Hydrogen peroxide preferentially enhances the tyrosine phosphorylation of epidermal growth factor receptor

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Abstract **We found that hydrogen peroxide (H_2O_2) enhances EGF receptor tyrosine phosphorylation in intact cells as well as solubilized membrane of an EGF receptor hyperproducing cell line NA. An antioxidant $MnCl_2$ effectively inhibited this enhancement. Interestingly, overall phosphorylation of the EGF receptor enhanced by H_2O_2 was half that of the EGF-enhanced phosphorylation when the receptor immunoprecipitated from [^{32}P]orthophosphate-labeled cells was examined. Tryptic phospho-peptide mapping of these receptors revealed that EGF enhanced the phosphorylation on five specific residues including serine 671, 1,046 and 1,047, threonine 669 and tyrosine 1,173, whereas H_2O_2 enhanced the phosphorylation remarkably on tyrosine 1,173 and three other residues and only moderately on serine 1,046 and 1,047 and threonine 669. Thus, H_2O_2 preferentially enhances the tyrosine phosphorylation of EGF receptor through oxidant stress.**

Key words: EGF receptor; Protein phosphorylation; Hydrogen peroxide; Oxidant stress

1. Introduction

The EGF receptor is a transmembrane glycoprotein of M_r 170,000 [1–3]. The binding of EGF to the receptor results in an increase in the tyrosine kinase activity of the receptor cytoplasmic domain [4] and autophosphorylation of the carboxyl-terminal tyrosine at residues 992, 1,068, 1,086, 1,148 and 1,173 [5–7]. Once activated, the receptor initiates a series of signal transduction events through tyrosine phosphorylation of interacting proteins of SH2 family [1]. Beside tyrosine phosphorylation, the in vivo EGF-activated receptors are abundant for the serine/threonine phosphorylation, by which physiological functions are attenuated. Various cellular protein kinases such as mitogen-activated protein kinase, casein kinase II, $p34^{cdc2}$ kinase and Ca^{2+}/calmodulin-dependent kinase II are considered to be involved in such phosphorylation and attenuation [8–11].

We previously showed that a perylenequinone compound calphostin-C enhances EGF receptor phosphorylation and 'early response gene' expression in a light-dependent manner [12,13]. Since calphostin-C easily accumulates in the membrane and induces oxidative modification of protein kinase C in the membrane [14], we hypothesized oxidant stress triggers the phosphorylation of EGF receptor. Thus, we examined the effect of hydrogen peroxide (H_2O_2) on cellular phosphorylation and found that H_2O_2 enhances the unique tyrosine phosphorylation on EGF receptor.

2. Materials and methods

2.1. EGF receptor phosphorylation

Human squamous carcinoma cell line NA [15] was grown in DMEM supplemented with 10% FBS (DMEM/FBS10) and kanamycin (100 μg/ml) in the presence or absence of [^{32}P]orthophosphate (3.7 MBq/ml, carrier-free, ICN Biomedicals, Irvine, CA, USA) as a tracer for 16 h at 5% CO_2 and 100% humidity. Then, the cells were treated with freshly prepared $MnCl_2$ (1 mM), H_2O_2 (1 mM) or EGF (50 ng/ml, ultra pure grade, Toyobo Co., Osaka, Japan). The cells were lysed with Tris-buffered saline containing 1% Triton X-100 and the EGF receptor was immunoprecipitated with monoclonal anti-human EGF receptor antibody B4G7 [12,16], electrophoresed on a SDS-polyacrylamide gel (7%) and transferred to a PVDF membrane (Immobilon-P, Millipore Co., Bedford, MA, USA). After incubation in non-fat skim milk (1%) for 30 min, various measurements including autoradiography, quantitative analysis of radioactivity by a BAS2000 Bio-imaging analyzer (Fuji Photo Film Co., Tokyo, Japan), immunostaining analysis and tryptic phospho-peptide mapping were carried out.

The membrane fraction was prepared and in vitro phosphorylation assay was carried out as previously described [17,18]. The cells were collected in the SAT buffer (0.25 M sucrose, 10 mM acetic acid, 10 mM triethanolamine, pH 7.4), and lysed in SEAT buffer (1 mM EDTA in SAT buffer) by pipetting with Gilson pipetman, followed by the centrifugation at 800 rpm for 5 min to pellet nuclei. The supernatant was centrifuged to pellet the membrane at 100,000 rpm for 5 min in a Beckman TLA100.2 rotor. The resulting pellet was resuspended in HEPES–Triton buffer (20 mM HEPES, 1% Triton X-100, 0.2 mM EDTA, 10% glycerol, pH 7.4) and centrifuged at 100,000 rpm for 5 min. The supernatant was used as the solubilized membrane fraction. The reaction mixture for in vitro phosphorylation assay (final volume: 30 μl) contained 20 mM HEPES (pH 7.4), 1 mM $MnCl_2$, 5 μg bovine serum albumin, 5 μM ATP, 10 μg membrane protein and 100 ng EGF or various concentration of H_2O_2. The reaction was initiated by the addition of ATP, incubated for 10 min on ice, and terminated by the addition of Laemmli's sample buffer.

2.2. Immunostaining analysis

The immunoprecipitated EGF receptor was transferred onto the membrane, and incubated with anti-phosphotyrosine antibody (mouse, 4G10, Upstate Biotechnology Inc., Lake Placid, NY, USA) or anti-human EGF receptor antibody (sheep, Upstate Biotechnology Inc.) for 2 h, with biotinylated second antibody (Zymed Lab., San Francisco, CA, USA) for 1 h, and then with avidin-biotin-horseradish peroxidase complex (Vector Lab., Burlingame, CA, USA) for 1 h, followed by staining.

2.3. Tryptic phospho-peptide mapping

The EGF receptor which was immunoprecipitated from [^{32}P]orthophosphate-labeled cells was transferred to the membrane. After autoradiography, the membrane region containing the EGF receptor was cut out and treated with 2 μg TPCK-trypsin in the presence of 40 μg bovine serum albumin at 37°C for 6 h and then treated with an additional 2 μg of TPCK-trypsin at 37°C overnight. The resulting peptides were fractionated by a linear acetonitrile gradient that ranged from 0% to 50% in the presence of trifluoroacetic acid on a Finepak SIL C18 column

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Abbreviations: EGF, epidermal growth factor; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum.

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(Japan Spectroscopic Co., Tokyo, Japan). Phospho-amino acid analysis was carried out as previously described [12].

3. Results

3.1. H_2O_2-enhancement of EGF receptor phosphorylation

We first examined tyrosine phosphorylation in an EGF receptor-hyperproducing cell line NA after treated with H_2O_2 (1 mM) or EGF by the immunostaining with anti-phosphotyrosine antibody. The protein of approximate M_r 170 K was detected in crude extracts and those immunoprecipitated by both anti-human EGF receptor antibody B4G7 and anti-phosphotyrosine antibody (Fig. 1A). Essentially the same amounts of EGF receptor protein were detected by the immunostaining with anti-EGF receptor antibody in crude extracts and B4G7-immunoprecipitates (Fig. 1B). Thus, it was suggested that H_2O_2 enhances tyrosine phosphorylation of the EGF receptor. The H_2O_2-enhanced tyrosine phosphorylation of EGF receptor was dose-dependent (maximal at 1 mM) and took place within 2.5 min (data not shown).

Next, NA cells were labeled with [^{32}P]orthophosphate for 16 h, and treated with EGF or H_2O_2, followed by the immunoprecipitation analysis. H_2O_2 enhanced [^{32}P]phosphate-incorporation into EGF receptor within 2.5 min and in a dose-dependent manner (Fig. 2). Interestingly, the level of H_2O_2-enhanced phosphorylation was only a half of the EGF-enhanced phosphorylation (Fig. 2).

3.2. Effective inhibition by antioxidant $MnCl_2$ of H_2O_2-enhanced EGF receptor tyrosine phosphorylation

We examined the effects of an antioxidant $MnCl_2$ on the EGF receptor tyrosine phosphorylation. H_2O_2 enhanced the EGF receptor phosphorylation when cells were labeled with [^{32}P]orthophosphate and treated with H_2O_2 in the absence of $MnCl_2$ (Fig. 3). However, when cells were treated with H_2O_2 in the presence of $MnCl_2$, the substantial decrease in tyrosine phosphorylation was observed (Fig. 3). $MnCl_2$ had no effect on the EGF-enhancement of receptor tyrosine phosphorylation.

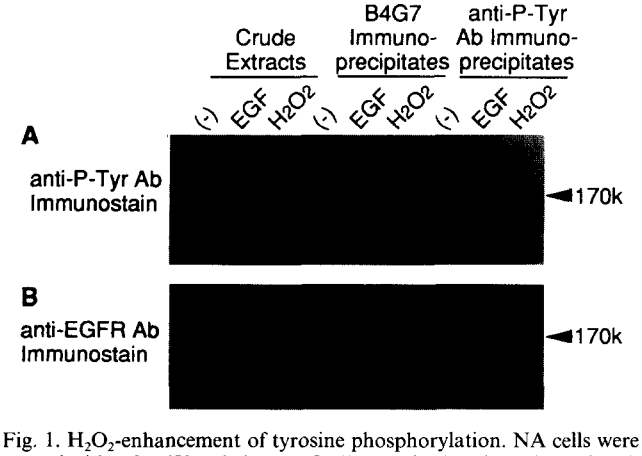

Fig. 1. H_2O_2-enhancement of tyrosine phosphorylation. NA cells were treated with EGF (50 ng/ml) or H_2O_2 (1 mM) for 15 min and then lysed. The extracts were subjected to immunoprecipitation by the anti-human EGF receptor antibody B4G7 and by anti-phosphotyrosine antibody. After electrophoresis of the immunoprecipitate on SDS-polyacrylamide gel (7%) and transfer to a PVDF membrane, immunostaining analysis by anti-phosphotyrosine antibody (A) and sheep anti-human EGF receptor antibody (B) was carried out.

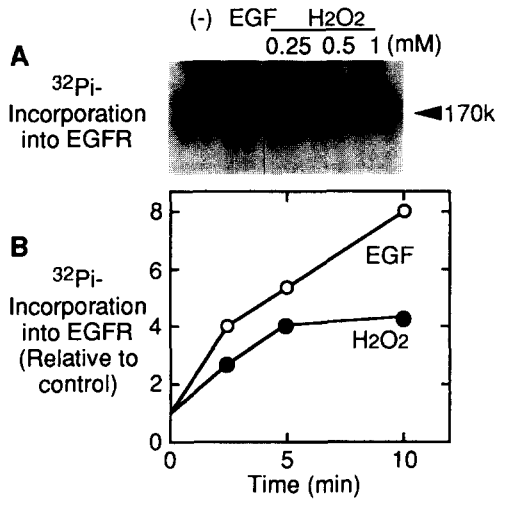

Fig. 2. H_2O_2-enhancement of EGF receptor phosphorylation. NA cells were labeled with [^{32}P]orthophosphate and treated with EGF or H_2O_2 at various doses (A) and at various times (B) in DMEM/FBS10. After immunoprecipitation of the EGF receptor with B4G7 antibody and transfer to a PVDF membrane, autoradiography and quantitative analysis was carried out using a BAS2000 Bio-imaging analyzer. Open circles, EGF; closed circles, H_2O_2.

3.3. Tryptic phospho-peptide mapping of H_2O_2-enhanced phosphorylation of EGF receptor

To identify the H_2O_2-enhanced phosphorylation sites in the EGF receptor, tryptic phosphopeptide mapping and phospho-amino acid analysis was carried out (Fig. 4). Each phosphorylated residue on the EGF receptor was identified from the comparison with previous works [5–7,12].

In control cells, the EGF receptor was phosphorylated on threonine 669 (fraction 67, Peptide 3 in Fig. 4) and moderately on serine 1,046 and 1,047 (fractions 80–82, Peptide 4). EGF-enhanced phosphorylation of the EGF receptor was remarkable on five specific residues including serine 671 (fraction 65, Peptide 3), serine 1,046 and 1,047 (fractions 78–82, Peptide 4), threonine 669 (fraction 67, Peptide 3) and tyrosine 1,173 (fraction 43, Peptide 2). In contrast, H_2O_2-enhanced phosphorylation was remarkable on serine 1,046 and 1,047 (fractions 77–82, Peptide 4), threonine 654 (fraction 31, Peptide 1) and 669 (fraction 67, Peptide 3) and tyrosine 1,173 (fraction 44, Peptide 2). In addition to tyrosine 1,173, three tyrosine-phosphorylations were detected in fractions 77–80, fraction 67, and fractions 54–57. Thus, H_2O_2-enhanced phosphorylation of the EGF receptor was extensive on tyrosine residues much more than EGF-enhanced phosphorylation.

3.4. Enhancement of in vitro phosphorylation of EGF receptor by H_2O_2

Finally, in vitro phosphorylation assay was carried out using the membrane fractions prepared from NA cells. Dose-dependent enhancement of EGF receptor tyrosine phosphorylation was observed when H_2O_2 was added (Fig. 5A), while the same amount of EGF receptor was present (Fig. 5B). Thus, H_2O_2-stimulation of EGF receptor tyrosine phosphorylation was reproduced in vitro.

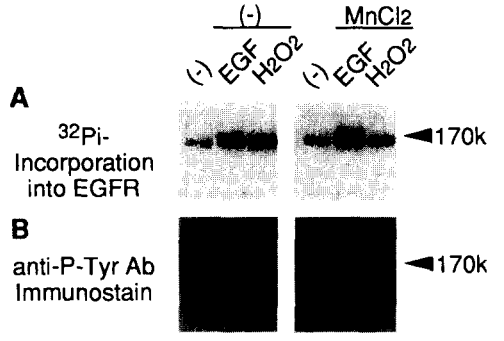

Fig. 3. Effects of antioxidant $MnCl_2$ on H_2O_2-enhanced tyrosine phosphorylation. NA cells were labeled with [^{32}P]orthophosphate for 16 h in DMEM/FBS10. The cells were pretreated with fresh $MnCl_2$ (1 mM) for 15 min, and then treated with EGF or H_2O_2 for 15 min. After immunoprecipitation and transfer to a membrane, autoradiography (A) and immunostaining using anti-phosphotyrosine antibody (B) was carried out as described in Fig. 1.

4. Discussion

In this study, we described the remarkable enhancement of EGF receptor tyrosine phosphorylation by H_2O_2 in an EGF receptor hyperproducing NA cells. This is not a peculiar phenomenon to NA cells because it was observed also in other cell lines including A431 (an EGF receptor hyperproducer derived from epidermoid carcinoma), HeLa (derived from epidermoid carcinoma) and A549 (derived from adenocarcinoma). The H_2O_2-treatment (1 mM for 15 min) induced membrane raffling, but resulted in neither receptor internalization nor cell growth inhibition (unpublished results).

The initial event after EGF binding is the autophosphorylation of EGF receptor on tyrosine residues. Previous studies under in vitro phosphorylation conditions demonstrated the presence of at least five tyrosine phosphorylated peptides in the EGF-activated receptor (Fig. 4B) [5–7]. In contrast, the studies under in vivo conditions demonstrated a single tyrosine phosphorylation site at 1,173 of the EGF receptor isolated from EGF-treated cells [1,12]. Besides tyrosine phosphorylation, serine/threonine phosphorylation is abundant and involved in the attenuation of the EGF-activated EGF receptor [8–11]. Thus, the half level of phosphorylation in H_2O_2-activated receptor, especially the low level of serine/threonine phosphorylation, may result in an insufficient attenuation of receptor function, such as lack of receptor internalization.

The H_2O_2-stimulation of EGF receptor tyrosine phosphorylation was reproduced using NA cells membrane fractions. This suggests a possibility that H_2O_2 directly activates the intrinsic tyrosine kinase of the EGF receptor and phosphorylate three additional tyrosine residues that are scarcely phosphorylated under physiological conditions for EGF stimulation (see Fig. 4B as summary). Alternatively, it is also possible that H_2O_2 activates an unidentified membrane tyrosine kinase(s) which is closely associated with EGF receptor.

It is known that $MnCl_2$ mimics a function of the intracellular scavenger superoxide dismutase [19]. In our study, freshly pre-

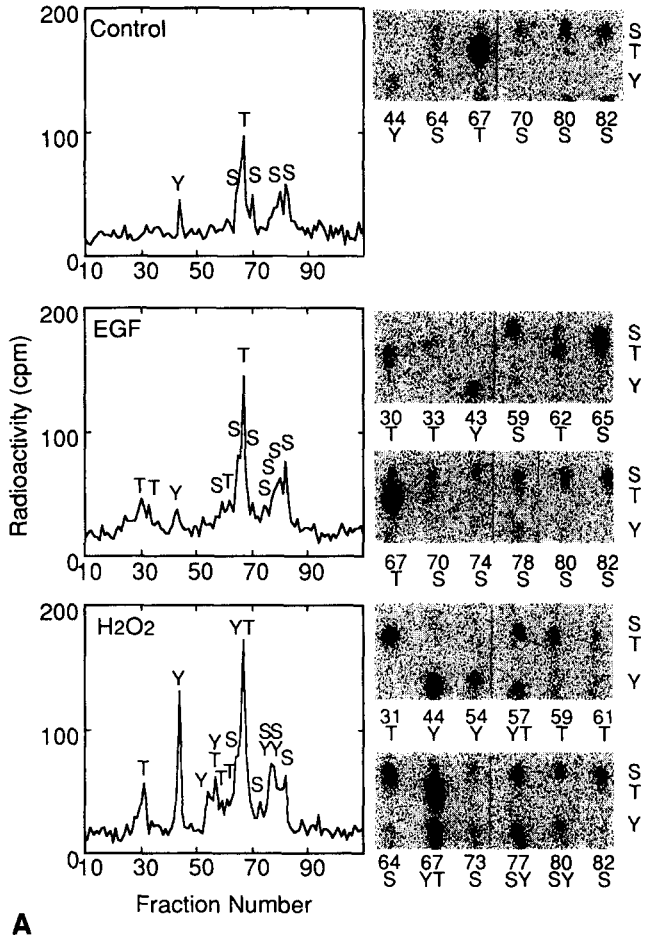

Fig. 4. Phospho-peptide mapping of H_2O_2-enhanced EGF receptor phosphorylation. (A) NA cells were labeled with [^{32}P]orthophosphate and treated with EGF or H_2O_2 in DMEM/FBS10. After immunoprecipitation and transfer to a membrane, the EGF receptor was digested with trypsin. The resulting peptide was fractionated with an acetonitrile gradient of 0% to 50% (fraction 10 through 110) in the presence of trifluoroacetic acid on C18 column (Left). Indicated fractions were subjected to phospho-amino acid analysis (Right). S, T and Y indicate phosphoserine, phosphothreonine and phosphotyrosine, respectively. (B) Summary of phosphorylation sites in the EGF receptor. Peptides 1 through 4 were identified by peptide mapping, whereas peptides 5 through 7 have not been mapped.

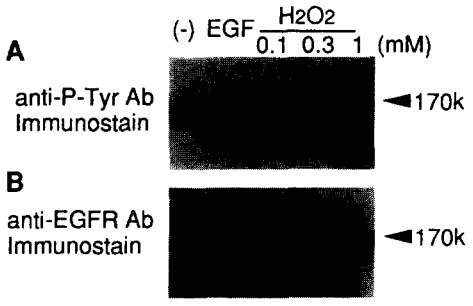

Fig. 5. In vitro phosphorylation of EGF receptor by H_2O_2. The membrane fraction was prepared from NA cells. In vitro phosphorylation assay was carried out in the presence of EGF (100 ng) and various concentrations of H_2O_2. After electrophoresis on SDS-polyacrylamide gel and transfer to PVDF membrane, immunostaining using anti-phosphotyrosine antibody (A) and sheep anti-human EGF receptor antibody (B) was carried out as described in Fig. 1.

pared $MnCl_2$ inhibited H_2O_2-enhancement of the EGF receptor tyrosine phosphorylation in vivo. This observation suggests that H_2O_2 effect on EGF receptor is through generation of oxidant stress. Interestingly, it was recently demonstrated that oxygen radicals generated by ultraviolet irradiation, H_2O_2 treatment or from xanthine by xanthine oxidase can activate *src* kinase, mitogen-activated protein kinase *raf*-1 kinase and ribosomal S6 protein kinase [20–22]. Those studies provided no evidence for the involvement of EGF receptor.

Oxidants are generated in tissues under various circumstances, including inflammation, hypoxia followed by reperfusion, and hyperoxia. The present study provided an additional viewpoint that the EGF receptor may be regulated by oxidant stress through the unique tyrosine phosphorylation mechanism.

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