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Inhibition of hypoxia inducible factor by phenethyl isothiocyanate

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ABSTRACT

Phenethyl isothiocyanate (PEITC), a natural dietary isothiocyanate, has anti-cancer activity in various in vitro and in vivo models. PEITC inhibits angiogenesis but the molecular mechanisms that underlie this effect are not known. We have now demonstrated that PEITC is an effective inhibitor of hypoxia inducible factor (HIF), a transcription factor that plays an important role in expression of pro-angiogenic factors. PEITC inhibited the activation of a HIF-dependent reporter construct following incubation of cells in hypoxia, or treatment with the hypoxia mimetic cobalt chloride. PEITC also interfered with the accumulation of HIF1α protein and induction of the endogenous HIF target genes, CAIX, GLUT1, BNIP3 and VEGF-A. The ability of PEITC to inhibit HIF activity was independent of the activity of prolyl hydroxylases, the Von-Hippel–Landau protein and the proteasome, all of which are required for the normal rapid turnover of HIF1α in normoxia. Decreased expression of HIF1α in PEITC treated cells was not associated with changes in the levels of HIF1α RNA suggesting that PEITC may inhibit HIF activity by decreasing translation of the HIF1α RNA. Consistent with this, PEITC decreased phosphorylation of the translation regulator 4E-BP1. Our data demonstrate that PEITC is an effective inhibitor of HIF activity. This may contribute to the anti-angiogenic and anti-cancer effects of PEITC.

1. Introduction

Isothiocyanates (ITCs) are a group of structurally related compounds with potential chemopreventive and anti-cancer activity [1–3]. Natural ITCs derived from cruciferous vegetables have anti-cancer activity in both in vitro and in vivo models, and increased dietary consumption of ITCs has been linked to reduced cancer risk in humans [4]. ITCs are derived by hydrolysis of specific β-thioglucoside *N*-hydroxysulfate (glucosinolate) precursors by the action of the plant enzyme myrosinase, activated following damage to the leaf (e.g., chopping or chewing). For example, broccoli is a rich source of glucoraphanin, the glucosinolate precursor of sulforaphane (SFN) and watercress is a rich source of gluconasturtiin, the precursor of phenethyl isothiocyanate (PEITC).

The ability of ITCs to inhibit the development of carcinogen-induced cancers is perhaps the best understood activity of ITCs [5–7]. ITCs are thought to inhibit carcinogen-induced carcinogenesis by modulating carcinogen metabolism via (i) inhibiting the activity of phase I cytochrome P450 enzymes and (ii) inducing phase II detoxifying and antioxidant gene expression, including glutathione-*S*-transferases, NAD(P)H:quinine oxidoreductase, UDP-glucuronosyl transferase and thioredoxin reductase. Induction of phase II gene expression is mediated by the Nrf2 transcription factor [8–10]. In the absence of inducers, Nrf2 is inactivated by association with the cysteine-rich Keap1 protein which inhibits Nrf2, at least in part, by acting as a ubiquitin ligase adaptor protein, targeting Nrf2 for ubiquitylation and proteasomal degradation [11–13]. SFN and PEITC are potent activators of Nrf2 and this appears to be dependent on ITC-mediated electrophilic attack and conjugation of several "sensor" cysteines within Keap1 [14–18]. This is thought to release Nrf2 from Keap1-mediated negative regulation. Nrf2 then accumulates in the nucleus where, in concert with the small Maf protein, it binds to antioxidant/electrophile response elements in target genes to activate transcription. Activation of MAP kinase signalling pathways and phosphorylation of Nrf2 may also contribute [17]. The ability of SFN to interfere with benzo[*a*]pyrene-induced gastric tumour formation is significantly reduced in Nrf2 deficient mice, demonstrating the importance of this pathway for ITC-mediated protection from carcinogen-induced carcinogenesis [19].

In addition to their established chemopreventive activity, ITCs also exert activity against established cancer cells [20,21]. For example, PEITC inhibits the growth of PC3 prostate cancer cells, associated with the downregulation of the positive cell cycle regulators, cdk1 and cdc25C, and SFN induces metaphase arrest in pancreatic cancer lines [22,23]. ITCs promote mitochondrial damage and apoptosis in various cells types and PEITC enhances apoptosis in

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primary leukaemia cells [24]. ITCs have been demonstrated to slow the growth of various human cancer and oncogene-transformed cell lines when grown as xenografts in immunocompromised animals, and to suppress tumour formation in Apc-deficient immunocompetent animals [22,25–29].

The mechanisms by which ITCs inhibit the growth and survival of established cancer cells are likely to be complex. Similar to effects on Keap1, conjugation of ITCs to key cell regulatory proteins is thought to be important [20]. At early time points following addition to cells, ITCs are bound predominantly to glutathione (GSH), a major cellular antioxidant [30,31]. ITC–GSH conjugates are exported from the cell where hydrolysis of these conjugates leads to regeneration of ITCs. The regenerated ITCs are then taken back up into cells and the net effect of this cycle is a rapid accumulation of ITCs within cells (perhaps up to 100 times over the extracellular concentration) and depletion of intracellular GSH [20]. Once GSH levels are depleted, ITCs conjugate to cellular proteins [20,32]. Presumably conjugation to cell cycle and cell death regulatory proteins leads to altered function, triggering induction of cell cycle arrest and apoptosis. However, the critical targets involved in growth inhibition by ITCs are not known and to our knowledge the only protein other that Keap1 that has been identified as a direct target for ITCs is α-tubulin [33]. SFN has also been demonstrated to inhibit the activity of NF-κB and AP-1 transcription factors [34,35]. Although these transcription factors contain reactive thiols, the mechanism of inhibition, and the contribution of these effects to the cell growth inhibitory and apoptosis-promoting effects of ITCs is unclear. In addition to direct conjugation of cellular proteins by ITCs, metabolites of PEITC and SFN may also play an important role, for example, via inhibition of histone deacetylase activity [1]. A further potential mechanism by which ITCs promote cell growth inhibition is via increasing cellular stress. For example, GSH-depletion leads to an increase in intracellular reactive oxygen species (ROS) and in some systems, ITC-induced growth inhibition is suppressed by antioxidants [20,25].

Angiogenesis, the formation of new blood vessels, plays a critical role in tumour development and metastasis and is considered one of the six hallmarks of the cancer cell [36]. Early stage tumours can grow independent of angiogenesis up to a size of 1–2 mm^2, however, inadequate supply of oxygen and nutrients and accumulation of toxic metabolites limits growth beyond this size. Thus, the switch to a pro-angiogenic state is a critical step in cancer development [37]. The molecular mechanisms that promote angiogenesis in cancer cells are complex, however, the hypoxia inducible factor (HIF) family of oxygen-sensitive transcription factors play a key role. The best studied member is HIF1α which forms a transcriptionally active heterodimeric complex with HIF1β [38,39]. In normoxic conditions, the expression of HIF1α is maintained at very low levels since it is effectively ubiquitinated and targeted for rapid proteasomal degradation by the Von-Hippel–Landau (VHL) protein, a component of an E3 ubiquitin ligase complex. Recognition of HIF1α by the VHL complex in normoxia is mediated by hydroxylation of specific proline residues (HIF1α Pro^{402} and Pro^{564}) by oxygen-dependent, Fe-containing prolyl hydroxylases. When oxygen levels are sufficiently reduced prolyl hydroxylase activity is inhibited, and HIF1α is no longer modified and is stabilised. HIF1α then translocates to the nucleus where, as part of a dimeric complex with the constitutively expressed HIF1β protein, it modulates expression of specific target genes involved in the regulation of angiogenesis, e.g., vascular endothelial growth factor-A (VEGF-A), metabolism (glucose transporter type 1 (GLUT1); SLC2A1) and apoptosis/survival (BCL2/adenovirus E1B 19 kd-interacting protein (BNIP3)) [38,39].

The activity of HIF is also influenced by ROS [40]. Treatment of cells with hydrogen peroxide is sufficient to stabilise HIF1α and induce expression of HIF target genes, even in normoxia [41]. Moreover, HIF activation in normoxia and hypoxia is inhibited by antioxidants such as GSH, its metabolic precursor *N*-acetylcysteine (NAC), and catalase [42–47]. Both cytosolic and mitochondrial sources of ROS have been implicated in HIF activation [43,48–51].

Several studies have demonstrated that ITCs interfere with angiogenesis pathways. PEITC decreased survival of human umbilical vein endothelial cells (HUVEC) and inhibited the formation of capillary-like tube structures and migration by HUVEC in vitro [52]. PEITC also inhibited ex vivo angiogenesis analysed using the chicken egg chorioallantoic membrane assay. SFN inhibited migration of human dermal microvascular endothelial cells (HMEC1) in an in vitro wound healing assay and inhibited tube formation of HMEC-1 cells on basement membrane [53]. SFN also inhibited the growth of HMEC-1 cells and bovine aortic endothelial cells [53,54].

Angiogenesis plays a critical role early in tumour development, and its inhibition may play a major role in the chemopreventive/anti-cancer effects of ITCs. Since HIF plays a central role in angiogenesis, we have investigated the effects of PEITC on HIF activity.

2. Materials and methods

2.1. Cell culture and chemicals

Human MCF7 breast cancer cells were obtained from American Type Culture Collection (Manassas, VA, USA). Human RCC4 renal cell carcinoma cells were obtained from CR-UK Research Services (London, UK). All cell lines were maintained in Dulbecco's Modified Eagle's medium (DMEM; Lonza group Ltd., Basel, Switzerland) supplemented with 10% (v/v) fetal calf serum (PAA Laboratories, Yeovil, UK), 1 mM L-glutamine and penicillin/streptomycin (Lonza group Ltd.). PEITC, NAC, catalase, Trolox, cycloheximide, desferrioxamine (DFO) and cobalt chloride ($CoCl_2$) were from Sigma (Poole, UK). MG132 was from Enzo Life Sciences (UK) Ltd., Exeter, UK. To induce hypoxia, cells were cultured in 5% CO_2, 94.9% Argon, 0.1% O_2 in a sealed chamber.

2.2. Cell growth inhibition and apoptosis assays

MCF7 cells were plated at a density of 1000 cells per well of a 96-well plate in 50 μl complete growth media. RCC4 cells were plated at a density of 5000 cells per well of a 96-well plate in 50 μl complete growth media. The following day cells were treated in triplicate with PEITC or dimethyl sulfoxide (DMSO) as a solvent control, or were left untreated. DMSO was used at a dilution equivalent to the highest concentration of PEITC tested in each assay. After 6 days, relative cell number was determined using the CellTiter 96® AQ_{ueous} One Solution Reagent (Promega, Southampton, UK) according to the manufacturer's instructions. Relative cell number was calculated as a percentage of untreated cells. Apoptosis was analysed using the fluorescein isothiocyanate (FITC) annexin V apoptosis detection kit II (BD Pharmingen, Oxford, UK) according to the manufacturer's instructions.

2.3. Reporter gene assays

MCF7 cells (grown in a 10 cm tissue culture dish) were transfected with 4 μg of the HIF-dependent luciferase reporter construct pGL2-TK-HRE [55] (a kind gift of Giovanni Melillo, Tumor Hypoxia Laboratory, NCI, USA) or pGL3-promoter (Promega) using Transfast (Promega) according to the manufacturer's instructions. After 24 h, cells were recovered and plated in 96-well plates (2000 cells/well). Cells were incubated for 5 h to allow cells to adhere before being treated to activate HIF in the presence or

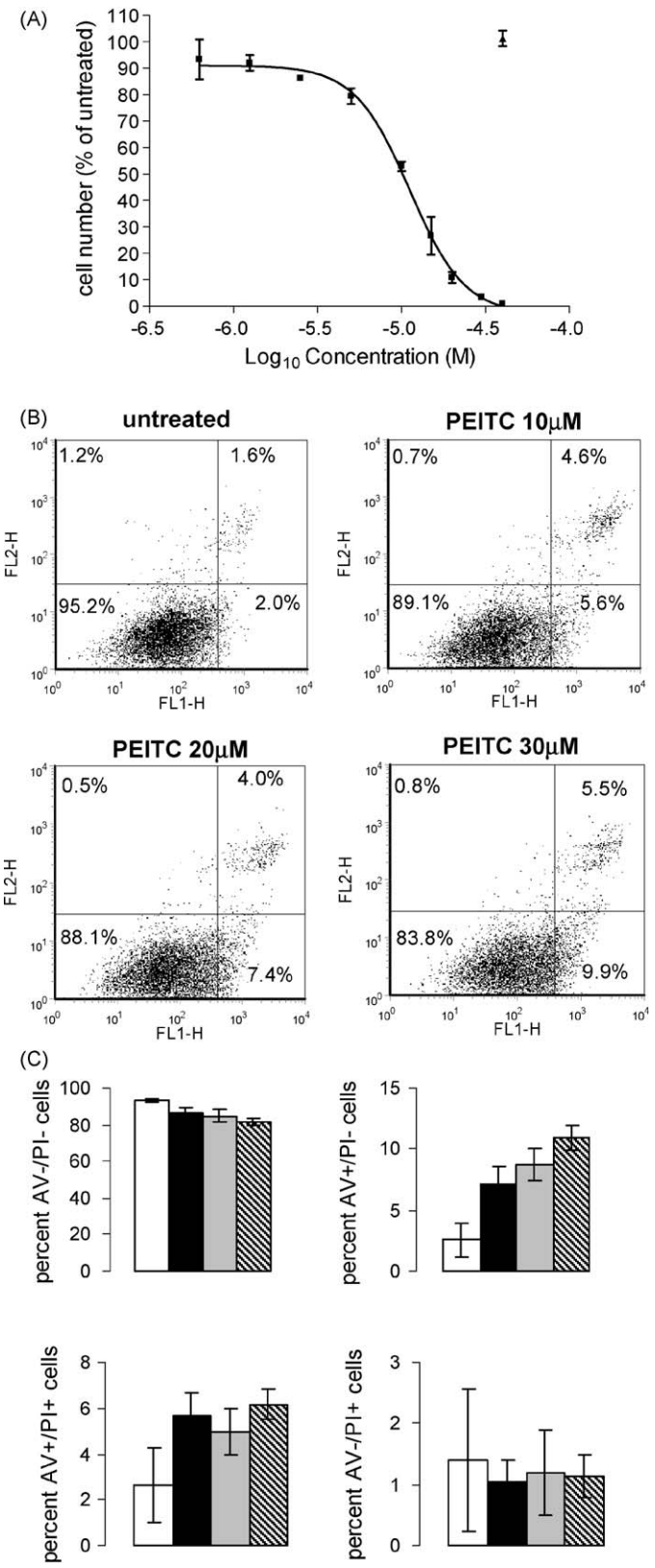

Fig. 1. PEITC-induced growth inhibition and apoptosis in MCF7 cells. (A) Representative growth inhibition experiments. MCF7 cells were incubated with the indicated concentrations of PEITC (■), or DMSO at a dilution equivalent to the highest concentration of PEITC (▲). After 6 days, relative cell numbers were determined using the CellTiter 96® AQ_{ueous} One Solution reagent. Results are derived from means of triplicate wells (±SD). (B) MCF7 cells were incubated with the indicated concentrations of PEITC for 24 h. The proportion of annexin V (FL1-H) and propidium iodide (PI; FL2-H) positive cells was determined by flow cytometry. A representative experiment is shown. (C) Quantitation of apoptosis in PEITC-treated MCF7 cells (24 h). AV (annexin V), PI (propidium iodide). Data are mean ± range derived from two independent experiments. Untreated cells (open bars); 10 μM PEITC (closed bars); 20 μM PEITC (grey bars); 30 μM PEITC (hatched bars).

absence of PEITC or other agents. In experiments using NAC, Trolox or catalase, cells were pretreated with 10 mM NAC, 100 μM Trolox or 2000 units catalase for 2 h prior to induction of HIF. Luciferase activity was measured after 24 h by addition of 100 μl of BrightGlow (Promega) according to the manufacturer's instructions.

2.4. Quantitative-reverse transcription-polymerase chain reaction (Q-RT-PCR)

Total RNA was isolated using Trizol (Invitrogen, Paisley, UK) and the quantity and quality of RNA was analysed using an Agilent 2100 Bioanalyser (Agilent Technologies Inc., South Queensferry, UK). cDNA was synthesised using oligo(dT) and MMLV reverse transcriptase (Promega) according to the manufacturer's instructions. cDNA was synthesised in a 25 μl reaction containing 1 μg of total RNA, oligo(dT) primer and MMLV reverse transcriptase, then diluted to 100 μl using nuclease free water. Q-RT-PCR was performed in 20 μl reactions containing 5 μl cDNA, 10 μl Universal Taqman PCR master mix (Applied Biosystems, Warrington, UK) and 1 μl of the Taqman Gene Expression Assay of interest (Applied Biosystems). Expression assays used for this study were: BNIP3 (Hs00969293_mH), VEGF-A (Hs00173626_m1), CAIX (Hs00154208_m1), GLUT1 (Hs00892681_m1) and β-actin (Hs99999903_m1). All reactions were performed in duplicate using the ABI PRISM 7500 Sequence Detection System (Applied Biosystems) according to the following thermal cycle protocol: 94 °C 10 min, followed by 40 cycles of 94 °C for 15 s and 60 °C for 1 min. Control reactions with no cDNA were run on each plate for each Taqman gene Expression Assay used and no amplification was detected in any control reaction. All expression values were normalised using expression of β-actin as a control.

2.5. Western immunoblotting

Immunoblots were performed as previously described [56] using a mouse monoclonal anti-HIF1α antibody (BD Biosciences, Oxford, UK), a mouse monoclonal anti-HIF2α antibody (Abcam, Cambridge, UK), a rabbit polyclonal anti-4E-BP1 antibody (Cell Signalling Technology, Danvers, MA, USA) and a rabbit anti-β-actin antibody (Sigma). Horseradish peroxidase conjugated secondary antibodies were from GE Healthcare UK (Amersham, UK) and bound immunocomplexes were detected using SuperSignal West Pico Chemiluminescent reagents (Perbio Science UK Ltd., Northumberland, UK).

2.6. Statistics

The statistical significance of any differences was analysed using Student's *t*-test with Bonferroni correction to correct for multiple testing.

3. Results

3.1. Growth inhibition and induction of apoptosis by PEITC in MCF7 cells

We selected human MCF7 breast cancer cells to investigate the effects of PEITC on HIF since their response to hypoxia has been well characterised [57,58]. We first characterised the effects of PEITC on the growth and survival of these cells. Consistent with

previous studies demonstrating growth inhibitory effects of PEITC in MCF7 cells [59,60], PEITC inhibited MCF7 cell growth with an IC_{50} of 10.8 ± 1.7 μM (mean ± SD) in 6 day assays (Fig. 1A). PEITC also increased the percentage of Annexin V positive cells (a marker of apoptosis), although overall the levels of PEITC-induced apoptosis in MCF7 cells were relatively modest (Fig. 1B and C).

3.2. PEITC inhibits HIF-dependent transcriptional activity

To investigate the effects of PEITC on HIF activity, we first analysed the effects of PEITC on activity of a HIF-dependent reporter construct [55]. As a control we also analysed the activity of the SV40-promoter based reporter plasmid pGL3-promoter. Cells were transfected with the HIF or control reporter constructs and cultured under normoxic or hypoxic (0.1% O_2) conditions, in the presence of increasing amounts of PEITC or DMSO as a solvent control.

As expected, hypoxia caused a strong induction of the HIF reporter construct, whereas the activity of the control construct was modestly decreased under hypoxic conditions (Fig. 2A). PEITC caused a statistically significant dose dependent decrease in the activity of the HIF reporter in cells under hypoxic conditions with an IC_{50} of ~3 μM. There was also a trend for PEITC to reduce basal activity of the HIF reporter in cells under normoxic conditions. (Although this is not evident from the data shown in Fig. 2A, due to the strong induction of reporter gene activity by hypoxia, it is clearly shown in subsequent experiments using $CoCl_2$ which gives a lower level of HIF reporter activation, Fig. 2B.) These effects were specific at PEITC concentrations up to 13 μM, since the activity of the control reporter construct was unaltered. However, in some experiments 26 μM PEITC reduced activity of the control reporter under both normoxic and hypoxic conditions (see Fig. 2B). This presumably reflects non-specific inhibition at this higher concentration.

To confirm these findings, we investigated the effects of PEITC on the ability of $CoCl_2$, a well studied hypoxic mimetic, to activate HIF in normoxic conditions. Similar to hypoxia, $CoCl_2$ increased expression of the HIF reporter construct, with little effect on the control reporter construct, and this was inhibited in a dose dependent manner by increasing concentrations of PEITC (Fig. 2B).

3.3. PEITC inhibits induction of HIF target genes

Activation of HIF is associated with increased expression of HIF target genes. To determine the effect of PEITC on HIF target genes, MCF7 cells were exposed to hypoxia for 16 h in the presence or absence of PEITC and expression of CAIX, VEGF-A, BNIP3 and GLUT1 were analysed by Q-RT-PCR. CAIX was relatively strongly induced by hypoxia (50-fold) and this was statistically significantly inhibited by PEITC in a dose dependent manner (Fig. 3A). PEITC also caused a statistically significant inhibition of the induction of BNIP3, VEGF-A and GLUT1, although these genes were less strongly induced by hypoxia alone (3–5 fold) and were less sensitive to the inhibitory effects of PEITC. PEITC also inhibited the induction of CAIX expression following treatment of MCF7 cells with $CoCl_2$ under normoxic conditions (Fig. 3B).

3.4. PEITC inhibits accumulation of HIF1α and HIF2α

To investigate the mechanism by which PEITC inhibited HIF-dependent transcription, we first analysed the effects of PEITC on

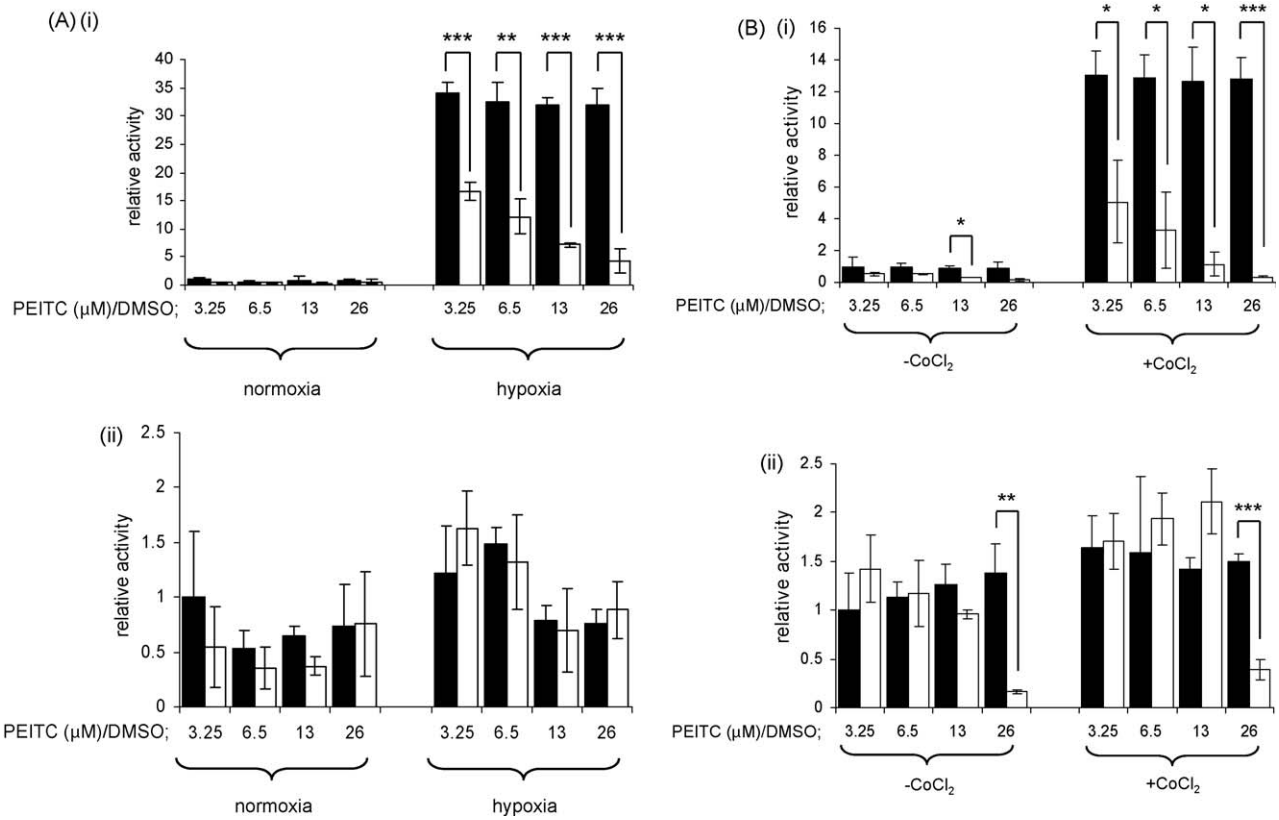

Fig. 2. Regulation of HIF-dependent transcription by PEITC. MCF7 cells were transfected with (i) pGL2-TK-HRE or (ii) control pGL3-promoter reporter constructs and treated with the indicated concentrations of PEITC (open bars) or equivalent amounts of DMSO as a solvent control (closed bars). HIF activity was induced by incubating cells (A) in hypoxic conditions or (B) treating cells with $CoCl_2$ (100 μM). Luciferase activity was analysed after 24 h. Data shown are means of triplicate determinations (±SD) and are representative of two individual experiments. Statistically significant differences between DMSO and PEITC treated cells are indicated (*$p < 0.05$; **$p < 0.01$; ***$p < 0.005$). All other differences were not statistically significant.

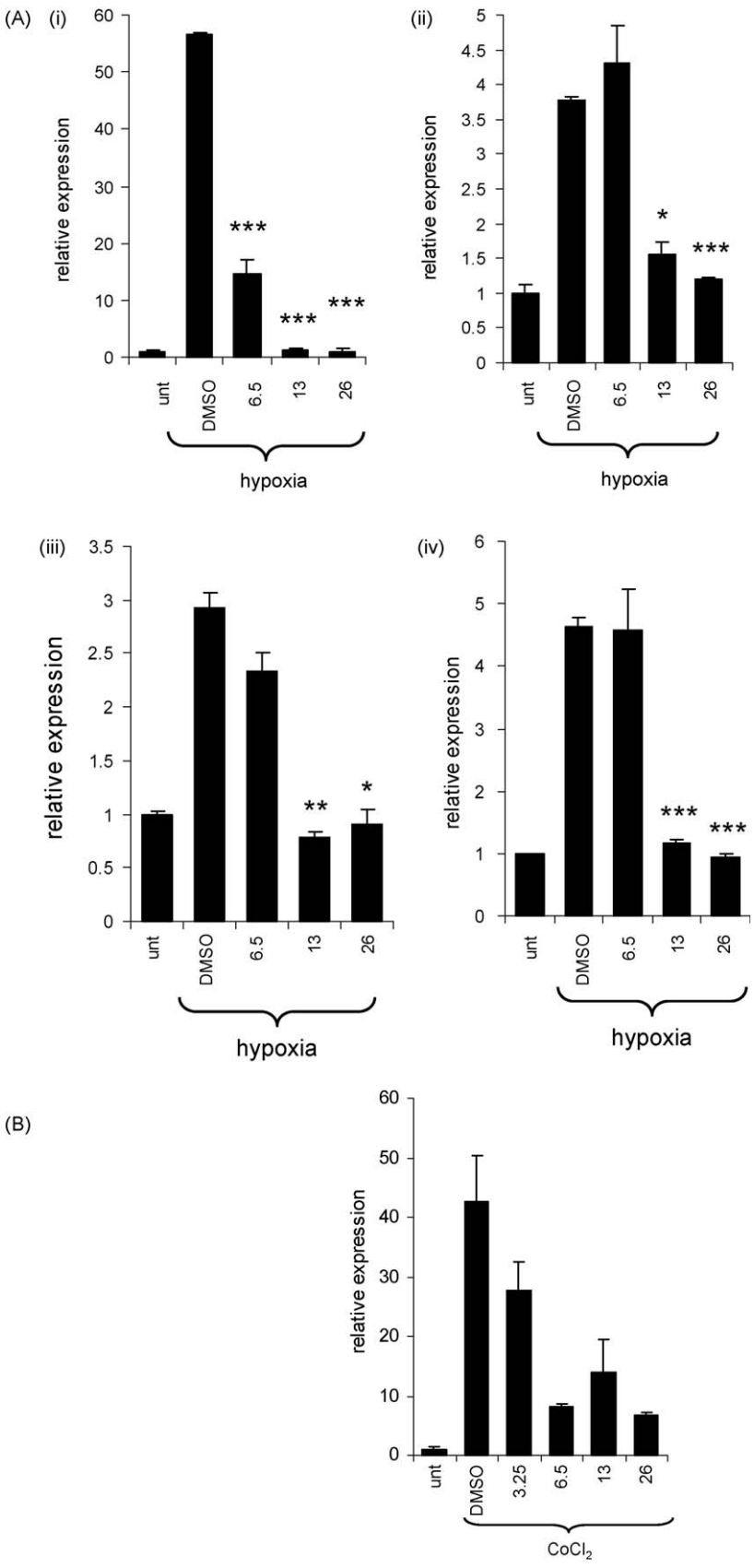

Fig. 3. Regulation of endogenous HIF target genes. (A) MCF7 cells were left untreated (unt) as a control or incubated in hypoxic conditions for 16 h in the presence or absence of the indicated concentrations of PEITC (μM), or DMSO as a control. Expression of (i) CAIX, (ii) VEGF-A, (iii) GLUT1 and (iv) BNIP3 were analysed by Q-RT-PCR. (B) MCF7 cells were left untreated (unt) as a control or treated with $CoCl_2$ (100 μM) for 24 h in the presence or absence of the indicated concentrations of PEITC (μM), or DMSO as a control. Expression of CAIX was analysed by Q-RT-PCR. In both experiments, the amount of DMSO used was equal to that in cells treated with 26 μM PEITC. Data are mean of duplicate determinations, normalised to expression of β-actin. Relative expression in untreated cells was set to 1.0. Statistically significant differences between DMSO and PEITC treated cells are indicated ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$). All other differences were not statistically significant.

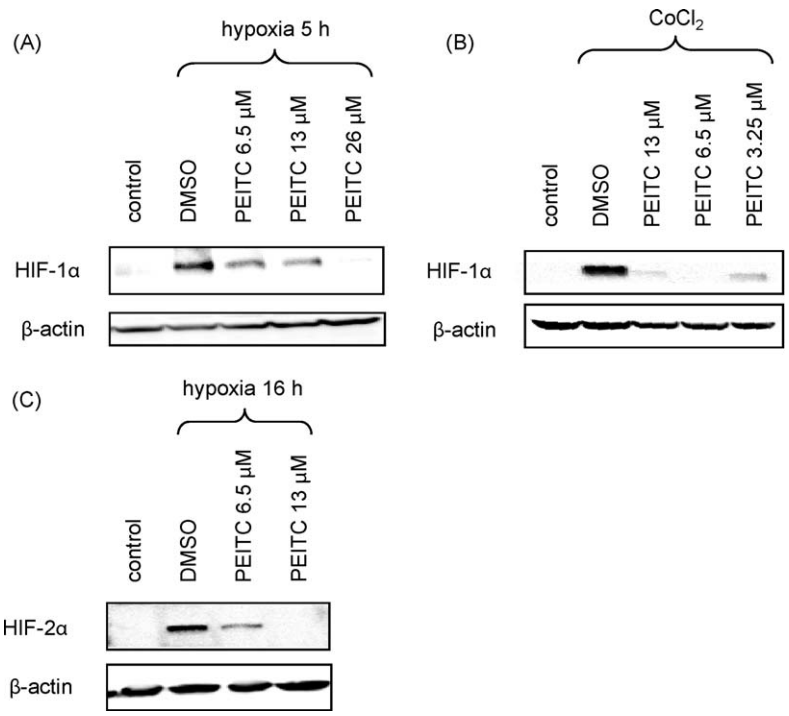

Fig. 4. Effect of PEITC on HIF1α and HIF2α protein expression. MCF7 cells were left untreated (control) or incubated in hypoxic conditions for 5 h (A) or 16 h (C) in the presence or absence of the indicated concentrations of PEITC, or DMSO (equivalent to 26 μM PEITC). (B) MCF7 cells were left untreated as a control or incubated with $CoCl_2$ (100 μM) for 24 h in the presence or absence of the indicated concentrations of PEITC (μM), or DMSO as a control (equivalent to 13 μM PEITC). Expression of HIF1α, HIF2α and β-actin as a loading control were analysed by immunoblotting.

accumulation of HIF1α in cells cultured under hypoxic conditions. MCF7 cells were exposed to hypoxia for 5 h in the presence or absence of PEITC (Fig. 4A). As expected, HIF1α protein levels were barely detectable in cells in normoxia, but were strongly increased in cells exposed to hypoxia, due to inhibition of proteasomal degradation. Consistent with the ability of PEITC to inhibit HIF-dependent transcription, PEITC decreased the accumulation of HIF1α. Similar results were obtained when HIF1α levels were increased in normoxia by treating cells with $CoCl_2$ (Fig. 4B).

In addition to HIF1α and HIF1β, the HIF family of transcription factors also contains HIF2α and HIF3α, which like HIF1α, are also induced by hypoxia and form transcriptionally active heterodimers with HIF1β [61,62]. Although the function of HIF3α has not been well characterised, HIF2α also appears to play a role in angiogenesis and carcinogenesis, and may regulate the expression of distinct, but overlapping, set of target genes compared to HIF1α [61,62]. Like HIF1α, HIF2α is targeted for rapid proteasomal degradation in normoxic conditions via the action of VHL and prolyl hydroxylases [63,64]. To investigate the effects of PEITC on HIF2α, MCF7 cells were exposed to hypoxia for 16 h in the presence or absence of PEITC (Fig. 4C). We selected the 16 h time point because accumulation of HIF2α was relatively slow, but was maintained for a protracted period in MCF7 cells compared to HIF1α. Differences in kinetics of activation of HIF1α and HIF2α have been reported in other cell types [65,66]. Similar to HIF1α, PEITC strongly decreased the accumulation of HIF2α.

3.5. Inhibition of HIF activity by PEITC is independent of prolyl hydoxylases

Ubiquitylation of HIF1α is dependent on the activity of prolyl hydroxylases and inhibition of these enzymes causes accumulation of HIF1α under normoxic conditions. To determine whether inhibition of HIF1α by PEITC required activity of prolyl hydroxylases, we investigated whether PEITC could also inhibit activation of HIF following prolyl hydroxylase inhibition. MCF7 cells were transfected with the HIF and control reporter constructs and treated with PEITC in the presence or absence of the prolyl hydroxylase inhibitor DFO. DFO inhibits prolyl hydroxylases by chelating the Fe atom that is present within the active site of these enzymes. As expected, DFO caused a strong induction in HIF reporter gene activity under normoxic conditions (Fig. 5). Treatment with PEITC caused a statistically significant and dose dependent reduction of HIF-dependent activity. Therefore, inhibition of HIF1α activity by PEITC is not dependent on the activity of prolyl hydroxylases.

3.6. Inhibition of HIF activity by PEITC is independent of VHL and the proteasome

The VHL protein plays a critical role in targeting HIF1α for proteasomal degradation downstream of prolyl hydroxylases. To determine whether the effects of PEITC were dependent on VHL, we analysed the activity of PEITC in VHL-deficient RCC4 renal carcinoma cells that have constitutive HIF1α activity in normoxia [67]. PEITC inhibited the growth of RCC4 cells with an IC_{50} of 44.0 ± 2.0 (mean ± SD). Therefore these cells are somewhat less sensitive to the growth inhibitory effects of PEITC compared to MCF7 cells. In RCC4 cells, PEITC caused a loss of HIF1α expression (Fig. 6A), decreased activity of the HIF reporter construct (Fig. 6B) and decreased expression of CAIX RNA (Fig. 6C), although somewhat higher concentrations of PEITC were required compared to MCF7 cells.

To determine whether the effects of PEITC were dependent on the proteasome, we analysed whether PEITC interfered with stabilisation of HIF1α by the proteasome inhibitor MG132 under normoxic conditions. Treatment of MCF7 cells with MG132 caused a strong increase in HIF1α expression (Fig. 7). However, this was still effectively reversed by PEITC, as well as the translation inhibitor, cycloheximide. Therefore, the ability of PEITC to decrease HIF1α expression does not require proteasome activity. Interestingly, a modest "recovery" of HIF1α expression was observed in

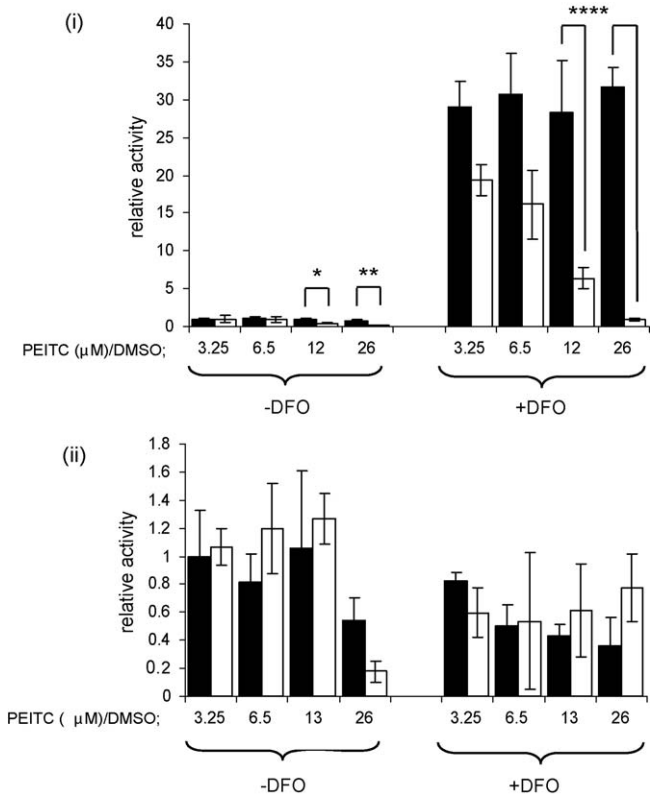

Fig. 5. Effect of PEITC on prolyl hydroxylase inhibitor-induced HIF activity. MCF7 cells were transfected with (i) pGL2-TK-HRE or (ii) control pGL3-promoter reporter constructs and treated with the indicated concentrations of PEITC (open bars) or equivalent amounts of DMSO as a solvent control (closed bars). HIF activity was induced by addition of DFO (100 μM). Luciferase activity was determined after 24 h. Data shown are means of triplicate determinations (±SD) and are representative of two individual experiments. Statistically significant differences between DMSO and PEITC treated cells are indicated ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$). All other differences were not statistically significant.

cells treated with MG132 and higher concentrations (30 μM) of PEITC, compared to cells treated with MG132 and lower concentrations of PEITC (Fig. 7). However, it is important to note that all concentrations of PEITC very effectively decreased HIF1α expression (by at least 80% compared to MG132/DMSO treated cells) and that PEITC at 30 μM was still as effective of cycloheximide.

3.7. Inhibition of HIF activity by PEITC is partially reversed by NAC

Treatment of cells with PEITC leads to a depletion of intracellular GSH and accumulation of ROS [20]. Enhancing cellular GSH levels by treatment with GSH or its metabolic precursor NAC can interfere with PEITC-induced growth inhibition [25,31,68]. Thus, we wanted to determine whether NAC also interfered with inhibition of HIF activity by PEITC. Activation of HIF is frequently inhibited by antioxidants [42–47] and consistent with this, pre-treatment of cells with NAC caused a modest but statistically significant ($p < 0.005$) reduction in HIF activity in hypoxic conditions. However, NAC significantly reversed the ability of PEITC to inhibit HIF activity (Fig. 8A).

The ability of NAC to counter the effects of PEITC could be due its activity as an antioxidant. By contrast, NAC, or its cellular product GSH could decrease PEITC levels by direct conjugation [30,31]. Therefore, to investigate further the potential role of ROS in inhibition of HIF activity by PEITC, we analysed the effects of Trolox and catalase, two non-thiol based antioxidants that would not conjugate with PEITC. Pre-treatment with Trolox alone did not inhibit HIF activity whereas pre-treatment with catalase, like NAC, caused a statistically significant ($p < 0.001$) reduction in HIF activity in hypoxic conditions (Fig. 8B and C). However, Trolox did not counter the ability of PEITC to inhibit HIF activity at any concentration, and catalase actually enhanced the ability of a lower concentration of PEITC (3.25 μM) to inhibit HIF activity. Thus, although NAC partially interferes with inhibition of HIF activity by PEITC, this appears to be due to direct conjugation of NAC/GSH to PEITC, rather than an antioxidant activity.

3.8. PEITC does not alter expression of HIF1α RNA

Although stabilisation of HIF1α plays a major role in the induction of HIF1 activity in hypoxic cells, HIF1α RNA transcription and translation are also subject to tight regulation [69]. We therefore analysed the effects of PEITC on the levels of HIF1α RNA (Fig. 9A). The expression of HIF1α RNA was not increased by hypoxia (even following protracted times – 24 h) and PEITC did not alter expression of HIF1α RNA. Similar results were obtained in $CoCl_2$ treated cells. Therefore, transcription of HIF1α RNA is not altered by PEITC.

3.9. PEITC decreases 4E-BP1 phosphorylation in MCF7 and RCC4 cells

Since PEITC did not effect HIF1α degradation or transcription it is likely that PEITC interferes with translation of HIF1α RNA. The 5′-untranslated region (5′-UTR) of the HIF1α RNA is highly structured and, like other RNAs with this feature, its translation is tightly dependent on the activity of the eIF4E translation factor [69]. eIF4E activity is regulated by binding to 4E-BP proteins (of which 4E-BP1 is the most prominent family member) and phosphorylation of 4E-BP1 prevents its interaction with eIF4E and therefore allows efficient translation of RNAs with complex 5′-UTRs, such as HIF1α. Therefore, inhibition of 4E-BP1 phosphorylation may contribute to the ability of PEITC to downregulate HIF1α expression. We therefore investigated the effects of PEITC on 4E-BP1 phosphorylation in MCF7 and RCC4 cells (Fig. 9B and C). In control cells, we detected multiple forms of 4E-BP1, consistent with the presence of phosphorylation [70]. When cells were treated with PEITC, the overall levels of 4E-BP1 were not substantially altered, but there was a clear loss of the more slowly migrating, phosphorylated isoforms. Therefore, PEITC treatment causes a loss of 4E-BP1 phosphorylation in MCF7 and RCC4 cells.

4. Discussion

Substantial evidence demonstrates that ITCs, such as PEITC, can exert an anti-cancer activity in diverse in vitro and in vivo models [1–3,20,21]. Consistent with this, dietary intake of cruciferous vegetables and urinary excretion of ITC metabolites has been associated with reduced cancer risk in multiple epidemiological studies [4]. Angiogenesis plays a key role in cancer development and is an attractive target for the development of novel anti-cancer therapies. For example, the VEGF neutralising antibody Bevacizumab is approved for treatment of metastatic colorectal cancer. PEITC and SFN reduce angiogenesis [52–54] and this effect may play an important role in the chemopreventive/anti-cancer effects of ITC.

Here, we have demonstrated that PEITC is a potent inhibitor of HIF, a master regulator of hypoxic responses. PEITC acts, at least in part, to prevent the accumulation of HIF1α and HIF2α protein in hypoxic cells. HIF1α is effectively destabilised by the prolyl hydroxylase/VHL/proteasome pathway in normoxia. However, this is not required for PEITC to reduce HIF1α expression since PEITC was effective in VHL-deficient cells, and PEITC could inhibit HIF activity induced directly by inhibition of prolyl hydroxylases or the

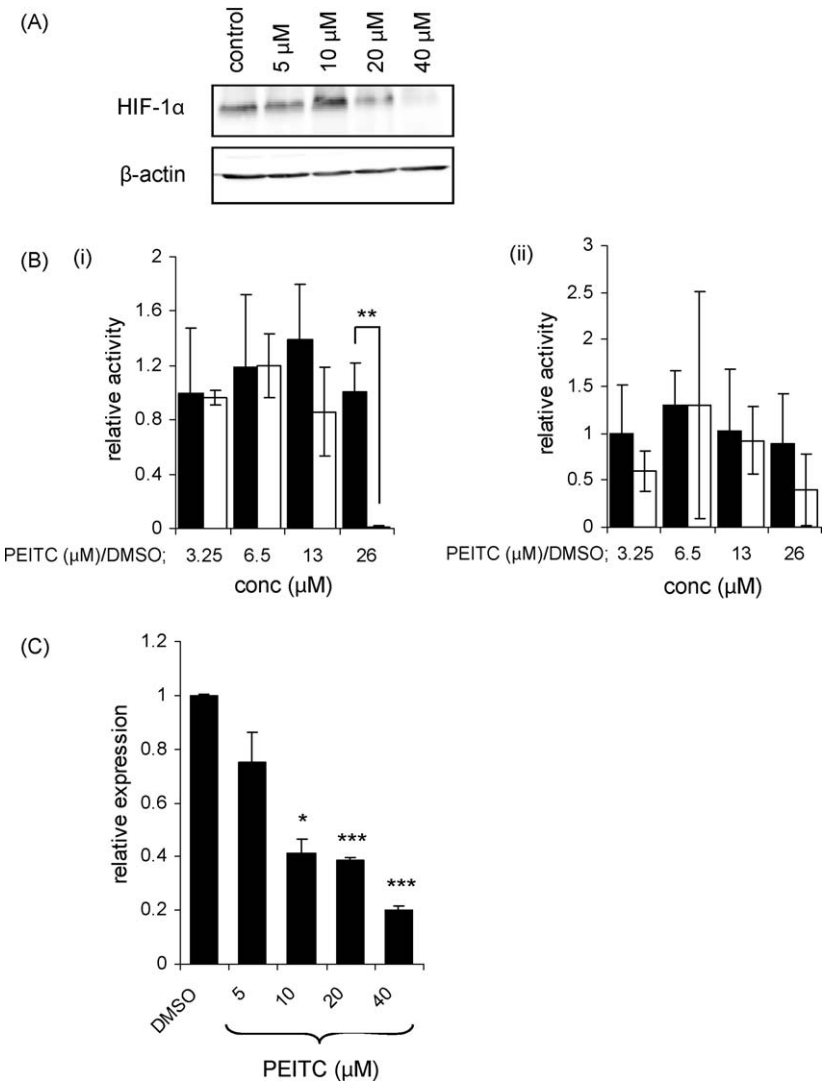

Fig. 6. Effect of PEITC on HIF activity and HIF1α expression in VHL-deficient RCC4 cells. (A) RCC4 cells were left untreated as a control or incubated with the indicated concentrations of PEITC (μM) for 24 h. Expression of HIF1α and β-actin were analysed by immunoblotting. (C) RCC4 cells were transfected with (i) pGL2-TK-HRE or (ii) control pGL3-promoter reporter constructs and treated with the indicated concentrations of PEITC (open bars) or equivalent amounts of DMSO as a solvent control (closed bars). Luciferase activity was measured after 24 h. Data shown are means of triplicate determinations (±SD) and are representative of two individual experiments. (D) RCC4 cells were treated with the indicated concentrations of PEITC (μM) or DMSO as a control (equivalent to 40 μM PEITC). After 24 h, expression of CAIX was analysed by Q-RT-PCR. Data are mean of duplicate determinations, normalised to expression of β-actin. Relative expression in untreated cells was set to 1.0. In (C) and (D), statistically significant differences between DMSO and PEITC treated cells are indicated ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$). All other differences were not statistically significant.

proteasome in normoxia. Moreover, PEITC did not alter expression of HIF1α RNA following hypoxia or treatment of cells with $CoCl_2$. Thus, PEITC appears to interfere with translation of HIF1α RNA. Recent data demonstrate that SFN, which also has anti-angiogenic activity, may also inhibit HIF activity by acting on HIF1α RNA translation in human tongue squamous cancer and prostate cancer cell lines [71]. By contrast, a reduction of HIF1α RNA levels by SFN was reported in human microvascular endothelial cells [53].

Although inhibition of HIF1α translation appears to be one mechanism by which PEITC interferes with HIF activity, it is possible that PEITC may act via multiple mechanisms. In some experiments (e.g., see Figs. 4B and 7) lower concentrations of PEITC appeared to be more effective than higher concentrations in decreasing HIF1α expression, although HIF activity remained effectively inhibited. This suggests that there may be complex, concentration-dependent effects of PEITC on HIF and further work is required to determine whether inhibition of HIF1α translation is the only mechanism by which PEITC targets HIF activity. Notably, the C-terminal transactivation domain of HIF1α contains a redox

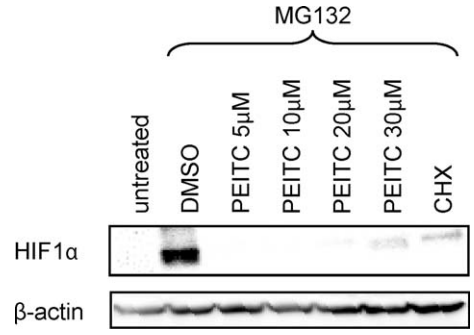

Fig. 7. Effect of PEITC on proteasome inhibitor-induced HIF activity. MCF7 cells were left untreated, or treated with DMSO or the indicated concentrations of PEITC or cycloheximide (10 μg/ml) in the presence of MG132 (25 μM) for 1 h. Expression of HIF1α and β-actin were determined by immunoblotting.

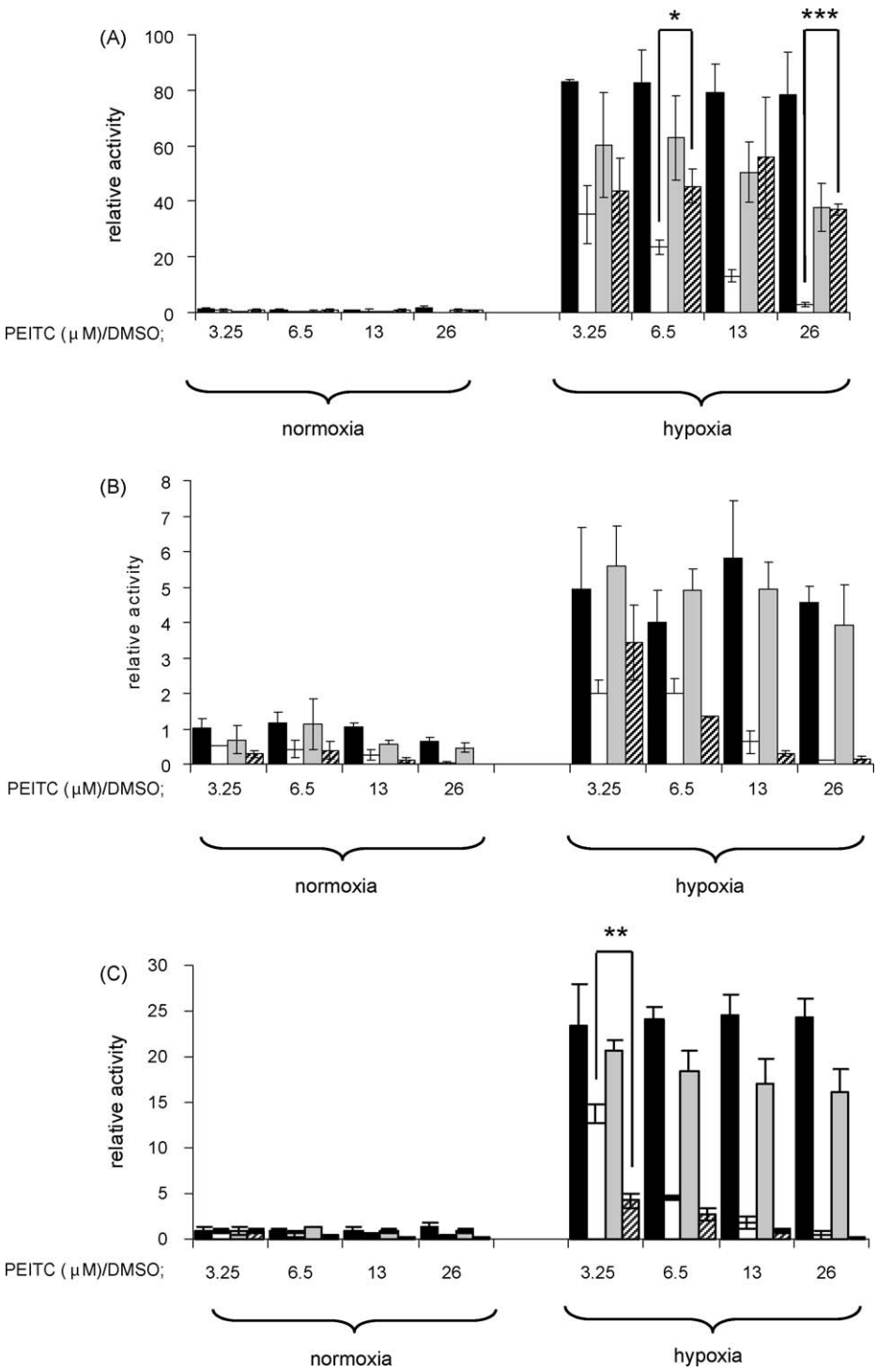

Fig. 8. Effect of NAC, Trolox and catalase on inhibition of HIF activity by PEITC. (A) MCF7 cells were transfected with the pGL2-TK-HRE reporter construct. Cells were then pretreated with NAC for 2 h or left untreated before being treated with the indicated concentrations of PEITC or equivalent amounts of DMSO as a solvent control. HIF activity was induced by incubating cells in hypoxic conditions for 24 h prior to analysis of luciferase activity. (B) As in (A), except cells were pretreated with Trolox (100 μM) for 2 h instead of NAC. (C) As in (A), except cells were pretreated with catalase (2000 units) for 2 h instead of NAC. Data shown are means of triplicate determinations (±SD) and are representative of two individual experiments. In all graphs; DMSO (closed bars), PEITC (open bars), antioxidant and DMSO (grey bars), antioxidant and PEITC (hatched bars). Statistically significant differences between PEITC and PEITC + antioxidant treated cells are indicated (*$p < 0.05$; **$p < 0.01$; ***$p < 0.005$). All other comparisons between PEITC and PEITC + antioxidant treated cells were not statistically significantly different.

regulated cysteine residue that is essential for HIF activity [72] and this may be targeted directly by PEITC.

Growth inhibitory effects of ITCs may involve direct conjugation and functional modification of key cell regulatory proteins and/or via increased ROS production following depletion of intracellular GSH [25,31,68]. ROS generally promote activation of HIF [41–47] and consistent with this, we demonstrated that NAC and catalase decreased induction of HIF activity in hypoxic conditions. However, only NAC, which contains a free thiol, reversed the ability of PEITC to inhibit HIF activity, whereas the non-thiol based antioxidants catalase and Trolox either had no effect, or enhanced the inhibitory effects of PEITC. These data

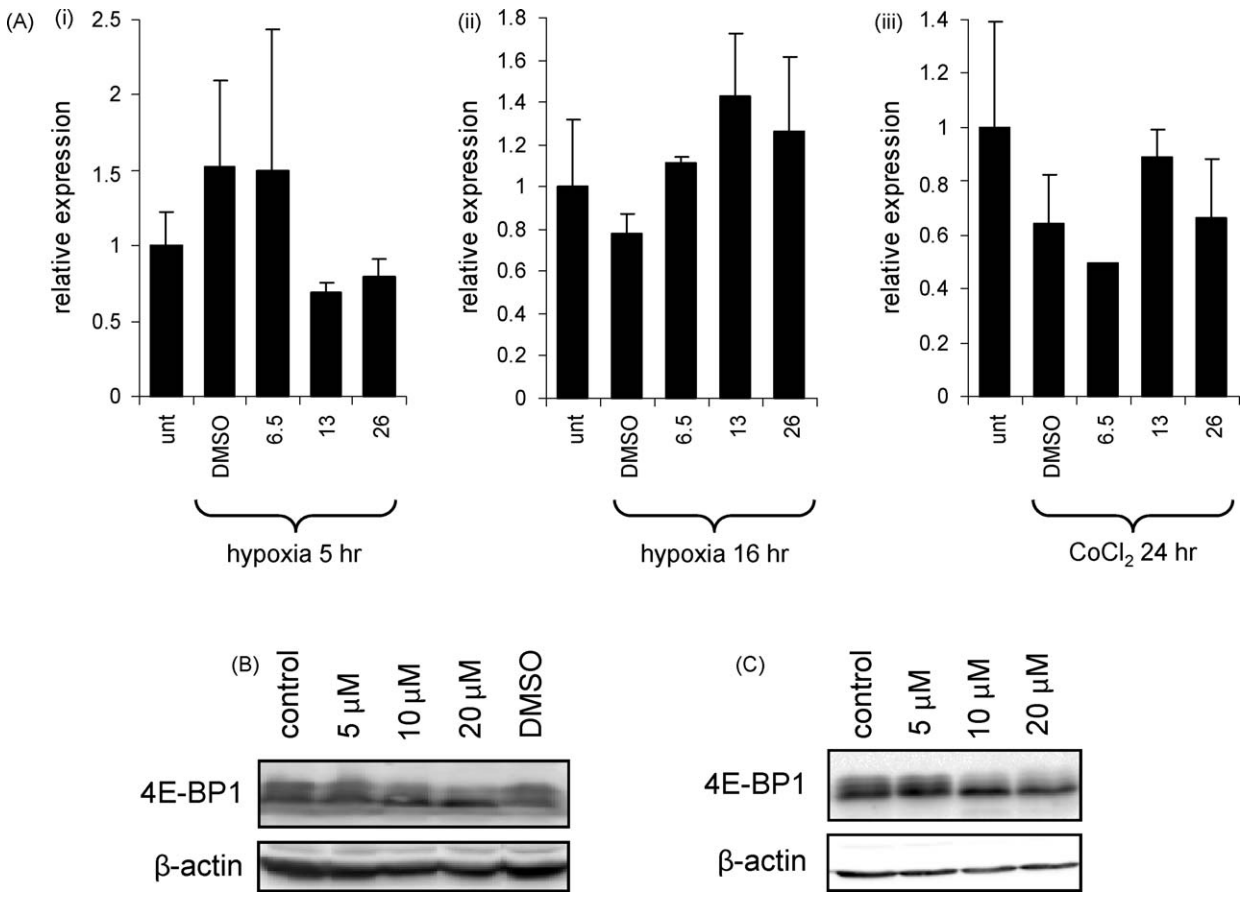

Fig. 9. Effect of PEITC on HIF1α RNA expression and 4E-BP1 phosphorylation. (A) MCF7 cells were left untreated or treated with DMSO (equivalent to 26 μM PEITC) or the indicated concentrations (μM) of PEITC. The expression of HIF1α RNA was determined after incubation for (i) 5 or (ii) 16 h in hypoxic conditions, or (iii) treatment with $CoCl_2$ (100 μM) for 24 h. Data are mean of duplicate determinations, normalised to expression of β-actin. Relative expression in untreated cells was set to 1.0. There were no statistically significant differences between PEITC and DMSO treated cells under any condition. (B) MCF7 and (C) RCC4 cells were left untreated as a control or incubated with the indicated concentrations of PEITC, or with DMSO, for 24 h. Expression of 4E-BP1 and β-actin were analysed by immunoblotting.

suggest that inhibition of HIF activity by PEITC does not directly involve modulation of intracellular ROS but rather may be due to conjugation of ITCs to cellular protein(s) involved in controlling HIF activity. Thus, we believe the inhibitory effects of NAC are due to its ability to protect cellular proteins from electrophilic attack, rather than acting to limit ROS *per se*. NAC may act directly as a "sink" to conjugate PEITC, or as a metabolic precursor to maintain cellular GSH levels.

PEITC also prevented the accumulation of HIF2α. HIF2α is closely related to HIF1α and is also induced by hypoxia [62]. However, there are important differences between HIF1α and HIF2α, both in terms of their regulation by hypoxia and in their function [62]. Knock-out experiments demonstrate that HIF1α and HIF2α are non-redundant, but both play roles in vascularisation in vivo, and HIF1α and HIF2α may target overlapping, but distinct sets of target genes. Interestingly, HIF2α appears to play a dominant role in growth/survival in VHL-defective renal cell carcinoma [73–75] and expression of HIF2α in the absence of HIF1α in VHL-deficient renal cell carcinoma is associated with elevated MYC activity [76].

A key question is how PEITC inhibits translation of HIF1α RNA. The 5′-untranslated region (5′-UTR) of the HIF1α RNA is highly structured and, like other RNAs with this feature, its translation is tightly dependent on the activity of the eIF4E translation factor [69]. eIF4E is an mRNA cap-binding protein that mediates the binding of the eIF4F complex to the 5′-cap structures of mRNA. eIF4E activity is regulated at multiple levels including phosphorylation and binding to 4EBP proteins (of which 4E-BP1 is the most prominent family member). 4E-BP1 is itself regulated by phosphorylation; 4E-BP1 phosphorylation prevents its interaction with eIF4E and therefore allows efficient translation of RNAs with complex 5′-UTRs, such as HIF1α. PEITC has previously been shown to inhibit 4E-BP1 phosphorylation in HCT-116 (colorectal cancer) and PC3 (prostate) cancer cells [70] and we observed similar effects in RCC4 and MCF7 cells. Thus, one potential mechanism by which PEITC may inhibit HIF activity is by reducing 4E-BP1 phosphorylation and HIF1α RNA translation.

Interestingly, the mTOR kinase, a major upstream upregulator of 4E-BP1 phosphorylation [77], contains several conserved cysteine-residues which have been suggested to play an important role in controlling mTOR activity [78]. Moreover, the mTOR inhibitor rapamycin also causes a decrease in HIF1α translation [79]. We are currently testing whether these cysteine-residues within mTOR are directly modified by PEITC and the role that this might play in inhibition of 4E-BP1 phosphorylation and HIF activity. However, it is important to note that HIF2α translation may be independent of mTOR since it is not affected by rapamycin [80]. Thus, PEITC may interfere with angiogenesis and HIF family protein function via multiple mechanisms.

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