivated by the phosphorylation on both threonine-14 and tyrosine-15 residues by Myt1 kinase [30]. When oocytes are stimulated by progesterone, it has been shown that Myt1 is phosphorylated by p90^{rsk} and thus inactivated [12]. The significant role of Mos in activation of this protein kinase cascade was confirmed using oocytes prepared from Mos knock-out mice [31,32]. In our experiment, the extent of activation of MAP kinase and p90^{rsk} due to protein phosphorylation was dependent on the translation of Mos and these kinase activities were kept low when Mos production was inhibited by EGCG. Recent evidence supports the idea that the inactive Cdc2 kinase is activated by a protein phosphatase Cdc25 under the decreased activity of Myt1 during the oocyte maturation by progesterone [12,33].

Although Mos is known as a proto-oncogene product [13,15,34], its function has not been confirmed yet in the biological system except for its role in the initiation of meiotic maturation and subsequent metaphase arrest in oocytes [13–15,28,29]. However, previous results indicate that the constitutive activation of MAP kinase kinase by Mos can induce cellular transformation [35,36]. One result from our laboratory indicates that extracellular signal regulated-type MAP kinase was inhibited by a relatively lower concentration of EGCG in vitro [8]. If this action of EGCG is demonstrated in the cellular system, this effect of EGCG on MAP kinase may relate to the tumor preventing effect of green tea [1]. The inhibitory effect of EGCG on the activity or the phosphorylation state of three types of MAP kinase was demonstrated using various stimulated or transformed cells [5–7]. More recently, the molecular mechanisms of Mos activation have been shown to be very complicated [37–39]. However, the exact target of EGCG in this process remains to be explored.

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