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Involvement of Nrf2, p38, B-Raf, and Nuclear Factor-κB, but Not Phosphatidylinositol 3-Kinase, in Induction of Hemeoxygenase-1 by Dietary Polyphenols

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ABSTRACT

The highly inducible enzyme, hemeoxygenase-1 (HO-1), metabolizes heme, thereby protecting a variety of cells against oxidative stress and apoptosis. Up-regulation by cancer chemopreventive agents has been reported, but its regulation and function in transformed cells are unclear. We compared induction by two dietary polyphenols, curcumin and epigallocatechin-3-gallate (EGCG), with that by the endogenous substrate hemin in epithelial and endothelial cells and examined the relevance to apoptosis. Curcumin or hemin (20 μM) induced HO-1 in breast cells from 5 to 24 h. Curcumin (5–40 μM) or hemin (5–100 μM) induced HO-1 and nuclear levels of nuclear factor (erythroid-derived 2)-related factor (Nrf2) in a dose-dependent manner. EGCG had no effect in breast cells, but at 30 μM, it induced nuclear translocation of Nrf2 and HO-1 expression in B-lymphoblasts. In all cases, induction was inhibited by pretreatment with the phosphatidylinositol 3-kinase (PI3K) inhibitor 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002) or the p38 inhibitor 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole (SB203580). The nuclear factor-κB (NF-κB)-DNA binding inhibitor helenalin (20 μM) also prevented induction. However, wortmannin had no effect, suggesting that PI3K was not involved. Curcumin and hemin also induced nuclear Nrf2 and HO-1 effectively in wild-type mouse embryo fibroblasts (wt MEFs) and in B-Raf$^{-/-}$ MEFs but not in Nrf2$^{-/-}$ MEFs. However, EGCG (5–20 μM) induced HO-1 only in wt MEFs. Results suggest that signaling through p38 mitogen-activated protein kinase, NF-κB, and Nrf2 as well as other unidentified molecules is involved in HO-1 induction by hemin and both polyphenols, but cell-specific factors also play a role, particularly with respect to EGCG. Induction of HO-1 by curcumin, EGCG, or low concentrations (5–10 μM) of helenalin did not protect MDA-MB468 breast cells or B-lymphoblasts from apoptosis.

The dietary polyphenols curcumin (diferuloylmethane) and epigallocatechin-3-gallate (EGCG) have significant potential as cancer-chemopreventive agents. Among their possible mechanisms of action are proapoptotic, antioxidant, and antiangiogenic effects (for review, see Manson, 2003, 2005; Surh, 2003). Among its many effects, curcumin induces hemeoxygenase-1 (HO-1), an enzyme with antioxidant and antiangiogenic properties, and with an influence on apoptosis, all of which could contribute to its chemopreventive efficacy. HO-1 is responsible for the conversion of heme to biliverdin and carbon monoxide, and its induction can prevent oxidative stress (Lee et al., 1996; Motterlini et al., 2000) and apoptosis (Ferris et al., 1999; Brouard et al., 2000; Inguaggiato et al., 2001; Tanaka et al., 2003; Fang et al., 2004; Liu et al., 2004) in a variety of cell types. It has also been proposed that HO-1 protects against vasoconstriction and cell proliferation during vascular injury (Duckers et al., 2001), whereas growth arrest in response to overexpression of HO-1 has been observed in human pulmonary epithelial cells (Lee et al., 1996). Deficiency of HO-1 in both humans and knockout mice results in an abundance of circulating heme and damage to the vascular endothelium (Poss and Tonegawa, 1997; Wiesel et al., 2000; Jeney et al., 2002; Kawashima et

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ABBREVIATIONS: EGCG, epigallocatechin-3-gallate; HO-1, hemeoxygenase-1; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase; Nrf2, nuclear factor (erythroid-derived 2)-related factor; NF-κB, nuclear factor-κB; AP-1, adaptor protein-1; TNF, tumor necrosis factor; IL, interleukin; ARE, antioxidant response element; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; ATF, activating transcription factor; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; SP600125, 1,9-pyrazoloanthrone anthra(1,9-cd)pyrazol-6(2*H*)-one; EMSA, electrophoretic mobility shift assay; MEF, mouse embryo fibroblast; wt, wild-type; CK2, casein kinase II.

al., 2002). Thus, this enzyme has a range of important functions in normal cells.

HO-1 can be regulated via various transcription factors, which include AP-1 (via ERK and/or JNK), NF-κB (Hill-Kapturczak et al., 2001; Juan et al., 2005) and Nrf2 (Alam et al., 1999, 2000; Balogun et al., 2003). In human renal proximal tubule cells, HO-1 induction by curcumin is blocked by an inhibitor of IκBα phosphorylation (Hill-Kapturczak et al., 2001). In human aortic smooth muscle cells, HO-1 is induced by another dietary polyphenol, resveratrol, an effect that is eliminated by inhibitors of NF-κB activation or IκBα phosphorylation (Juan et al., 2005). Deletion of NF-κB binding sites on the HO-1 promoter also strongly reduces activity. Nrf2 binds to the ARE in promoter regions of protective enzymes involved in xenobiotic metabolism. In mice where the Nrf2 gene has been disrupted, induction of enzymes such as glutathione *S*-transferase and NAD(P)H:quinone oxidoreductase by various chemopreventive or antioxidant compounds is largely eliminated in the liver and intestine (Itoh et al., 1997; McMahon et al., 2001).

Depending on cell type and inducer, signaling pathways reported to regulate Nrf2 transcriptional activity and HO-1 expression include those important for proliferation and survival, involving mitogen-activated protein kinases (MAPKs—ERK, JNK, and p38) and phosphatidylinositol 3-kinase (PI3K). Curcumin up-regulates HO-1 in endothelial cells, renal epithelial cells, and astrocytes (Motterlini et al., 2000; Hill-Kapturczak et al., 2001; Scapagnini et al., 2002) as well as in rat kidney in vivo (Jones et al., 2000). In renal cells, up-regulation via Nrf2 and the ARE has been reported to involve p38 MAPK (Balogun et al., 2003). PI3K has been implicated in HO-1 induction in two dopaminergic cell lines in response to 6-hydroxydopamine (Salinas et al., 2003) or hemin (Nakaso et al., 2003) and more recently in rat pheochromocytoma PC12 cells in response to a plant-derived phenol, carnosol (Martin et al., 2004). A very recent study, reporting on EGCG-induced differential gene expression in wild-type mice compared with $Nrf2^{-/-}$ mice, noted induction of HO-1 in the small intestine but not the liver of wild-type mice (Shen et al., 2005).

After microarray analysis of the effects of EGCG in B-lymphoblasts, and curcumin in breast cells, we observed induction of HO-1 mRNA. Because both polyphenols can influence signaling through several pathways and induce apoptosis, we wished to examine whether the mechanism by which they induce HO-1 is similar to that for the endogenous substrate hemin and whether increased enzyme levels protect the cell types in question from apoptosis.

Materials and Methods

Materials

All chemicals were purchased from Sigma Chemical (Poole, Dorset, UK), and all solvents were from Fisher Scientific (Loughborough, UK), unless otherwise stated. Anti-α-tubulin antibody was from Oncogene Research Products (Darmstadt, Germany), anti-β-actin was from Abcam (Cambridge, UK), anti-Akt was from BioSource (Nivelles, Belgium), anti-HO-1 was from Stressgen (Yorkshire, UK), anti-phospho ATF2 was from Cell Signaling Technology (Beverly, MA), and anti-Nrf2 and anti-p65 (supershift) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Fluorescein isothiocyanate-conjugated Annexin V was from Bender Medsystems (Vienna, Austria). Inhibitors were obtained as follows: LY294002 and U0126 were from Promega (Mannheim, Germany); wortmannin, SB203580, and SP600125 were from Calbiochem (Darmstadt, Germany); and helenalin was from BIOMOL Research Laboratories (Hamburg, Germany). Curcumin (50 mM), EGCG, (100 mM) and hemin (10 mM) stock solutions were prepared in dimethyl sulfoxide and stored at −20°C in the dark.

Cell Lines

Human breast cell lines HBL100 and MDA-MB468, kindly provided by Prof. Rosemary Walker (Breast Cancer Research Unit, Glenfield Hospital, Leicester, UK), were originally obtained from American Type Culture Collection (Manassas, VA). Lymphoblastoid cell lines immortalized with Epstein Barr virus, (normotensive C143 and hypertensive H308), originally derived from patient blood samples, were a kind gift from Prof. Leong Ng (Department of Cardiovascular Sciences, University of Leicester, Leicester, UK). Mouse embryo fibroblasts, Nrf2-wild type and Nrf2-null, were kindly provided by Prof. John Hayes (Biomedical Research Centre, Ninewells Hospital and Medical School, University of Dundee, UK). The mouse embryo fibroblasts for wild-type and Raf-null cells were kindly provided by Dr. Catrin Pritchard (Department of Biochemistry, University of Leicester).

Methods

Cell Treatments. Breast cells were maintained as described previously (Squires et al., 2003). Mouse fibroblasts were grown in Dulbecco's modified Eagle's medium or Iscove's modified Dulbecco's medium in gelatin-coated flasks. Cells were treated with the appropriate concentration of agent and/or inhibitor as described under *Results*, with matching controls containing an equivalent volume of dimethyl sulfoxide (not greater than 0.1%).

Western Blotting. Whole cell and nuclear lysates were prepared as described previously (Squires et al., 2003). Protein concentrations were determined using the Bio-Rad assay. Thirty micrograms of protein per lane was separated by SDS-polyacrylamide gel electrophoresis and blotted onto Hybond-N nitrocellulose. After incubation with appropriate antibodies, blots were developed using ECL (Amersham Biosciences Inc., Little Chalfont, Buckinghamshire, UK). Results presented are representative of at least three separate experiments.

Electrophoretic Mobility Shift Assay for NF-κB DNA Binding. Nuclear extracts were prepared from cells treated with helenalin at the concentrations indicated. EMSAs were performed as described previously (Plummer et al., 1999) using a ^{32}P-end-labeled NF-κB consensus oligonucleotide (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) and an excess of either unlabeled NF-κB oligonucleotide or an unrelated AP-1 consensus sequence (5′-CGC TTG ATG AGT CAG CCG GAA-3′). To detect a supershift, samples were preincubated with 1 μl (200 μg/0.1 ml) of anti-p65 supershift antibody.

Determination of Apoptosis. The amount of apoptosis in response to various treatments was determined using the Annexin V staining method (Vermes et al., 1995). After treatments, floating cells were reserved and then combined with adherent cells after trypsinization. After staining, all cells were pelleted and analyzed by flow cytometry using a FACScan (Becton Dickinson, Oxford, UK), using Cell Quest software.

Results

Induction of HO-1. Levels of HO-1 protein were very low or undetectable in untreated cells (Fig. 1). We showed previously, using microarray analysis, that curcumin (10 μM) induced HO-1 mRNA very effectively in MDA-MB468 breast cells after treatment for 3 h. This effect had disappeared by 24 h and was not observed in the HBL100 cell line, which was only examined at 24 h (Squires, 2000). EGCG (25 μM) induced HO-1 mRNA in B-lymphoblastoid cells after treatment

for 4 or 24 h (Atherfold, 2003). These mRNA data are validated in the present study.

Curcumin (20 μM) caused a time-dependent induction of HO-1 protein levels in two breast cell lines, HBL100 and MDA-MB468, apparent from 5 h, but decreasing again by 48 h. Induction was also dose-dependent, being maximal between 15 and 30 μM, whereas not occurring at doses higher than 40 μM (Fig. 1A, data for MDA-MB468 line only).

In a similar manner, hemin (20 μM) induced the enzyme from 5 to 24 h in both breast cell lines with levels declining again at later time points. However, unlike curcumin, hemin induced HO-1 at concentrations up to 100 μM (Fig. 1B). Higher doses were not examined.

EGCG (20 μM over 48 h or 5–60 μM for 6 h) did not induce HO-1 in breast cells (data not shown). However, in agreement with the microarray data, EGCG (30 μM) induced HO-1 protein in lymphoblasts over a similar time course to the other two inducers (Fig. 1C).

EGCG (15 μM), in combination with curcumin (15 μM) or hemin (30 μM), did not inhibit HO-1 induction by either of these agents in breast cells (Fig. 1D). However, 40 to 50 μM curcumin, which on its own was ineffective at inducing HO-1, also inhibited induction by hemin (Fig. 1E).

Effect of Signaling Inhibitors on HO-1 Induction. Curcumin and EGCG can influence a range of signaling pathways, including those involving ERK and JNK MAPKs, PI3K, and NF-κB (for review, see Manson, 2005), which could contribute to either induction or inhibition of HO-1. To assess whether any of these pathways are involved in regulating HO-1 under the conditions used here, we investigated the effect of a number of well known signaling inhibitors.

Induction of HO-1 in breast cells after treatment for 4.5 h with 20 μM curcumin was substantially inhibited by 30-min pretreatment of cells with 10 μM SB203580, a fairly specific inhibitor of p38α and β signaling (Davies et al., 2000). However, expression was inhibited even more effectively by 50 μM LY294002, an inhibitor of PI3K and casein kinase II (Fig. 2A). U0126 (30 μM), an inhibitor of mitogen-activated protein kinase kinase, and therefore of ERK activation, was partially effective at inhibiting HO-1 induction in MDA MB468 cells (Fig. 2A), whereas no inhibition was observed with the JNK inhibitor SP600125. These two inhibitors had no apparent effect on induction of HO-1 by curcumin in HBL100 cells. To confirm that PI3K activity was important for HO-1 induction, the experiment was repeated with wortmannin. However, no inhibition of HO-1 was observed with 100 nM (Fig. 2B) or 1 μM wortmannin. At these concentrations, levels of pAkt were significantly decreased or eliminated, confirming that the inhibitor was active.

To determine whether NF-κB activity might be important, helenalin, which blocks p65 binding to DNA (Garcia-Piñeres et al., 2001), was also used. At 20 μM, this inhibitor, which was chosen because both breast cell lines have constitutive nuclear localization of several NF-κB subunits, completely blocked induction of HO-1 in both breast lines (Fig. 2C). At this concentration, it also eliminated phosphorylated Akt in the HBL100 cell line, whereas it caused a slight decrease in the MDA-MB468 line. However, at lower concentrations, helenalin induced HO-1 (see below).

Similar results with respect to the effect of wortmannin, LY294002, helenalin, and SB203580 on HO-1 induction were obtained in breast cells treated with hemin for 5.5 h after a 30-min pretreatment with inhibitors (Fig. 2, B and D).

EGCG-induced HO-1 in lymphoblasts was also inhibited partially by SB203580 (1 μM) and completely by LY294002 (50 μM) after 30-min pretreatment with the inhibitors (Fig. 2E). SP600125 or U0126 had no inhibitory effect in lymphoblastoid cells.

Effect of Curcumin on p38 Signaling. Because induction of HO-1 was significantly inhibited by SB203580, a fairly specific inhibitor of p38, the effect of curcumin on this pathway was investigated. No phosphorylated p38 was detected

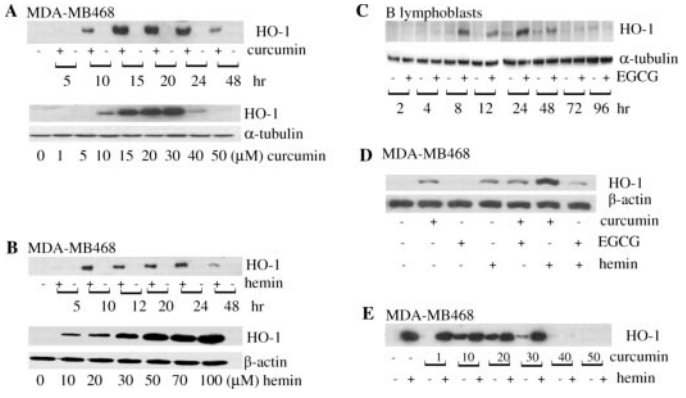

Fig. 1. Induction of HO-1 by curcumin, hemin, and EGCG. A, time course with 20 μM curcumin and dose response at 6 h. B, time course with 20 μM hemin and dose response at 16 h. C, time course with 30 μM EGCG. D, effect of combination treatments with curcumin (15 μM), EGCG (15 μM), and hemin (30 μM) on HO-1 induction after 6 h. E, effect on HO-1 induction of combination treatments with increasing doses of curcumin and 50 μM hemin.

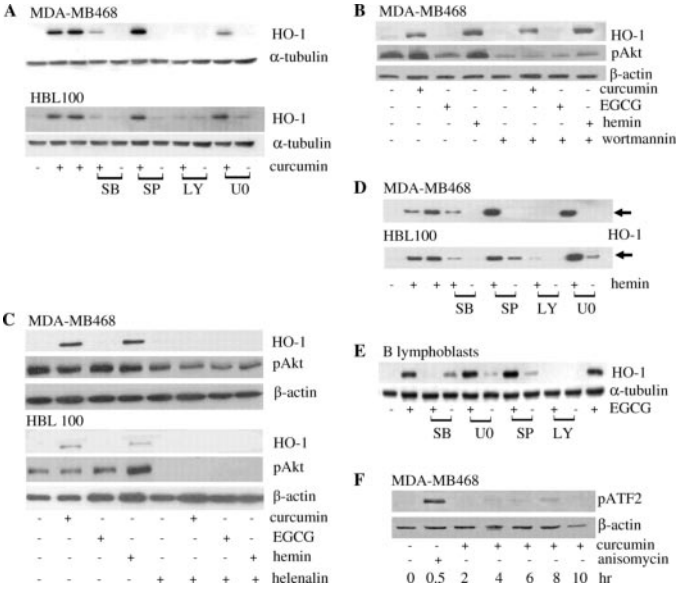

Fig. 2. Effect of inhibitors on HO-1 expression and of curcumin on p38 signaling. A, breast cells were pretreated with inhibitor for 30 min before addition of curcumin for 4.5 h. B, MDA-MB468 cells were pretreated with wortmannin for 25 min before addition of inducer for 4.5 h. C, breast cells were treated with helenalin for 4 h before treatment with inducers for 6 h. D, breast cells were pretreated with inhibitors for 30 min before addition of hemin for 5.5 h. E, B-lymphoblasts were pretreated with inhibitor for 30 min before treatment with EGCG for 10 h. F, MDA-MB468 cells were treated with curcumin for up to 10 h, after which lysates were probe for phospho-ATF2. Anisomycin (100 nM for 30 min) was used as a positive control. Curcumin (20 μM); EGCG (20 μM); hemin (30 μM); helenalin (20 μM); LY, LY294002 (50 μM); SP, SP600125 (10 μM); SB203580 (SB; A and D, 10 μM; E, 1 μM); U0126 (U0; 30 μM); and wortmannin (0.1 μM).

in either breast line by Western blotting in untreated cells or after treatment with 20 μM curcumin for up to 10 h (data not shown). However, on investigating a possible downstream target of p38, ATF2, time-dependent phosphorylation in response to curcumin was observed (Fig. 2F), which was dose-dependent up to 60 μM (data not shown). Anisomycin was included in each of these experiments as a positive control.

Effect of Helenalin on NF-κB-DNA Binding. To assess the effect of helenalin on NF-κB-DNA binding, EMSAs were carried out. In both of the breast cell lines, helenalin (20 μM, from 2 h) effectively inhibited nuclear protein binding to an oligonucleotide containing the NF-κB consensus sequence (Fig. 3A). The banding pattern was specific, as determined by the use of competitive and noncompetitive oligonucleotides, and the lower of the protein bands was identified as containing p65 by supershift. It is noteworthy that at helenalin concentrations of 1 and 5 μM, which did not inhibit DNA binding, HO-1 was induced (Fig. 3B). At 10 μM, induction was less marked in the HBL100 cells, which was mirrored by less DNA binding in the EMSA (compare Fig. 3, A and B). With HBL100 nuclear extracts, helenalin also decreased the upper band in the EMSA more so than with MDA-MB468 extracts.

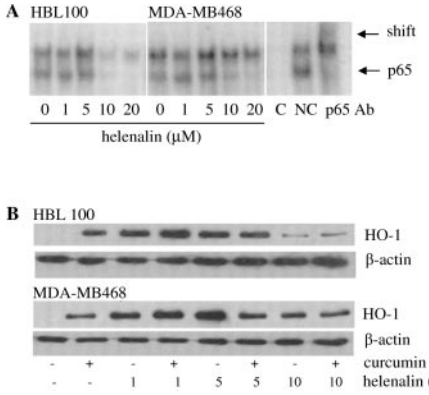

Fig. 3. Effect of helenalin on NF-κB DNA binding and HO-1 induction in breast cells. A, EMSA showing the effect of treatment for 5 h with increasing doses of helenalin on NF-κB-DNA binding in HBL100 and MDA-MB468 cells. Specificity of the binding pattern was determined by the use of competitive (C) and noncompetitive (NC) oligonucleotides. The presence of p65 in the lower band was determined by supershift. EMSAs were performed as described under *Materials and Methods* and are representative of at least three independent experiments. B, dose response for HO-1 induction by helenalin, with and without 20 μM curcumin. Cells were pretreated for 3 h with the indicated doses of helenalin, before addition of curcumin and a further 6-h incubation.

Treatment of MDA-MB468 cells with LY294002 (50 μM) did not inhibit NF-κB-DNA binding at times up to 6 h, and this inhibitor did not decrease nuclear levels of p65 in either cell line (data not shown).

Induction/Stabilization of Nrf2 and Its Nuclear Translocation. To establish whether the transcription factor Nrf2 was involved in HO-1 induction in the cell types used here, we examined protein levels and nuclear localization in response to each of the agents and inhibitors.

Curcumin and Hemin. Low levels of Nrf2 were present in nuclear extracts from HBL100 or MDA-MB468 cells in the absence of any treatment, but exposure to either agent for 6 h, with doses that induced HO-1, caused a significant increase in Nrf2 nuclear protein levels. The dose-dependent increase in Nrf2 paralleled the increase in HO-1 in response to both curcumin and hemin (Fig. 4A). However, there was little evidence that either LY294002 or SB203580 inhibited nuclear accumulation of Nrf2 (Fig. 4B).

EGCG did not significantly increase nuclear Nrf2 levels in breast cells, where no induction of HO-1 occurred (data not shown), but elevated nuclear expression was observed in lymphoblasts, which was partially inhibited by treatment with LY294002 or SB203580 at 2 h but only by LY294002 at 4 and 8 h (Fig. 4C).

HO-1 and Nrf2 Expression in Wild-Type and Knockout Mouse Embryo Fibroblasts. Curcumin and hemin induced HO-1 very effectively in wild-type (wt) MEFs, with a dose response similar to that seen in breast cells (Fig. 5, A and C). Induction by EGCG exhibited a similar narrow dose range (5–20 μM) to that seen for curcumin (Fig. 5D). No significant induction with any agent was observed in fibroblasts from mice that were null for Nrf2 (Fig. 5A, showing curcumin data only). However, HO-1 was readily, albeit somewhat less efficiently, induced by curcumin or hemin in MEFs that were null for B-Raf, a key upstream component of the ERK signaling pathway (Fig. 5, B and C). In contrast, EGCG was completely ineffective in B-Raf-null MEFs (data not shown).

Both curcumin and hemin increased nuclear levels of Nrf2 in wt MEFs and $Raf^{-/-}$ MEFs (Fig. 5E) but not in $Nrf2^{-/-}$ MEFs (data not shown), with a similar dose response as seen for the increase in HO-1 in these cells.

Curcumin, EGCG, and Helenalin Induce Apoptosis Despite Induction of HO-1. There is considerable evidence that HO-1 induction by hemin prevents cells from undergo-

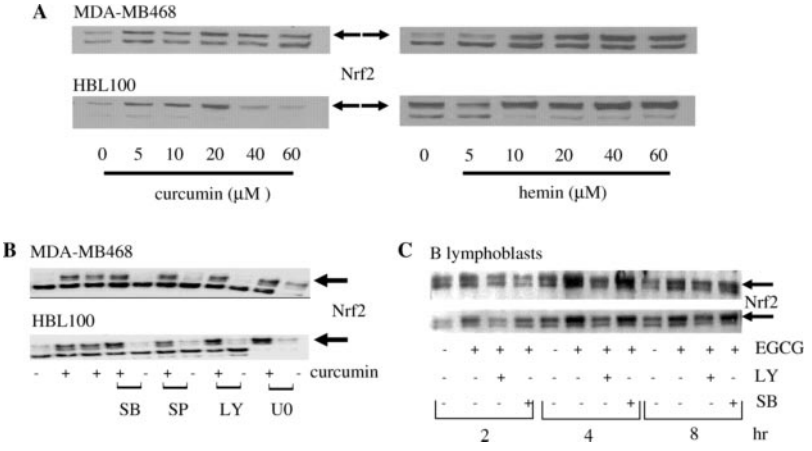

Fig. 4. Nuclear localization of Nrf2 in response to treatments. A, breast cells were treated with indicated concentrations of inducing agent for 6 h, and expression of Nrf2 in nuclear extracts was examined. B, inhibitors were used in 30-min pretreatments at the following concentrations: LY294002, 50 μM; SB230580, 10 μM; SP600125, 10 μM; and U0126, 30 μM, followed by treatment with curcumin (20 μM) for 4.5 h. C, before treatment with EGCG (30 μM) for the times indicated, inhibitors were used in 30-min pretreatments at the following concentrations: LY294002, 50 μM; and SB230580, 1 μM.

ing apoptosis. Conversely, it is also well established that both curcumin and EGCG can induce apoptosis in a range of cell types. We therefore examined whether cells used in this study underwent apoptosis after treatments that induced HO-1.

We showed previously that curcumin (20 μM) induces apoptosis (47%) in MDA 468 cells after a 48-h treatment (Squires et al., 2003), and a further 49% of cells had undergone (possibly secondary) necrosis. HBL100 cells were less sensitive with only around 10% of apoptotic cells at this dose. However, after treatment with 40 μM curcumin, which is less effective at inducing HO-1, around 30% of HBL100 cells were apoptotic, and most of the remainder had undergone necrosis. Therefore, HO-1 induction did not seem to protect MDA-MB468 breast cells from undergoing apoptosis, but it may have afforded some protection to HBL100 cells.

EGCG caused growth inhibition of lymphoblast cultures derived from normotensive or hypertensive patients. The IC_{50} was in the range of 10 to 18 μM (Atherfold, 2003). At a dose of 10 μM, EGCG also induced apoptosis (~30%), after 48-h treatment (Fig. 6A). Thus, induction of HO-1 during the first 24 h of treatment did not protect this cell type either.

The response of breast cells to helenalin treatment (Fig. 6B) was similar to their response to curcumin. MDA-MB468 cells were more sensitive and underwent significant apoptosis (~40%) at doses that induced HO-1. The HBL100 cells showed significant levels of necrosis at doses where NF-κB-DNA binding was largely inhibited. Hemin at doses up to 70 μM did not induce apoptosis in breast cells (data not shown).

Discussion

Results from this study suggest that induction of HO-1 by curcumin or hemin is very similar in epithelial cells and fibroblasts. However, although EGCG can also induce this enzyme in fibroblasts and lymphoblasts, it is ineffective in breast epithelial cells.

The main difference between curcumin and hemin was the dose response, in that hemin induced HO-1 at all concentrations investigated, whereas curcumin showed a very narrow activity range. Scapagnini et al. (2002) reported a similar strict dose-response in astrocytes and suggested that lack of induction at higher concentrations (50–100 μM) was because of curcumin-induced loss of cell viability linked to a failure to increase glutathione levels.

Terry et al. (1998) reported that curcumin (20 μM) inhibited induction of HO-1 mRNA by TNF-α or IL-1, and they attributed this to inhibition of AP-1. Motterlini et al. (2000) considered that although HO-1 can be regulated by AP-1 and NF-κB, it is unlikely that these transcription factors are involved in induction by curcumin because it is a potent inhibitor of both. EGCG has also been shown to inhibit NF-κB activation (for review, see Manson 2005).

In the present study, concentrations of 40 to 50 μM curcumin did not induce HO-1 and were able to inhibit induction by hemin. EGCG also exhibited restricted activity in fibroblasts. These results together with a lack of induction by hemin or low doses of curcumin in the presence of 20 μM helenalin suggested that NF-κB transactivation might be important. Most of the cells used in this study had some constitutive nuclear localization of NF-κB subunits. Therefore, to block transactivation in these cells, inhibition of DNA binding might be more effective than inhibition of upstream signaling and nuclear translocation. Curcumin, EGCG, and helenalin have all been reported to block NF-κB-DNA binding independently of an effect on translocation of the sub-

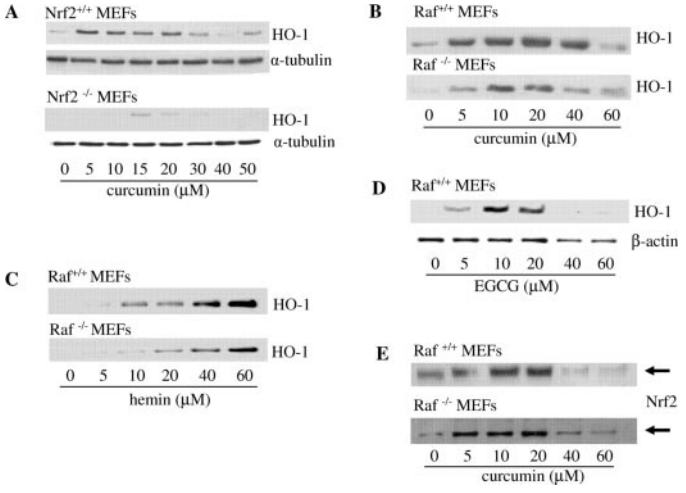

Fig. 5. HO-1 induction and Nrf2 nuclear localization in MEFs. A, Wild-type and Nrf2$^{-/-}$ MEFs were treated with the indicated concentrations of curcumin for 6 h. B to D, wild-type and Raf$^{-/-}$ MEFs were treated with indicated concentrations of curcumin for 6 h (B), hemin for 16 h (C), or EGCG for 6 h (D). E, wild-type and Raf$^{-/-}$ MEFs were treated with curcumin for 6 h, and nuclear extracts were probed for Nrf2.

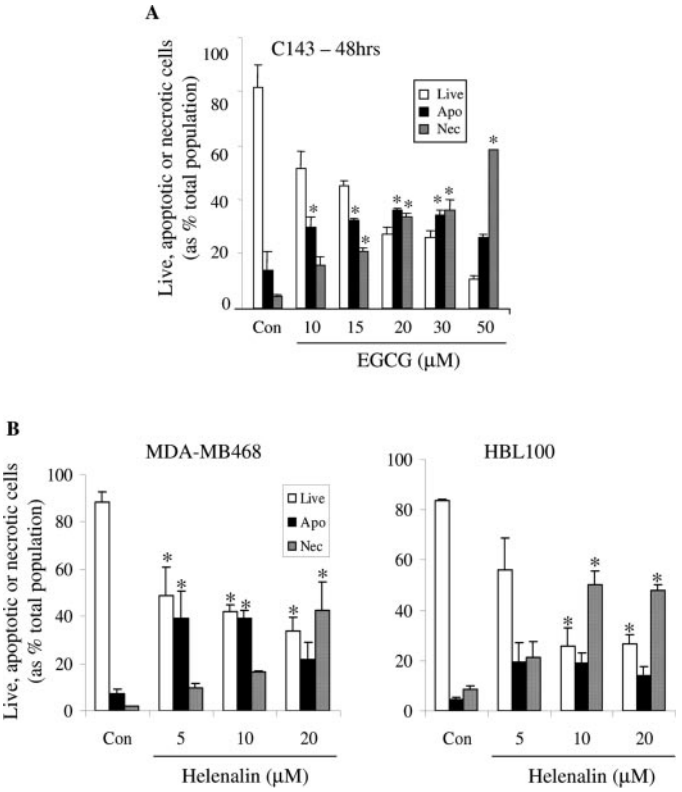

Fig. 6. Induction of apoptosis by helenalin and EGCG. A, C143 lymphoblasts were treated with EGCG for 48 h, and levels of apoptosis were measured by Annexin V binding. Percentages of viable, apoptotic, and necrotic cells are indicated. B, breast cells were treated with helenalin for 24 h, and levels of apoptosis were measured as described above. *, significant difference from control ($p < 0.05$; $n = 3$) as determined by balanced analysis of variance, followed by Tukey's least significant difference test.

units. Helenalin works by alkylating the cysteine 38 residue in the DNA binding domain of the p65 subunit (Garcia-Piñeres et al., 2001). Curcumin can react directly with the p50 subunit (Brennan and O'Neill, 1998), and EGCG can inhibit phosphorylation of the p65 subunit (Wheeler et al., 2004). It has recently been shown that another polyphenol, resveratrol, induces HO-1 in human aortic smooth muscle cells only at low concentrations. Induction was dependent on NF-κB being activated by low doses but inhibited by higher doses of resveratrol (Juan et al., 2005). Thus, the narrow HO-1-inducing dose range exhibited by curcumin, EGCG, and helenalin may reflect inhibition of NF-κB at higher concentrations, possibly coupled with activation at lower doses.

Although induction of HO-1 was completely inhibited by LY294002, it was unaffected by wortmannin, suggesting that signaling through PI3K was not involved. Further evidence in support of this came from experiments using 20 μM helenalin, which only effectively inhibited phosphorylation of Akt in HBL100 cells, but completely prevented HO-1 induction in both breast cell lines.

In addition to inhibiting PI3K, LY294002 is also an effective inhibitor of casein kinase II (CK2), an enzyme that has been shown to phosphorylate NF-κB. Thus, inhibition of CK2 activity can affect NF-κB transcriptional activity (Romieu-Mourez et al., 2002; Viatour et al., 2005). It is therefore possible that the observed inhibition by LY294002 may involve reduced phosphorylation of p65 by CK2. However, in EMSAs LY294002 did not obviously affect NF-κB-DNA binding in MDA-MB468 cells (L. M. Howells, unpublished data).

Neither curcumin nor hemin required Raf for induction of HO-1, although induction seemed somewhat less efficient in $Raf^{-/-}$ MEFs. But this signaling component of the growth factor receptor/MAPK pathway seemed to be essential for induction by EGCG in MEFs, although in lymphoblasts treatment with the mitogen-activated protein kinase kinase inhibitor U0126 did not block HO-1 induction by EGCG.

Experiments with Nrf2-null MEFs showed that this transcription factor is required for induction by all three compounds. The use of inhibitors in breast cells did not identify a pathway that was responsible for curcumin- or hemin-induced Nrf2 activation. LY294002 and SB203580 treatment resulted in only partial inhibition of EGCG-induced nuclear Nrf2 levels in lymphoblasts. In some circumstances, p38 is upstream of CK2 and NF-κB activation (Viatour et al., 2005), allowing the possibility that in the present study signaling through p38 may be important for NF-κB rather than Nrf2 activation.

Induction of HO-1 by curcumin has been reported to involve Nrf2 and p38 in renal epithelial cells (Balogun et al., 2003). Results from a study by Alam et al. (2000) suggested that HO-1 induction by cadmium occurred via sequential activation of the p38 pathway and Nrf2, whereas MAPK pathways involving ERK and JNK were not required.

Several studies have implicated PI3K signaling in HO-1 induction. Heme induced Akt phosphorylation in neutrophils, and inhibition of Ras/ERK and PI3K pathways abolished heme-protective effects in this cell type (Arruda et al., 2004). Cells transfected with membrane-targeted Akt exhibited increased HO-1 expression (Salinas et al., 2003). In a neuroblastoma cell line, PI3K inhibitors blocked nuclear translocation of Nrf2 (Nakaso et al., 2003). The plant phenol carnosol induced HO-1 in PC12 cells via a mechanism involving Nrf2, and similar to our results, the inhibitor LY294002 blocked induction (Martin et al., 2004). These authors did not use wortmannin, but they reported that overexpression of active PI3K caused induction of HO-1, whereas a dominant negative mutant of Akt had the opposite effect. As in the present study, inhibition of p38 by SB203580 also significantly reduced the response, whereas ERK and JNK were dispensable for HO-1 up-regulation. A recent study using mouse embryo fibroblasts found that hemin-induced HO-1 activity was blocked by a CK2 inhibitor and by LY294002 but not by wortmannin, PD98059, or SB203580 (Abate et al., 2005).

HO-1 is reported to protect against cell death. Cells from mice with a targeted deletion of HO-1 were much more sensitive to apoptosis induced by staurosporine, etoposide, or serum deprivation, an effect that was greatly reduced by overexpression of HO-1 (Ferris et al., 1999). Induction of HO-1 by hemin or cadmium in gastric cancer cells that was inhibited by the p38 inhibitor SB203580 or by the ERK inhibitor PD098059, and was dependent on activation of NF-κB, was involved in resistance to apoptosis (Liu et al., 2004). In the present study, however, although hemin did not induce apoptosis in any of the cell lines tested, induction of HO-1 by curcumin or EGCG did not protect MDA-MB468 breast tumor cells or lymphoblasts, respectively, from apoptosis. HBL100 cells (derived from normal tissue) were more resistant to apoptosis at doses of curcumin that induced HO-1.

A number of studies on endothelial cells have implicated CO, released by HO-1 during breakdown of heme, as the main protective moiety (Brouard et al., 2000, 2002; Otterbein et al., 2000; Zhang et al., 2003). In RAW macrophages, CO inhibited expression of lipopolysaccharide-induced proinflammatory cytokines TNF-α, IL-1β, and macrophage inflammatory protein-1b, whereas at the same time increasing the expression of anti-inflammatory IL-10, effects that were dependent on activation of p38-MAPK (Otterbein et al., 2000). These authors speculated that this might represent a mechanism by which HO-1 and CO are amplified by p38 to exert functional anti-inflammatory effects. Brouard et al. (2002), working with various endothelial cells, found that CO protected against TNFα-induced apoptosis via a mechanism involving activation of p38 and NF-κB and expression of the antiapoptotic genes *c-IAP2* and *A1*. Zhang et al. (2003), using an ischemia-reperfusion model, showed that CO modulated Fas/Fas ligand, activated caspases-8, -3, and -9, and up-regulated BclII, dependent on activation of p38α and mitogen-activated protein kinase kinase 3.

One possibility for the similar induction of HO-1 by curcumin and hemin is the initial requirement for release of heme from heme-containing proteins by curcumin, in which case induction by curcumin might be expected to be significantly slower. However, in the cell types investigated here, timing of induction of HO-1 by curcumin was similar to hemin. If release of CO is important for protecting against apoptosis, induction of HO-1 by curcumin or EGCG may be ineffective if they do not generate enough heme. It would be of interest to determine whether curcumin and EGCG generate CO/biliverdin and whether pretreatment with hemin can protect cells from induction of apoptosis by the polyphenols.

Owuor and Kong (2002) proposed a model whereby at low

concentrations, EGCG or tea polyphenols activate MAPK signaling pathways, leading to activation of Nrf2 and ARE with subsequent induction of phase II and other defensive enzymes (including HO-1), which protect cells against toxic insults, thereby enhancing cell survival, a beneficial homeostatic response. But at higher concentrations, they suggested that the same agents activate caspase-dependent pathways, leading to apoptosis. This does not seem to be the case in the MDA-MB468 breast tumor cells or lymphoblasts where apoptosis is occurring despite the activation of the Nrf2 gene battery.

This study has identified a number of key molecules required for HO-1 induction, but also implicates other unidentified components as being essential. Expression of HO-1 was very low or undetectable in untreated MDA-MB468 or HBL100 breast cells, which already contain significant levels of phosphorylated Akt and ERK as well as constitutive nuclear NF-κB. This argues against any or all of these being sufficient to induce the enzyme or to stabilize or induce translocation of Nrf2. This was borne out by the low levels of Nrf2 in untreated whole cell or nuclear extracts.

In conclusion, we have shown that induction of HO-1 by curcumin and hemin seems to be very similar, involving signaling through the MAPK, p38, the transcription factor Nrf2 and a pathway blocked by LY294002 but not wortmannin. Our results also suggest that NF-κB activity is important and that a reason for the strict dose dependence exhibited by curcumin, EGCG, and helenalin might be related to their ability to activate NF-κB-DNA binding at lower concentrations but inhibit it at higher concentrations. Our data also indicate that induction of HO-1 does not guarantee protection from apoptosis.

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