Involvement of Nrf2, p38, B-raf and NF-κB, but not PI3K, in induction of hemeoxygenase (HO-1) by dietary polyphenols

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Abbreviations: ARE: antioxidant response element; EGCG: epigallocatechin-3-gallate; ERK: extracellular regulated kinase; JNK: c-jun N-terminal kinase; MAPK: mitogen activated protein kinase; MEF: mouse embryo fibroblast; MEK: mitogen activated protein kinase kinase; NF-κB: nuclear factor kappa B; Nrf2: nuclear factor (erythroid-derived 2) related factor; PI3K: phosphatidylinositol-3 kinase
Abstract

The highly inducible enzyme, hemeoxygenase-1 (HO-1), metabolizes heme, thereby protecting a variety of cells against oxidative stress and apoptosis. Upregulation by cancer chemopreventive agents has been reported, but its regulation and function in transformed cells is 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 5-24hrs. Curcumin (5-40µM) or hemin (5-100 µM) induced HO-1 and nuclear levels of Nrf2 in a dose-dependent manner. EGCG had no effect in breast cells, but at 30µM induced nuclear translocation of Nrf2 and HO-1 expression in B-lymphoblasts. In all cases induction was inhibited by pretreatment with the PI3K inhibitor, LY294002, or the p38 inhibitor, SB203580. The 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 (5-20µM) induced HO-1 only in wt MEFs. Results suggest that signaling through p38 MAPK, 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 (5-10µM) 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 pro-apoptotic, antioxidant, and anti-angiogenic effects (reviewed in Surh, 2003; Manson, 2003 & 2005). Amongst its many effects, curcumin induces hemeoxygenase-1 (HO-1), an enzyme with antioxidant and anti-angiogenic 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; Liu et al, 2004; Fang 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), while growth arrest in response to over-expression 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 & Tonegawa, 1997; Wiesel et al., 2000; Jeney et al., 2002; Kawashima et 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 which 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 antioxidant response element (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, signalling 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 upregulates 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 upregulation via Nrf2 and the antioxidant response element (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 to Nrf2-/- mice, noted induction of HO-1 in the small intestine, but not the liver of wild-type mice (Shen et al, 2005).

Following microarray analysis of the effects of EGCG in B-lymphoblasts, and curcumin in breast cells, we observed induction of HO-1 mRNA. Since 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-Aldrich Company Ltd, Poole, UK, and all solvents from Fisher Scientific, Loughborough, UK, unless otherwise stated. Anti-α-tubulin antibody was from Oncogene, (Darmstadt, Germany), anti-β-actin from ABCAM (Cambridge, UK), anti-Akt from Biosource (Nivelles, Belgium), anti-HO-1 from Stressgene (Yorkshire, UK), anti-phospho ATF2 from Cell Signaling Technology (Beverly, MA) and anti-Nrf2 and anti-p65 (supershift ) antibodies from Santa-Cruz Biotechnology, (Santa Cruz, CA). FITC-conjugated annexin V was from Bender Medsystems (Vienna, Austria). Inhibitors were obtained as follows: LY294002 and U0126 from Promega (Manheim, Germany), wortmannin, SB 203580 and SP600125 from Calbiochem (Darmstadt, Germany), and helenalin from Biomol (Hamburg, Germany). Curcumin (50mM), EGCG, (100mM) and hemin (10mM) stock solutions were prepared in DMSO 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, 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, UK).

**Methods**

*Cell treatments*

Breast cells were maintained as described previously (Squires et al., 2003). Mouse fibroblasts were grown in Dulbecco’s Modified Eagles 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 in results, with matching controls containing an equivalent volume of DMSO, (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 BioRad assay. Routinely 30µg protein per lane was separated by SDS-PAGE and blotted onto Hybond-N nitrocellulose. Following incubation with appropriate antibodies, blots were developed using ECL (Amersham, Little Chalfont, UK). Results presented are representative of at least 3 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 32P-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’). In order to detect a supershift, samples were preincubated with 1µl (200µg/0.1ml) 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). Following treatments, floating cells were reserved, and combined with adherent cells following trypsinization. Following staining, all cells were pelleted and analyzed by flow cytometry using a FACscan (Becton Dickinson, Oxford, UK), using the Cell Quest software.
RESULTS

Induction of HO-1

Levels of HO-1 protein were very low or undetectable in untreated cells (Figure 1).

We showed previously, using microarray analysis, that curcumin (10µM) induced HO-1 mRNA very effectively in MDA-MB468 breast cells following treatment for 3 hrs. This effect had disappeared by 24 hrs and was not observed in the HBL100 cell line, which was only examined at 24 hrs (Squires, 2000, PhD Thesis, University of Leicester). EGCG (25µM) induced HO-1 mRNA in B lymphoblastoid cells following treatment for 4 or 24 hrs (Atherfold, 2003, PhD Thesis, University of Leicester). 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 5hrs, but decreasing again by 48hrs. Induction was also dose-dependent, being maximal between 15 and 30 µM, while not occurring at doses higher than 40µM (Figure 1A, data for MDA-MB468 line only).

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

EGCG (20µM over 48hrs or 5-60 µM for 6 hrs) did not induce HO-1 in breast cells (data not shown). However, in agreement with the microarray data, EGCG (30µM) induced HO-1 protein in lymphoblasts over a similar time course to the other two inducers (Figure 1C). EGCG (15µM), in combination with curcumin (15µM) or hemin (30µM), did not inhibit HO-1 induction by either of these agents in breast cells (Figure 1D). However, 40-50µM of curcumin, which on its own was ineffective at inducing HO-1, also inhibited induction by hemin (Figure 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 (reviewed in 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 hours with 20 µM curcumin, was substantially inhibited by 30 minute 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 50µM LY294002, an inhibitor of phosphatidylinositol-3-kinase (PI3K) and casein kinase II (Figure 2A). U0126 (30 µM) an inhibitor of mitogen activated protein kinase kinase (MEK), and therefore of ERK activation, was partially effective at inhibiting HO-1 induction in MDA MB468 cells (Figure 2A), while 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 100nM (Figure 2B) or 1µM wortmannin. At these concentrations, levels of pAkt were significantly decreased or eliminated, confirming that the inhibitor was active.

In order 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 20µM 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 (Figure 2C). At this concentration it also eliminated phosphorylated Akt in the HBL100 cell line,
while causing 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 hrs following a 30 min pretreatment with inhibitors (Figure 2B and D).

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

**Effect of curcumin on p38 signaling**

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

**Effect of helenalin on NF-κB-DNA-binding**

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

Treatment of MDA-MB468 cells with LY294002 (50 µM) did not inhibit NF-κB-DNA-binding at times up to 6 hr, nor did this inhibitor decrease nuclear levels of p65 in either cell line (data not shown).

**Induction/stabilization of Nrf2 and its nuclear translocation**

In order 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 hours, 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 (Figure 4A). However, there was little evidence that either LY294002 or SB203580 inhibited nuclear accumulation of Nrf2 (Figure 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 hrs, but only by LY294002 at 4 and 8 hrs (Figure 4C).
HO-1 and Nrf2 expression in wild type and knock-out mouse embryo fibroblasts (MEFs)

Curcumin and hemin induced HO-1 very effectively in wild-type (wt) MEFs, with a dose-response similar to that seen in breast cells (Figure 5A and C). Induction by EGCG exhibited a similar narrow dose range (5-20µM) to that seen for curcumin (Figure 5D). No significant induction with any agent was observed in fibroblasts from mice which were null for Nrf2 (Figure 5A, showing curcumin data only). However, HO-1 was readily, albeit somewhat less efficiently, induced by curcumin or hemin in MEFs which were null for B-Raf, a key upstream component of the ERK signaling pathway (Figure 5B 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 (Figure 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 undergoing 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 following treatments which induced HO-1.

We showed previously that curcumin (20µM) induces apoptosis (47%) in MDA 468 cells following a 48hr 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, following treatment with 40µM 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 appear to protect...
MDA-MB468 breast cells from undergoing apoptosis, but 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 10-18 $\mu$M (Atherfold, 2003, PhD Thesis).

At a dose of 10$\mu$M, EGCG also induced apoptosis (~30%), following 48 hrs treatment (Figure 6A). Thus induction of HO-1 during the first 24hrs of treatment did not protect this cell type either.

The response of breast cells to helenalin treatment (Figure 6B) was similar to their response to curcumin. MDA-MB468 cells were more sensitive and underwent significant apoptosis (~40%) at doses which induced HO-1. The HBL100 cells showed significant levels of necrosis at doses where NF-$\kappa$B-DNA-binding was largely inhibited.

Hemin at doses up to 70$\mu$M 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, while 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, while 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 (50-100µM) was due to curcumin-induced loss of cell viability linked to a failure to increase GSH levels. Terry et al. (1998) reported that curcumin (20µM) inhibited induction of HO-1 mRNA by TNFα or IL-1 and attributed this to inhibition of AP-1. Motterlini et al., (2000) considered that while HO-1 can be regulated by AP-1 and NF-κB, it is unlikely that these transcription factors are involved in induction by curcumin since it is a potent inhibitor of both. EGCG has also been shown to inhibit NF-κB activation (reviewed in Manson 2005).

In the present study concentrations of 40-50µM 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 20µM 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 in order 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 subunits. Helenalin works by alkylating the cysteine 38 residue in the DNA-binding
domain of the p65 subunit (Garcia-Pineres 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). Interestingly, it was recently shown that another polyphenol, resveratrol, induced 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.

While induction of HO-1 was completely inhibited by LY294002, it was unaffected by wortmannin, suggesting that signalling 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, while completely preventing 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 which 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 (unpublished data).

Neither curcumin nor hemin required Raf for induction of HO-1, although induction appeared somewhat less efficient in Raf−/− MEFs. But this signalling component of the growth factor receptor/MAPK pathway appeared to be essential for induction by EGCG.
in MEFs, although in lymphoblasts treatment with the MEK 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 which 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 signalling 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, while MAPK pathways involving ERK and JNK were not required.

Several studies have implicated PI3K signalling 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 reported that overexpression of active PI3K caused induction of HO-1, while 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, while ERK and JNK were
dispensable for HO-1 upregulation. 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 which was greatly reduced by overexpression of HO-1 (Ferris et al., 1999). Induction of HO-1 by hemin or cadmium in gastric cancer cells which 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, while 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 tumour cells or lymphoblasts respectively from apoptosis. HBL100 cells (derived from normal tissue) were more resistant to apoptosis at doses of curcumin which 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 (Otterbein et al., 2000; Brouard et al., 2000; 2002; Zhang et al., 2003). In RAW macrophages, CO inhibited expression of LPS-induced proinflammatory cytokines TNFα, IL-1β and macrophage inflammatory protein-1b, while at the same time increasing the expression of anti-inflammatory IL10, effects which were dependent on activation of p38MAPK (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 anti-apoptotic genes c-IAP2 and A1. Zhang and colleagues (2003a and b), using an ischemia-reperfusion model,
showed that CO modulated Fas/Fas Ligand, activated caspases 8, 3 and 9, and upregulated Bcl2, dependent on activation of p38α and M KK3.

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 obviously 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 & Kong (2002) proposed a model whereby at low concentrations, EGCG or tea polyphenols activate MAPK signalling 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 tumour 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 appears to be very similar, involving signalling 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, while inhibiting at higher concentrations. Our data also indicate that induction of HO-1 does not guarantee protection from apoptosis.

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FIGURE LEGENDS

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

Figure 2. Effect of inhibitors on HO-1 expression and of curcumin on p38 signaling.
A) Breast cells were pretreated with inhibitor for 30mins, before addition of curcumin for 4.5hrs. B) MDA-MB468 cells were pretreated with wortmannin for 25mins, prior to addition of inducer for 4.5hrs. C) Breast cells were treated with helenalin for 4hrs prior to treatment with inducers for 6hrs. D) Breast cells were pretreated with inhibitors for 30 mins before addition of hemin for 5.5hrs. E) B-lymphoblasts were pretreated with inhibitor for 30mins prior to treatment with EGCG for 10hrs. F) MDA-MB468 cells were treated with curcumin for up to 10hrs, following which lysates were probe for phosphoATF2. Anisomycin (100nM for 30min) was used as a positive control.
curcumin (20μM); EGCG (20μM); hemin (30μM); helenalin (20μM); LY = LY294002 (50μM); SP = SP600125 (10μM); SB = SB203580 (A & D 10μM; E 1μM ); U0 = U0126 (30μM); wortmannin (0.1μM).

Figure 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 hrs 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 non-competitive (NC)
oligos. The presence of p65 in the lower band was determined by supershift. EMSAs were performed as described in Materials and Methods and are representative of at least 3 independent experiments.

B) Dose response for HO-1 induction by helenalin, with and without 20µM curcumin. Cells were pretreated for 3 hrs with the indicated doses of helenalin, before addition of curcumin and a further 6hrs incubation.

**Figure 4. Nuclear localization of Nrf2 in response to treatments.**

A) Breast cells were treated with indicated concentrations of inducing agent for 6hrs and expression of Nrf2 in nuclear extracts examined. B) Inhibitors were used in 30min pretreatments at the following concentrations: LY294002, 50µM; SB230580, 10µM; SP600125, 10µM; U0126, 30µM, followed by treatment with curcumin (20µM) for 4.5 hrs. C) Prior to treatment with EGCG (30µM) for the times indicated, inhibitors were used in 30min pretreatments at the following concentrations: LY294002, 50µM; SB230580, 1µM.

**Figure 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 hrs. B-D) Wild type and Raf−/− MEFs were treated with indicated concentrations of B) curcumin for 6hrs, C) hemin for 16hrs, or D) EGCG for 6hrs. E) Wild type and Raf−/− MEFs were treated with curcumin for 6hrs and nuclear extracts probed for Nrf2.

**Figure 6 Induction of apoptosis by helenalin and EGCG**

A) C143 lymphoblasts were treated with EGCG for 48 hrs and levels of apoptosis measured by annexin V binding. Percentages of viable, apoptotic and necrotic cells are indicated. B) Breast cells were treated with helenalin for 24hrs and levels of apoptosis measured as above.

* indicates significant difference from control (p<0.05, n=3) as determined by balanced ANOVA, followed by Tukey’s least significant difference test.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6