EGFR-independent activation of p38 MAPK and EGFR-dependent activation of ERK1/2 are required for ROS-induced renal cell death

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Dong, Jing, Sampath Ramachandiran, Kulbhushan Tikoo, Zhe Jia, Serrine S. Lau, and Terrence J. Monks. EGFR-independent activation of p38 MAPK and EGFR-dependent activation of ERK1/2 are required for ROS-induced renal cell death. Am J Physiol Renal Physiol 287: F1049–F1058, 2004. First published June 29, 2004; doi:10.1152/ajprenal.00132.2004.—2,3,5-Tris-(glutathionyl)hydroquinone (TGHQ), a reactive metabolite of the nephrotoxicant hydroquinone, induces the ROS-dependent activation of MAPKs, followed by histone H3 phosphorylation and oncotic cell death in renal proximal tubule epithelial cells (LLC-PK1). Cell death and histone H3 phosphorylation are attenuated by pharmacological inhibition of p38 MAPK or ERK1/2 pathways. Because TGHQ, but not epidermal growth factor (EGF), induces histone H3 phosphorylation and cell death in LLC-PK1 cells, we hypothesized that there are differences in the mechanisms by which TGHQ and EGF induce activation of the EGFR receptor (EGFR). We therefore compared the relative ability of TGHQ, H2O2, and EGF to activate EGFR and MAPKs and found that p38 MAPK activation is EGFR independent, whereas ERK1/2 activation occurs mainly through EGFR activation. TGHQ, H2O2, and EGF induce different EGFR tyrosine phosphorylation profiles that likely influence the subsequent differential kinetics of MAPK activation. We next transfected LLC-PK1 cells with a dominant negative MAPK or ERK pathway. Because TGHQ, but not EGF, induces histone H3 phosphorylation and cell death in LLC-PK1 cells, we hypothesized that there are differences in the mechanisms by which TGHQ and EGF induce activation of the EGFR receptor (EGFR). We therefore compared the relative ability of TGHQ, H2O2, and EGF to activate EGFR and MAPKs and found that p38 MAPK activation is EGFR independent, whereas ERK1/2 activation occurs mainly through EGFR activation. TGHQ, H2O2, and EGF induce different EGFR tyrosine phosphorylation profiles that likely influence the subsequent differential kinetics of MAPK activation. We next transfected LLC-PK1 cells with a dominant negative p38 MAPK-expressing plasmid (pcDNA3-DNp38). TGHQ failed to induce phosphorylation of p38 MAPK and its substrate, MK-2, in pcDNA3-DNp38-transfected cells, indicating loss of function of p38 MAPK. In untransfected, pcDNA3 or pcDNA3-p38 (native)-transfected LLC-PK1 cells, Hsp27 was intensively phosphorylated after TGHQ treatment, whereas in pcDNA3-DNp38-transfected cells, TGHQ failed to induce Hsp27 phosphorylation. Thus p38 MAPK and ERK1/2-dependent ERK1/2 activation by TGHQ lead to the activation of two downstream signaling factors, i.e., histone H3 and Hsp27 phosphorylation, which have in common the potential ability to remodel chromatin.

REACTIVE OXYGEN SPECIES (ROS) are associated with a variety of human diseases and toxicities associated with exposure to redox-active chemicals and/or their metabolites (4). 2,3,5-Tris-(glutathionyl)hydroquinone (TGHQ) is a metabolite of hydroquinone (HQ) and contributes to HQ-mediated hepatotoxicity and nephrotoxicity (21). Renal proximal tubule epithelial cells are especially susceptible to TGHQ-induced toxicity, probably due to the high activity of γ-glutamyl transpeptidase (γ-GT), which catalyzes the metabolism of TGHQ and facilitates the subsequent cellular uptake of the corresponding cysteine conjugates (27). Quinone-methides induce ROS-dependent DNA damage and cell death in renal proximal tubule epithelial cells (LLC-PK1) (14, 28, 45).

Oxidative stress is known to activate MAPKs (7). The MAPK family comprises three major subgroups: ERK, JNK/SAPK, and p38 MAPK (7). ERKs behave mainly as mitogen-activated proliferation/differentiation factors, whereas JNK/SAPK and p38 MAPK are mainly stress-activated proteins related to apoptotic cell death. The MAPKs are all rapidly activated by TGHQ in LLC-PK1 cells, and either inhibition of ERK1/2 activation with PD-98059 or inhibition of p38 MAPK activation with SB-202190 attenuates cell death induced by TGHQ (32). In contrast, the JNK inhibitor SP-600125 has no effect on TGHQ-induced cell death (32). On activation, after phosphorylation of tyrosine and threonine residues, MAPKs stream target of the MAPKs is histone H3, which becomes phosphorylated after TGHQ-induced ERK activation (41). ERK1/2 activation may contribute to both cell proliferation and cell death in a variety of cells. In a few cases, activated ERK1/2 may act as a cell death-inducing factor. Thus ERK activation is related to vanadate-induced oncotic cell death of vascular smooth muscle cells (8), in H2O2-induced cell death of oligodendrocytes (1), in T cells (42), and in pleural mesothelial cells (16). Therefore, the inappropriate phosphorylation of histone H3 by ERK1/2 may lead to premature chromatin condensation (PCC) and cell death in LLC-PK1 cells (41). Both p38 MAPK and JNK1/2 are more responsive to cellular stress, such as that mediated by inflammatory cytokines, heat shock, and ROS (31). Interestingly, in certain cell systems, p38 MAPK and JNK1/2 can be activated in an EGFR-dependent manner (5, 17).

The events that couple ROS generation to MAPKs activation in TGHQ-treated LLC-PK1 cells are unknown. Oxidants can activate growth factor–linked signaling pathways (26), but the initial events of this activation are not clear. Two possible mechanisms may exist: 1) oxidants mimic epidermal growth factor (EGF)-EGFR receptor (EGFR) interactions by modification of the cysteine residues on the receptors (6); or 2) oxidants inactivate phosphatases, thereby inhibiting dephosphorylation of the EGFR (18). The EGFR, platelet-derived growth factor (PDGF) receptor, and T cell receptor complexes are all activated after oxidative stress and are coupled to the subsequent
activation of ERKs (11, 26, 36). Binding of EGF to the EGFR induces the dimerization, autophosphorylation, and transactivation of the tyrosine kinase activity of EGFR, providing a variety of binding sites for a series of proteins, thereby initiating the activation of downstream signaling pathways. For example, phospho-tyrosine 992 (pY992) within the EGFR provides a binding motif for phospholipase C-γ (PLC-γ), initiating downstream signaling, including PKC activation and subsequent ERK activation (9). Phospho-tyrosine 1068 (pY1068) within the EGFR provides a binding motif for Grb2/SH2 domain binding, which also leads to ERK activation (35). Phospho-tyrosine 1173 (pY1173) represents a motif for PLC-γ and Shc, both of which can initiate activation of the ERK cascade (40). Interestingly, pY1068, pY1148, and pY1173 are essential for EGFR internalization and degradation, as well as for tyrosine kinase activity (12). Additional phospho-tyrosine sites on the EGFR (pY845, pY1148, pY1086, pY1101) permit the differential activation of a number of other proteins in response to EGF stimulation (2, 3, 22, 37, 39). Although all MAPKs appear to be EGFR-dependent in different cellular systems (5, 17), whether TGHQ-mediated MAPK activation occurs through EGFR activation and which tyrosine phosphorylation site(s) are being targeted by TGHQ remain unclear.

After TGHQ-induced MAPK activation, histone H3 is phosphorylated in LLC-PK1 cells (41). Because histone H3 phosphorylation is usually associated with mitosis, inappropriate histone H3 phosphorylation will provide a signal that conflicts with the cell cycle arrest induced by TGHQ (14), resulting in premature chromatin condensation and subsequent oncotic cell death. Both ERK1/2 and p38 MAPK have the potential to induce histone H3 phosphorylation by activating downstream histone kinases (41). Another downstream signaling factor of p38 MAPK is MAPKAP kinase-2 (MK-2), which can phosphorylate heat shock protein 27 (Hsp27). Hsp27 is a chaperone that regulates actin stabilization (19) and apoptosis (29). Both histone H3 and Hsp27 have the potential to remodel chromatin structure and to possibly contribute to TGHQ-induced premature chromatin condensation and oncotic cell death. The present study was therefore designed to determine how MAPKs are activated by TGHQ and the roles of MAPK activation in TGHQ-induced oncotic cell death.

MATERIALS AND METHODS

Chemicals and reagents. TGHQ was freshly synthesized and purified in our laboratory according to established protocols (20). TGHQ is nephrotoxic and nephrocarcinogenic in rats and therefore must be handled with protective clothing and in a well-ventilated hood. EGF and H2O2 were obtained from Sigma (St. Louis, MO). Antibodies for phospho-p44/42 MAPK (p-ERK1/2), p44/42 MAPK (ERK1/2), phospho-p38 MAPK, phospho-SAPK/JNK, phospho-EGFR (Y992, 1068), phospho-Hsp27 (Ser82), phospho-MK-2 (Thr222, and Thr334), and EGFR antibodies were purchased from Cell Signaling Technology (Beverly, MA). The antibody for phospho-EGFR (Y1173) was from Upstate Biotechnology (Lake Placid, NY). Antibodies for phospho-EGFR (Y1086), phospho-Hsp27 (Ser15), JNK1/2, actin, and HRP-conjugated IgGs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for phospho-Hsp27 (Ser78), Hsp27 were from Stressgen (Victoria, BC). Antibody for phospho-EGFR (Y1148) was from BioSource International (Camarillo, CA). PD-98059, SB-202190, and SB-203580 were obtained from Calbiochem (San Diego, CA). AG-1478 was purchased from Biomol Biomolecules (Plymouth Meeting, PA). FuGENE 6 Reagent for transfection was purchased from Roche Molecular Biochemicals (Indianapolis, IN). All other chemicals were from Sigma or Fisher Scientific (Houston, TX) and of the highest grades available.

Cell culture and treatment regimen. LLC-PK1 cells (American Type Culture Collection, Rockville, MD), a renal proximal tubule epithelial cell line derived from the New Hampshire minipig, were maintained in DMEM containing 10% fetal bovine serum (FBS) at 37°C in a humidified incubator with 5% CO2-95% air. Cells were plated in 24-well plates at a density of 5 × 10^4 cells/well, or in 100-mm dishes at a density of 1 × 10^6 cells/dish, and grown to 70–80% confluency. Cells were then washed once with DMEM containing 25 mM HEPES and treated with the various agents (TGHQ 200 μM in 10 ml medium or 400 μM in 5 ml medium, 0–4 h; H2O2 88.2 μM, 0–4 h; EGF 50 ng/ml, 0–4 h, with or without inhibitors or modulators). The dose of TGHQ used to treat cells is a more accurate comparison between protocols rather than concentration (45). Thus when examining signaling pathways induced by TGHQ, we maintained a constant concentration of TGHQ (200 μM in 10 ml of treatment medium; 2 μmol total dose). In contrast, when the metabolic labeling and histone extractions were performed, TGHQ was provided at 400 μM in 5 ml medium (2 μmol total dose) to maintain the same dose per cell and to reduce radioactive waste. In both protocols, the dose of TGHQ remained the same: 2 μmol/100 mm dish. Activation of the signaling pathways and histone H3 phosphorylation in both treatments were thus comparable. At the end of each treatment, cells were washed three times with DMEM containing 25 mM HEPES.

Western blot analysis. Cells were plated in 100-mm dishes at a density of 1 × 10^6 cells/dish or 6-well plates at a density of 3 × 10^5 cells/well and grown for 36 h (70–80% confluence) before treatment. Cells were washed and lysed with 1× lysis buffer (Cell Signaling Technology) containing (in mM) 20 Tris HCl (pH 7.5), 150 NaCl, 1 Na2EDTA, 1 EGTA, 2.5 sodium pyrophosphate, 1 β-glycerophosphate, and 1 Na2VO4 as well as 1% Triton, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Aliquots of 25–50 μg of lysate were separated by 10–15% SDS-PAGE and transferred to nitrocellulose membranes. The blots were blocked in 5% nonfat dry milk (Bio-Rad, Hercules, CA) in Tris-buffered saline (TBS) with 0.1% Tween 20 for 1 h and then incubated with primary antibodies overnight at 4°C or for 1 h at room temperature in blocking solution. The dilution factors for the primary antibodies were p-p44/42 MAPK (1:1,000), p44/42 MAPK (1:1,000), p-JNK/SAPK (monoclonal, 1:2,000), JNK/SAPK (1:1,000), p-p38 MAPK (monoclonal, 1:2,000), p38 MAPK (1:1,000), p-EGFR (Y992, 1068, 1086, 1148, 1173, 1:1,000), EGFR (1:500), p-Hsp27 (Ser15) (1:500), p-Hsp27 (Ser78, Ser82) (1:1,000), p-MK-2 (Thr222, Thr334) (1:1,000), and Hsp27 (1:5,000). Secondary antibodies, goat anti-rabbit, and goat anti-mouse antibodies conjugated with HRP were diluted to 1:2,000 in blocking solution (5% nonfat dry milk in TBS containing 0.1% Tween 20). The blots were then washed and incubated with secondary antibodies for 1 h at room temperature in blocking solution. Blots were finally developed with the ECL (enhanced chemiluminescence), Western blotting detection reagents, and exposed to Hyperfilm (Amersham Pharmacia Biotech).

Metabolic labeling of cells and histone extraction for phosphorylation studies. To determine the effects of TGHQ-induced oxidative stress on histone phosphorylation, LLC-PK1 cells were labeled with 40 μCi/ml of [3H]thymidine phosphoric acid in DMEM with 25 mM HEPES for 4 h. Radioactivity was removed and cells were treated with 50 ng/ml of EGF, or 400 μM of TGHQ in DMEM containing 25 mM HEPES. Cells were washed in ice-cold low-salt buffer (LSB; 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 2.5 mM EDTA) and then lysed in lysis buffer (LSB containing 0.25 mM sucrose, 1% Triton X-100). Histones were extracted with 0.25 M HCl and precipitated in 20%
trichloroacetic acid. The precipitate was washed twice, once with 0.25 M HCl containing acetone, and then with acetone only. Proteins were separated on a 13.5% SDS polyacrylamide gel by electrophoresis. After electrophoresis, the proteins were stained with Coomassie blue, dried, and then exposed to Kodak XAR film.

Neutral red assay. To determine the viability of LLC-PK1 cells after challenge with various agents, Neutral red uptake was determined as previously described (25). Neutral red is a sensitive measure of lysosomal membrane integrity, which in the present cell model precedes changes in markers of cell death, measured by loss of plasma membrane integrity [cytosolic enzyme (LDH) leakage] by 30–90 min (25).

Transfection of LLC-PK1 cells. LLC-PK1 cells were seeded in 6-well plates or 24-well plates and grown for 16 h, followed by transient transfection with empty vectors (pcDNA3), kinase-dead dominant negative construct pcDNA3-DNp38-expressing vectors, or native p38-expressing vectors, using FuGENE 6 Reagent according to the manufacturer's instructions. Cells were incubated with a mixture of DMEM medium, transfection reagent, and vectors at 37°C for 4 h, and then DMEM medium, containing 10% FBS, was added to each well and incubated for 48 h before treatment.

Statistical analysis. All data are expressed as means ± SD. Analysis of variance with a post hoc Student-Newman-Keuls test was used to compare the mean values, and P < 0.05 was considered to be significant.

RESULTS

TGHQ but not EGF induces histone H3 phosphorylation. TGHQ, epidermal growth factor (EGF), and H2O2 all induce EGF receptor (EGFR)-dependent ERK1/2 phosphorylation, but with varying kinetics. LLC-PK1 cells were treated with TGHQ (200 μM), H2O2 (88.2 μM), or EGF (50 ng/ml) for different periods of time (0, 0.25, 0.5, 1, 2, 4 h). Some cells were pretreated with AG-1478 (AG; 0.25 μM) for 1 h, followed by treatment with TGHQ (1 h), H2O2 (1 h), or EGF (15 min). DMSO-treated samples represent the solvent control for AG-1478. Catalase was used at a concentration of 10 U/ml in double-distilled H2O and was coadministered with the various agents. Whole cell lysates were extracted and electrophoretically resolved on a 10% SDS-PAGE gel, followed by Western blot analysis. A: TGHQ-treated cells. B: EGF-treated cells. C: H2O2-treated cells. Numbers shown represent hours of treatment. Total ERK levels did not change during the treatment time (data not shown).

Fig. 1. 2,3,5-Tris-(glutathion-S-yl)hydroquinone (TGHQ), epidermal growth factor (EGF), and H2O2 all induce EGF receptor (EGFR)-dependent ERK1/2 phosphorylation, but with varying kinetics. LLC-PK1 cells were treated with TGHQ (200 μM), H2O2 (88.2 μM), or EGF (50 ng/ml) for different periods of time (0, 0.25, 0.5, 1, 2, 4 h). Some cells were pretreated with AG-1478 (AG; 0.25 μM) for 1 h, followed by treatment with TGHQ (1 h), H2O2 (1 h), or EGF (15 min). DMSO-treated samples represent the solvent control for AG-1478. Catalase was used at a concentration of 10 U/ml in double-distilled H2O and was coadministered with the various agents. Whole cell lysates were extracted and electrophoretically resolved on a 10% SDS-PAGE gel, followed by Western blot analysis. A: TGHQ-treated cells. B: EGF-treated cells. C: H2O2-treated cells. Numbers shown represent hours of treatment. Total ERK levels did not change during the treatment time (data not shown).

Fig. 2. TGHQ, but not EGF, induces histone H3 phosphorylation in renal epithelial cells. 32P-labeled LLC-PK1 cells were treated with TGHQ (400 μM) or EGF (50 ng/ml) for different periods of time (0, 2, 4, 6, 8, 12, 24 h). Histones were extracted and electrophoretically resolved on a 13.5% SDS polyacrylamide gel. A: Coomassie-stained gel. B: Autoradiography. Numbers shown represent hours of treatment.
ERK1/2 activation may also be crucial for the subsequent phosphorylation of histone H3.

EGFR tyrosine phosphorylation is induced by EGF, TGHQ, and H2O2. To determine whether the differences in the downstream consequences of ERK1/2 activation arise as a consequence of the differential interaction between EGF or TGHQ and the EGFR, we compared the effects of EGF and TGHQ on the pattern of EGFR phosphorylation. Because TGHQ induces ERK1/2 activation in a ROS-dependent manner, the effects of H2O2 were also compared with TGHQ and EGF. The dose of H2O2 (88.2 μM) was selected based on the dose at which it induced the same level of loss of cell viability as TGHQ (200 μM) after 2 h of treatment (Fig. 3, A and B). EGF (50 ng/ml) induced rapid ERK1/2 phosphorylation (Fig. 1B) without subsequent loss of cell viability (Fig. 3C). Time-response curves of TGHQ and H2O2 are shown in Fig. 3, D and E.

Phosphorylation of EGFR at Y992, Y1068, Y1086, Y1148, and Y1173 was specifically investigated, because phosphorylation at any of these sites could result in MAPK/ERK activation, but through different downstream signaling events (see Fig. 12). EGF, as a competent ligand for EGFR, was able to bind to EGFR, caused dimerization of EGFR, and induced autophosphorylation of EGFR and tyrosine kinase transactivation at all the pY sites examined (Fig. 4). TGHQ also induced rapid and intensive EGFR phosphorylation at Y992 and Y1068, and weaker EGFR phosphorylation at Y1086 and Y1148, but not at Y1173 (Fig. 4). In contrast, H2O2 induced significant amounts of EGFR phosphorylation only at Y992, and trace amounts of EGFR phosphorylation at Y1086 and Y1148 (Fig. 4). EGF-induced EGFR phosphorylation reached maximal levels as early as 15 min, whereas both TGHQ- and H2O2-induced EGFR phosphorylation peaked at 1 h (Fig. 4).

Differences in the patterns and kinetics of EGFR phosphorylation among TGHQ, EGF, and H2O2 (see Fig. 11) likely determine the activation of the subsequent downstream signaling pathways engaged by EGFR phosphorylation. The pattern of TGHQ-induced EGFR phosphorylation was also different from that seen with H2O2, suggesting a ROS-independent component to TGHQ-induced EGFR phosphorylation. EGFR-mediated EGFR phosphorylation was followed by decreases in total EGFR levels (Fig. 4) probably due to the rapid internalization and degradation of the activated receptor required to desensitize the system (23, 34, 44).

TGHQ, EGF, or H2O2-mediated ERK1/2 phosphorylation is EGFR dependent, whereas TGHQ- and H2O2-induced p38 MAPK and JNK1/2 phosphorylation is EGFR independent. TGHQ, EGF, and H2O2 all induced ERK1/2 phosphorylation very rapidly (Fig. 1). However, the kinetics of ERK1/2 phosphorylation by each of these agents varied. EGF induced extremely rapid and relatively sustained ERK1/2 phosphorylation, peaking at 15 min, whereas TGHQ- and H2O2-induced ERK1/2 phosphorylation was more rapid and more sustained than TGHQ, consistent with its effects on EGFR tyrosine phosphorylation at Y992. H2O2 induced phosphorylation of relatively fewer tyrosine residues of EGFR, but more intensive ERK1/2 phosphorylation, implying the presence of additional tyrosine phosphorylation sites on EGFR for H2O2. Whether differences in the kinetics of both EGFR and ERK1/2 phosphorylation by EGF, TGHQ, or H2O2 contribute to the different effects on the downstream targets is not clear and needs further investigation.

An EGFR inhibitor, AG-1478, totally blocked constitutive ERK1/2 phosphorylation in untreated cells (Fig. 1A). AG-1478...
completely blocked EGF induced ERK1/2 phosphorylation (Fig. 1A), whereas it reduced TGHQ- and H2O2-induced ERK1/2 phosphorylation to levels lower than those seen in untreated cells (Fig. 1B and C). The mechanism of ERK1/2 activation by either EGF or TGHQ/H2O2 is likely different. EGF is an EGFR ligand, causing dimerization and autophosphorylation. In contrast, TGHQ/H2O2 probably stimulates EGFR phosphorylation by either modifying the cysteine residues and mimicking EGF-EGFR interaction or by inactivating phosphatases, thereby inhibiting dephosphorylation of the EGFR. Thus residual ERK1/2 phosphorylation may be a consequence of the inhibition of phosphatase(s). DMSO, the solvent for AG-1478, had no effect on ERK1/2 phosphorylation. Thus ERK1/2 phosphorylation caused by TGHQ, EGF, and H2O2 appeared to be EGFR dependent. We subsequently determined whether p38 MAPK and JNK1/2 phosphorylation requires EGFR activation. TGHQ and H2O2, induced phosphorylation of p38 MAPK and JNK1/2 (Fig. 5), but EGF did not (data not shown). AG-1478 failed to block either TGHQ- or H2O2-induced p38 MAPK and JNK1/2 phosphorylation, indicating that both TGHQ- and H2O2-induced p38 MAPK and JNK1/2 phosphorylation occurs in an EGFR-independent manner.

Histone H3 is phosphorylated through both ERK and p38 MAPK pathways. TGHQ induced the activation of all three subfamilies of MAPKs (Figs. 1 and 5), among which ERK1/2 and p38 MAPK could potentially induce histone H3 phosphorylation (41). To determine whether TGHQ-induced histone H3 phosphorylation occurs through ERK1/2 and p38 MAPK pathways, we used PD-98059, an ERK1/2 pathway inhibitor, and SB-202190, a p38 MAPK pathway inhibitor. Cells were pretreated with each of the inhibitors and were subsequently exposed to TGHQ. Treatment of LLC-PK1 cells with either inhibitor alone had no effect on histone H3 phosphorylation (Fig. 6). Histone H3 phosphorylation was significantly increased by TGHQ treatment (400 μM, 1 h), and both PD-98059 and SB-202190 attenuated TGHQ-induced histone H3 phosphorylation, PD-98059 being more effective than SB-202190 (Fig. 6). The data imply that histone H3 phosphorylation occurs probably through the activation of both ERK and p38 MAPK pathways. The combination of the two inhibitors had no additive effects and did not block TGHQ-induced decreases in neutral red uptake (32). Equal loading of the extract is illustrated in the Coomassie-stained gel.

TGHQ-induced phosphorylation of MK-2 and Hsp27 is p38 MAPK dependent. One downstream target of p38 MAPK is MK-2, which is known to phosphorylate Hsp27. MK-2 was phosphorylated by TGHQ (200 μM, 1 h) at both phosphoserine residues: Thr222 and Thr334 (Fig. 7). Both SB-202190 and SB-203580 are inhibitors of the p38 MAPK pathway, and pretreatment of LLC-PK1 cells with each of these inhibitors for 1 h significantly decreased or completely blocked TGHQ-induced MK-2 phosphorylation at Thr222 and Thr334, respec-
tively, indicating that MK-2 activation occurs via the p38 MAPK pathway (Fig. 7). Equal loading was confirmed by Western blot analysis for actin on the same blots after stripping (Fig. 7).

We subsequently investigated whether Hsp27 phosphorylation occurs through the p38 MAPK pathway. Hsp27 is a chaperone protein that regulates actin polymerization and subcellular localization in response to ROS generation (24). Interestingly, in an independent study using two-dimensional electrophoresis, followed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF) peptide mass mapping and postsource decay (PSD), we found that TGHQ altered the relative intensity of three spots, each identified as Hsp27, LLC-PK1 cells, such that the more intense spot migrated with a more acidic pI (8a). We confirmed that the shift in pI was due to the phosphorylation of Hsp27 at three serine residues (Ser15, Ser78, Ser82), using phospho-specific antibodies for phosphorylated Hsp27 by Western blot analysis (Fig. 8). The phosphorylation of Hsp27 at all three sites increased in as early as 15 min, and reached a peak between 1 and 2 h. Total levels of Hsp27 did not change during the period of treatment (Fig. 8). Phosphorylation of Hsp27 was similarly attenuated or blocked by inhibition of the p38 MAPK pathway (by SB-202190, SB-203580), but not by inhibition of the ERK pathway (PD-98059) (Fig. 9). Instead, PD-98059 appeared to slightly enhance TGHQ-induced phosphorylation of Hsp27 at the Ser78 residue (Fig. 9). Expression levels of Hsp27 did not change in all samples examined (Fig. 9).

To confirm the effects of pharmacological inhibitors of the p38 MAPK pathway on TGHQ-induced Hsp27 phosphorylation, LLC-PK1 cells were transfected with dominant negative mutant p38 MAPK-expressing vectors (pcDNA3-DNp38), or empty vectors pcDNA3, or native p38 MAPK-expressing vectors (pcDNA3-p38). In untransfected, pcDNA3-transfected, or native p38-transfected cells, TGHQ was capable of inducing intensive p38 MAPK phosphorylation. Indeed, in pcDNA3-p38-transfected cells, phosphorylation of p38 MAPK was higher than in untreated cells, and TGHQ induced even more p38 MAPK phosphorylation in these cells (Fig. 10), probably due to the significantly increased levels of p38 MAPK in these cells. In contrast, in DNp38-transfected cells, TGHQ failed to induce p38 MAPK phosphorylation (Fig. 10). Consistent with these findings, only in DNp38-transfected cells did TGHQ fail to induce MK-2 (both Thr222 and Thr334) and Hsp27 (Ser15, Ser78, Ser82) phosphorylation (Fig. 10). Again, total Hsp27 expression levels were similar in all samples. The data there-
fore confirm that phosphorylation of both MK-2 and Hsp27 occurs via the p38 MAPK pathway and that Hsp27 phosphorylation is independent of the ERK pathway.

DISCUSSION

Although we confirmed that TGHQ induces MAPK phosphorylation robustly and in a time-dependent fashion (Figs. 1 and 5), the upstream and downstream targets during TGHQ-mediated activation of the MAPK pathway remained unclear. Because MAPK activation is EGFR dependent in most cells, we examined whether TGHQ-induced MAPK activation in LLC-PK1 cells also occurs through the EGFR. TGHQ induced histone H3 phosphorylation rapidly in LLC-PK1 cells, whereas EGF does not induce histone H3 phosphorylation for at least 24 h (Fig. 2). Histone H3 phosphorylation induced by TGHQ is ROS dependent and is associated with TGHQ-induced oncotic cell death (41). TGHQ, H2O2, and EGF all induce ERK1/2 phosphorylation in an EGFR-dependent manner, because AG-1478, an inhibitor of EGFR, blocks ERK1/2 phosphorylation induced by each agent (Fig. 1). In contrast, TGHQ- and H2O2-induced p38 MAPK and JNK1/2 activation are EGFR independent (Fig. 5), and EGFR fails to induce p38 MAPK or JNK1/2 phosphorylation in LLC-PK1 cells. Because pharmacological inhibitors of the p38 MAPK and ERK pathways decrease histone H3 phosphorylation (Fig. 6) and protect LLC-PK1 cells from TGHQ-induced cell death (32), we conclude that both p38 MAPK and ERK pathways contribute to TGHQ-induced histone H3 phosphorylation and cell death. Thus in contrast to the general concept that p38 MAPK is an inflammation/apoptosis-related kinase, p38 MAPK also participates in ROS-induced oncotic renal proximal tubule epithelial cell death.

Several tyrosine residues on the EGFR that are prone to autophosphorylation have been identified, including three major sites (Y1068, Y1148, and Y1173) and two minor ones (Y992 and Y1086) (2). On phosphorylation, these residues promote the binding of a number of downstream signaling proteins that possess SH2 domains, such as Shc, PLC-γ, Grb2, and SHP1. Binding of these molecules to the EGFR results in the activation of multiple signaling pathways that enhance DNA synthesis and promote cell division. Interestingly, TGHQ, H2O2, and EGF induce differential phosphorylation of EGFR tyrosine residues and subsequent different kinetics of MAPK activation in LLC-PK1 cells (Figs. 1 and 4). EGF, the endogenous ligand of EGFR, induces rapid (<15 min) EGFR tyrosine phosphorylation at all residues examined (Y992, Y1068, Y1086, Y1148, Y1173) (Fig. 4, and summarized in Fig. 11). In contrast, TGHQ induces much slower (1 h) EGFR
tyrosine phosphorylation, and only at residues Y992 and Y1068. Surprisingly, H2O2 only induces significant EGFR tyrosine phosphorylation at Y992, but H2O2-mediated ERK1/2 phosphorylation is dependent on EGFR activation (Fig. 1).

Fig. 10. LLC-PK1 cells transfected with a dominant negative mutant p38 MAPK-expressing vector failed to respond to TGHQ-induced responses. LLC-PK1 cells were transfected with pcDNA3 (empty vector), pcDNA3 vector expressing native p38 MAPK (p38), or pcDNA3 vector expressing dominant negative mutant p38 MAPK (DNP38) as described in MATERIALS AND METHODS. Transfected cells or untransfected cells were treated with TGHQ (200 μM) for 1 h, and cell lysates were separated by 10% SDS-PAGE gels, followed by Western blot analysis using phospho-specific antibodies for p38 MAPK, MK-2, and Hsp27. Total p38 MAPK expression levels were higher in p38- and DNP38 MAPK-expressing vector-transfected cells. Total Hsp27 expression level was examined, and no change was revealed.

Fig. 11. Profiles of EGFR tyrosine phosphorylation by TGHQ, EGF, and H2O2. LLC-PK1 cells were treated with TGHQ (200 μM), EGF (50 ng/ml), or H2O2 (88.2 μM) for different periods of time (0, 0.25, 0.5, 1, 2, 4 h). Western blot analysis revealed the levels of specific phospho-EGFR at Y992, Y1068, Y1148, and Y1173. EGFR tyrosine phosphorylation was determined semiquantitatively by densitometric scanning of each Western blot.

Fig. 12. Possible signaling pathways that contribute to TGHQ-induced oncotic cell death in LLC-PK1 cells. TGHQ-generated reactive oxygen species (ROS) phosphorylate EGFR tyrosine residues, mainly at Y992 and Y1068, and induce the activation of the ERK pathway. ERK1/2 activation leads to histone H3 phosphorylation. TGHQ-generated ROS also induce the activation of p38 MAPK, which phosphorylates MK-2 and Hsp27. p38 MAPK also leads to histone H3 phosphorylation. Inappropriate histone H3 phosphorylation leads to premature chromatin condensation and oncotic cell death. Meanwhile, Hsp27 phosphorylation induced by p38 MAPK activation might also be associated with TGHQ-induced chromatin remodeling and oncotic cell death. Thus multiple pathways contribute to TGHQ-induced oncotic cell death.
Additional, as yet unidentified, phosphorylation site(s) may exist on EGFR that are responsive to H2O2. Both TGHQ and H2O2 induce weaker EGFR tyrosine phosphorylation at residues Y1086 and Y1148, compared with EGF (Fig. 4). Phosphorylation of Y1068, Y1148, and Y1173 can lead to the degradation of the EGFR, but they are also required for maximum kinase activity (12). Absence or weak phosphorylation of EGFR Y1173 in TGHQ- and H2O2-treated cells may prolong the activation of EGFR by these two agents. Moreover, H2O2 poorly phosphorylates the EGFR at Y1045, the major docking site for the ubiquitin ligase c-Cbl (34). Consequently, H2O2-activated EGFR fails to recruit c-Cbl and does not undergo ubiquination and endocytosis (34).

Because TGHQ generates ROS in LLC-PK1 cells (28), it is likely that there exist(s) common EGFR phosphorylation site(s) for both TGHQ and H2O2. Src phosphorylation sites, Y845 and Y1101, could represent additional phosphorylation targets of TGHQ and H2O2. Differential EGFR tyrosine phosphorylation profiles induced by different agents will likely contribute to the differential recruitment of signaling factors to the EGFR, resulting in different cellular responses (38). Based on this view, TGHQ-, EGF-, or H2O2-induced EGFR tyrosine phosphorylation likely results in the recruitment of different signaling proteins to the EGFR that determines the differences in cellular response. Additionally, AG-1478 blocks TGHQ-, H2O2-, and EGF-induced EGFR Y992 phosphorylation (data not shown), verifying its ability to block EGFR phosphorylation. However, AG-1478 does not protect cells from TGHQ-induced cell death (data not shown). We conclude that although AG-1478 blocks ERK1/2 activation, it could also interfere with other signaling pathways, compromising its effects on cell death.

Oxidative stress induces the phosphorylation of heat shock protein 27 (Hsp27), and both oligomerization and phosphorylation of Hsp27 may play an important role in the regulation of actin dynamics in response to growth factors and stress (10). Overexpression of Hsp27 prevents kidney ischemia-reperfusion-induced functional injury (30). Because SB-202190, an inhibitor of the p38 MAPK pathway, protects LLC-PK1 cells against TGHQ-induced cell death (32), TGHQ-induced p38 MAPK-mediated Hsp27 phosphorylation might contribute to ROS-induced cell death. In support of this view, MK-2−/− mice are protected from ischemic brain injury (43), indicating that the p38 MAPK pathway is involved in ROS-induced cell death. Although Hsp27 overexpression or induction is associated with cytoprotection, the role of Hsp27 phosphorylation in ROS-induced renal cell death is unclear. Hsp27 serves as a molecular chaperone that associates with certain proteins, such as actin, Akt (33), and the inhibitory protein IkBo (29), to stabilize and regulate their activation/deactivation. Inappropriate phosphorylation of Hsp27 might therefore convert Hsp27 from a cytoprotective to an apoptosis-inducing protein (13). TGHQ-induced phosphorylation of MK-2 and Hsp27 is totally blocked by the p38 MAPK pathway inhibitors SB-202190 or SB-203580 (Fig. 8, A and B), or by transfection of LLC-PK1 cells with dominant negative mutant p38 MAPK-expressing vectors (Fig. 10). Thus TGHQ-induced p38 MAPK pathway activation mediates Hsp27 phosphorylation. The potential significance of Hsp27 phosphorylation was examined by immunostaining of phospho-Hsp27, which was only observed within damaged or dying cells (data not shown). Moreover, in post-confluent LLC-PK1 cells lacking stress-inducible Grp78, TGHQ failed to induce Hsp27 phosphorylation, indicating a link between ER stress and the p38 MAPK pathway and subsequent Hsp27 phosphorylation (15). We therefore conclude that Hsp27, another downstream effector of the MAPKs, is phosphorylated by TGHQ through the p38 MAPK pathway and that in preconfluent cells this modification is associated with TGHQ-induced oncotic cell death. The specific role of HSP27 in cell death and cell survival remains to be established.

In summary, our data indicate that TGHQ induces selective EGFR phosphorylation, leading to the activation of the ERK pathway, and induces EGFR-independent p38 MAPK pathway activation. Both the ERK and p38 MAPK pathways contribute to histone H3 phosphorylation and oncotic cell death. In addition, TGHQ induces Hsp27 phosphorylation via the p38 MAPK pathway. Both histone H3 and Hsp27 phosphorylation have the potential ability to remodel chromatin. Because inappropriate chromatin condensation has been linked to TGHQ-induced cell death, the activation of both p38 MAPK and ERK1/2, and subsequent downstream signaling factors, likely plays a key role in ROS-mediated oncotic cell death in renal epithelial cells. The mechanisms of TGHQ-induced oncotic cell death are summarized in Fig. 12. The role of p38 MAPK in histone H3 phosphorylation remains to be resolved, as does the functional role of Hsp27 phosphorylation in TGHQ-induced oncotic cell death. Ongoing experiments are addressing these questions. Finally, immunoprecipitation of the EGFR coupled to mass spectrometry may identify different EGFR binding partners in response to stimulation with EGF, H2O2, or TGHQ.

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