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Hypoxia-inducible factor-1 (HIF-1) pathway activation by quercetin in human lens epithelial cells

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ABSTRACT

Quercetin is a dietary bioflavonoid which has been shown to inhibit lens opacification in a number of models of cataract. The objectives of this study were to determine gene expression changes in human lens epithelial cells in response to quercetin and to investigate in detail the mechanisms underlying the responses. FHL-124 cells were treated with quercetin (10 μM) and changes in gene expression were measured by microarray. It was found that 65% of the genes with increased expression were regulated by the hypoxia-inducible factor-1 (HIF-1) pathway. Quercetin (10 and 30 μM) induced a time-dependent increase in HIF-1α protein levels. Quercetin (30 μM) was also responsible for a rapid and long-lasting translocation of HIF-1α from the cytoplasm to the nucleus. Activation of HIF-1 signaling by quercetin was confirmed by qRT–PCR which showed upregulation of the HIF-1 regulated genes EPO, VEGF, PGK1 and BNIP3. Analysis of medium taken from FHL-124 cells showed a sustained dose-dependent increase in VEGF secretion following quercetin treatment. The quercetin-induced increase and nuclear translocation of HIF-1α was reversed by addition of excess iron (100 μM). These results demonstrate that quercetin activates the HIF-1 signaling pathway in human lens epithelial cells.

1. Introduction

Flavonoids are a group of polyphenolic compounds ubiquitously distributed throughout the plant kingdom. They are major contributors to total dietary antioxidant intake (Hollman et al., 1999). The flavonol quercetin is the most widely consumed flavonoid in the human diet (Lamson and Brignall, 2000) and is particularly abundant in onions, kale, apples, tea and red wine (Hertog et al., 1992). Quercetin is a potent antioxidant, the most potent antioxidant of the entire flavonoid group (Rice-Evans et al., 1996). Dietary flavonoids have numerous proposed benefits to human health, which has been largely attributed to their antioxidant properties. Significantly, there is epidemiological evidence that a quercetin-rich diet is inversely associated with lower incidences of cancer and cardiovascular diseases (Formica and Regelson, 1995; Hertog et al., 1993; Knekt et al., 1996, 2002). In relation to cataract, it has long been recognised that oxidative stress has a significant role to play in the pathophysiology of the disease (Spector, 1995; Truscott, 2005) and recent experiments clearly show increased protein oxidation in human lenses with cataract compared to age-matched controls (Hains and Truscott, 2008). Furthermore, diets rich in antioxidants have been linked with a reduced risk for cataract in several epidemiological studies (Jacques and Chylack, 1991; Tavani et al., 1996). Whilst much research relating to protection against cataract by antioxidants has focussed on the antioxidant vitamins, it is becoming increasingly apparent that the role of flavonoids in protecting against cataract requires further consideration (Sanderson and McLauchlan, 2002).

Quercetin and other flavonoids have been shown to have protective effects against lens opacification in in vivo and in vitro models of cataract (Beyer-Mears and Farnsworth, 1979; Lija et al., 2006; Sanderson and McLauchlan, 2002; Sanderson et al., 1999). In a galactosemic neonatal rat model of cataract, both topical and dietary administration of quercetin was found to diminish cataractogenesis (Beyer-Mears and Farnsworth, 1979). In another rat model, the occurrence and maturation of selenite-induced cataract was lowered by quercetin (Lija et al., 2006). We have previously demonstrated that quercetin effectively inhibited H_2O_2-induced lens opacification in a lens organ culture oxidative model of cataract (LOCH) (Sanderson et al., 1999). As a potent antioxidant, quercetin would be expected to be effective in this model as a result of direct antioxidant action. However, we also showed that protection occurred in lenses that had been pre-treated with quercetin for 24 h, but were exposed to oxidative stress in the

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absence of quercetin. This suggests that in addition to direct antioxidant effects, quercetin is also able to induce protective mechanisms within the cells.

It is known that antioxidants can cause changes in gene expression. Much research in this area has focussed on stimulation of antioxidant defences via the transcription factor Nrf-2 acting at the antioxidant response element (ARE) (Li and Kong, 2009) and quercetin has been shown to stimulate this pathway (Hanneken et al., 2006). However, a number of other pathways have also been shown to be upregulated by the flavonoid in a variety of cell types (Gong et al., 2009; Jeong et al., 2009; Kampkotter et al., 2008; Triantafyllou et al., 2007). In order to explain the mechanism of how lens cells could be protected by an exposure to quercetin prior to, but not during, oxidative stress, then such changes in gene expression must be considered. In the research presented here, we undertook global gene expression analysis of human lens epithelial cells exposed to quercetin. Of the genes that were upregulated, the majority were found to be controlled by the transcription factor hypoxia-inducible factor-1 (HIF-1). We demonstrate the stimulation of the HIF pathway in lens epithelial cells by quercetin and show the activation and synthesis of downstream effectors including vascular endothelial growth factor (VEGF) and erythropoietin (EPO).

2. Methods

All chemicals and reagents were from Sigma (Poole, UK) unless otherwise stated.

2.1. Cell culture

The human lens epithelial cell line (FHL-124) was a kind gift from Dr. J. R. Reddan (University of Michigan, USA). FHL-124 is a non-virally transformed cell line generated from human capsule-epithelial explants (Reddan et al., 1999) showing a 99.5% homology (in transcript profile) with the native lens epithelium (Wormstone et al., 2004). FHL-124 were routinely cultured at 35 °C in a humidified atmosphere of 95% air and 5% CO_2, in Eagle's Minimal Essential Medium (EMEM) (Gibco, Paisley, UK) supplemented with 5% v/v foetal bovine serum (Gibco) and 0.005% w/v gentamicin. Experiments were conducted on cells grown to 90% confluency. Prior to experimentation, cells were serum starved for 24 h. The cells were then exposed to quercetin (10 or 30 μM) in serum-free EMEM. These concentrations of quercetin have previously been shown to protect lens transparency when rat lenses were pre-incubated with quercetin for 24 h prior to exposure to oxidative stress (Sanderson et al., 1999). DMSO was required to solubilise quercetin and as a result DMSO (0.1% v/v) was present in all solutions including controls.

2.2. Microarrays

FHL-124 cells were grown in 60 mm culture dishes. Total RNA was isolated from untreated (control) and 10 μM quercetin-treated cells (24 h) using RNeasy Mini column extraction kits (Qiagen, Crawley, UK). Transcription profiles were analyzed using 2-colour oligonucleotide DNA arrays printed on epoxy-coated slides (Eady et al., 2005). Microarrays were produced using a commercial set of 14 000 oligonucleotides (Operon human oligonucleotide set version 1.0; Operon Biotechnologies, Cologne, Germany). Further details and validation of the microarrays has been previously described (Eady et al., 2005). All test samples were hybridised to the array with an FHL-124 reference sample that was common to all arrays. Hybridised arrays were scanned using an Agilent G2565BA microarray scanner system (Agilent Technologies South Queensferry, UK) and images were analyzed with Agilent G2567AA feature extraction software (Agilent Technologies). Aberrant features were detected by visual inspection and manually flagged. Data were analyzed using GeneSpring (Agilent Technologies) and normalized using locally weighted (LoWess) linear regression (Listgarten et al., 2003). Statistical analysis was performed using Welch's one way ANOVA with Benjamini and Hochberg multiple test corrections with a significance level set at $p < 0.05$. Analysis was performed using data from 2 independent experiments each with 4 replicates. The independent experiments were analyzed separately then the final list was derived from the quercetin-regulated genes that were common to both experiments.

2.3. Western blot analysis

Whole cell lysates from FHL-124 cells were prepared using Daub lysis buffer (Daub et al., 1997) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 μg/ml aprotinin for 20 min on ice and centrifuged at 13 000 rpm for 10 min. The protein concentration in the supernatant was determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL). Equal amounts of proteins were separated on 8% SDS-polyacrylamide gels and transferred to PVDF membrane using a semi-dry transfer cell (Bio-Rad, Herts, UK). The membrane was blocked with PBS containing 5% w/v non fat dry milk and 0.1% v/v Tween-20, hybridized with primary antibody followed by incubation with secondary antibody (Amersham Biosciences, Bucks, UK) and bands were detected using an ECL^+ chemiluminescent blot analysis system (Amersham Biosciences). Primary monoclonal antibodies to HIF-1α (BD Transduction Laboratories, UK) and β-actin (New England BioLabs, Hitchin, UK) were used a 1:600 and 1:1000 respectively. Statistical analysis was performed by unpaired, two-tailed Student's t test using Minitab release 14 (Minitab Inc, State College, PA). The significance level was set at $p < 0.05$.

2.4. Immunocytochemistry

FHL-124 cells were grown onto sterile glass coverslips and treated with 30 μM quercetin for 0.5, 4 and 24 h. Cells were fixed with 4% v/v formaldehyde in PBS for 30 min and permeabilised with PBS containing 0.5% v/v Triton X-100 for 30 min. All washes were made in PBS-BSA-Igepal (0.02% w/v and 0.05% v/v respectively). Non-specific sites were blocked with normal goat serum (1:50 in 1% w/v BSA in PBS). Anti-HIF-1α (1:100) or IgG1 control (R&D Systems, Abingdon, UK) (both diluted in 1% w/v BSA in PBS at same concentration of 2.5 μg/ml) was added to the coverslips for 1 h at 35 °C. They were visualised with ALEXA 488-conjugated secondary antibody (Molecular Probes, Leiden, Netherlands). Chromatin was stained with DAPI to reveal nuclei (1:100 in 1% w/v BSA in PBS) (Molecular Probes). The stained preparations were mounted in hydromount mounting medium (National Diagnostics, Hull, UK). Images were obtained using a CCD Upright Zeiss fluorescent microscope and Zeiss Axio vision 4.5 software. All images were captured using the same time of exposure. Images were processed to optimise to the peak fluorescence.

2.5. Quantitative Real Time PCR (qRT–PCR)

Total RNA was isolated from quercetin-treated FHL-124 cells using RNeasy mini column (Qiagen). RNA was reverse-transcribed by reverse transcriptase (Superscript™ II, Invitrogen, Paisley, UK) with random primers (Promega). The 25 μl PCR reactions were performed using an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) under the following conditions: 50 °C for 2 min, 95 °C for 10 min, and then 40 cycles, each

consisting of 15 s at 95 °C and 1 min at 60 °C. Validated primers and probes were obtained from Applied Biosystems (Assay-on-demand, Applied Biosystems) for the following genes: *BNIP3* (assay identification number Hs00969289_m1), *EPO* (Hs00171267_m1), *PGK1* (Hs99999906_m1) and *VEGF* (Hs00173626_m1). In order to identify stable genes for normalization, the geNorm method was used (Vandesompele et al., 2002). Expression of the genes EIF4A2, RPL13A and 18S was found to be the most stable. The geometric mean of C_T values of these three genes was used to normalize input for each sample analyzed. The calculations were made using the comparative C_T method ($\Delta\Delta C_T$) as previously reported (Livak and Schmittgen, 2001; User Bulletin #2 Applied Biosystems). The data were expressed as the relative-fold increase in gene expression for quercetin-treated samples compared with the untreated control. Statistical analysis was performed by unpaired, two-tailed Student's *t* test using Minitab release 14 (Minitab Inc, State College, PA). The significance level was set at $p < 0.05$.

2.6. Quantification of VEGF protein

FHL-124 cells were grown onto 60 mm dishes and treated with quercetin (24–72 h). A human VEGF ELISA kit (R&D Systems, Minneapolis, MN, USA) was employed to measure secreted VEGF concentrations from a sample (200 µl) of medium. Total proteins were extracted from each dish and used to normalize VEGF levels. Statistical analysis was performed by unpaired, two-tailed Student's *t* test using Minitab release 14 (Minitab Inc, State College, PA). The significance level was set at $p < 0.05$.

3. Results

In order to investigate changes in gene expression in lens cells caused by quercetin, gene array analyses were carried out using FHL-124 cells exposed for 24 h to 10 µM quercetin. A comparison of transcript profiles identified 20 genes for which increased expression was highly significant ($p < 0.01$) and 10 genes for which expression significantly decreased ($p < 0.01$) (Table 1). Interestingly, it was observed that 13/20 (65%) of the genes that showed increased expression are reported in the literature to be regulated by hypoxia and HIF-1 (Table 1).

Given that several HIF-1 regulated genes were upregulated in response to quercetin in FHL-124 cells (Table 1), we looked at the effects of quercetin on HIF-1α protein levels in these cells. HIF-1α is the oxygen-sensitive subunit of the HIF-1 transcription factor (Wang et al., 1995). It is a cytoplasmic protein which during hypoxia shows an increase in cellular levels and translocation to the nucleus. In the nucleus it dimerises with HIF-1β to give the active transcription factor HIF-1. In FHL-124 cells treated with 10 and 30 µM quercetin for 4 and 24 h, a large accumulation of HIF-1α protein was induced. HIF-1α accumulation was greatest after 4 h at the higher concentration (30 µM) but was detected after 24 h following exposure to both 10 and 30 µM (Fig. 1A). A band of lower molecular weight than the main band was observed at the higher concentration of quercetin, which is likely to be due to a post-translational modification of the HIF-1 protein. HIF-1α band intensities were quantified and normalized to β-actin (Fig. 1B). An almost 70-fold increase in HIF-1α protein levels was measured after 4 h incubation with 30 µM quercetin. At the 24 h time point, levels were increased by approximately 15-fold with both 10 and 30 µM quercetin.

The accumulation and translocation of HIF-1α to the nucleus would indicate activation of HIF-1α and the induction of the HIF-1 signaling pathway. Immunocytochemistry experiments were carried out to look at the location of HIF-1α proteins within the cells following quercetin treatment. When cells were exposed to 30 µM quercetin, there was a rapid change in HIF-1α status, with a 30 min exposure being sufficient to induce a large accumulation of HIF-1α in the cells, with the highest density inside the nucleus (Fig. 2). This accumulation was highest after 4 h, and persisted for at least 24 h. HIF-1α was detectable in the untreated cells, but at much lower levels than in quercetin-treated cells. These data confirm that there is an increase in HIF-1α with quercetin, but more importantly, the data clearly show that HIF-1α is translocated to the nucleus indicating HIF-1 pathway activation in response to quercetin exposure.

To confirm the activation of the HIF-1 signaling pathway, we investigated the changes in transcript levels of four HIF-1 regulated genes following quercetin treatment. BNIP3 and PGK1 had been identified from the microarray study, whilst erythropoietin (EPO) and vascular endothelial growth factor (VEGF or VEGF-A) were selected because they are well-studied HIF-1 regulated genes. Cells were exposed to 10 µM quercetin for 8, 24 or 48 h and the changes in mRNA expression were measured by quantitative PCR. Significant increases were seen in expression of each of the genes at the 24 and/or 48 h time points compared to controls (Fig. 3).

Table 1
Genes for which mRNA levels were up- or down-regulated by quercetin ($p < 0.01$).

GenBank Accession No.	Common Name	Description	Fold change[a]
Upregulated genes			
X60673	**AK3L1**	**adenylate kinase 3-like 1**	**2.99**
AF002697	**BNIP3**	**BCL2/adenovirus E1B 19kDa interacting protein 3**	**2.84**
AK000996	**TMEM45A**	**transmembrane protein 45A**	**2.43**
NM_000291	**PGK1**	**phosphoglycerate kinase 1**	**2.14**
D87953	**NDRG1**	**N-myc downstream regulated gene 1**	**1.80**
AK000507	**DDIT4**	**DNA-damage-indubible transcript 4**	**1.70**
K03515	**GPI**	**glucose sulfate isomerase**	**1.69**
AB037857	PTGFRN	prostaglandin F2 receptor negative regulator	1.67
D25328	**PFKP**	**phosphofructokinase, platelet**	**1.63**
AF109735	**PFKFB3**	**6-phosphofructo-2-kinase/fructose-2, 6-biphosphatase 3**	**1.55**
U25997	**STC1**	**stanniocalcin 1**	**1.55**
L07956	**GBE1**	**glucan (1,4-alpha-), branching enzyme 1**	**1.54**
J05032	DARS	aspartyl-tRNA synthetase	1.52
AL162078	KLC4	kinesion light chain 4	1.50
L19686	**MIF**	**macrophage migration inhibitory factor (glycosylation-inhibiting factor)**	**1.50**
AL110269	**WSB1**	**WD repeat and SOCS box-containing 1**	**1.46**
AB032251	BPTF	bromodomain PHD finger transcription factor	1.44
AB014731	DENR	density-regulated protein	1.41
AB023235	MTMR15	myotubularin related protein 15	1.41
AL137714	WASH2P	WAS protein family homolog 2 pseudogene	1.40
Downregulated genes			
M87507	CASP1	caspase 1, apoptosis-related cysteine peptidase (inteleukin 1, beta, convertase)	0.64
U29344	FASN	fatty acid synthase	0.61
AB000220	SEMA3C	sema domain, immunoglobulin domain (Ig) short basic domain, secreted, (semaphorin) 3C	0.59
U70451	MYD88	myeloid differentiation primary response gene (88)	0.58
X14787	THBS1	thrombospondin 1	0.57
X17025	IDI1	isopentenyl-diphosphate delta isomerase 1	0.54
AF026941	RSAD2	radical S-adenosyl methionine domain containing 2	0.53
AK000080	SAMD9	sterile alpha motif domain containing 9	0.51
X66435	HMGCS1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	0.47
M37984	TNNC1	troponin C type 1 (slow)	0.45

Genes highlighted in bold are regulated by HIF-1.
[a] Data are expressed as the relative -foldchange in gene expression for quercetin-treated samples compared with the untreated control.

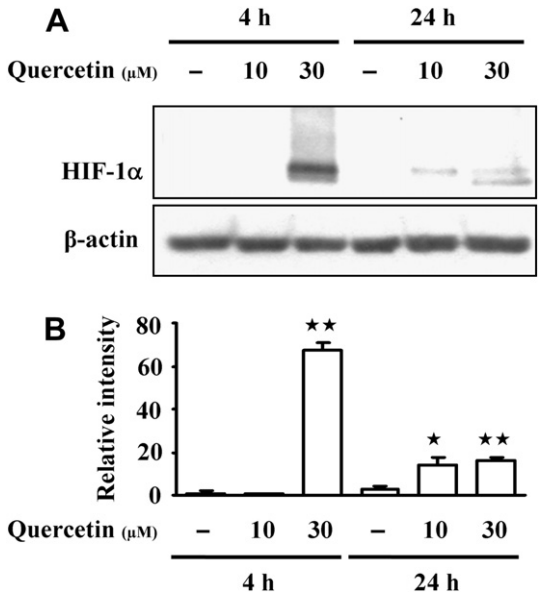

Fig. 1. Accumulation of HIF-1α in response to quercetin. FHL-124 cells were exposed to quercetin (10 and 30 μM) for 4 and 24 h (A) HIF-1α protein levels were examined by Western blot showing increased levels of HIF-1α with quercetin compared to the normalising protein β-actin. (B) Amount of HIF-1α assessed by densitometric analysis of Western blot data. Level is normalized to that of β-actin. Each bar represents the mean ± S.E.M. (arbitrary units) of 4 independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ relative to untreated cells at each time point.

The greatest upregulation of BNIP3 and VEGF (4-fold and 2.3-fold respectively) was observed after 24 h quercetin exposure. For PGK1 and EPO, upregulation was highest after 48 h (2.6-fold and 1.8-fold respectively). These data confirm the activation of the HIF-1 signaling pathway by quercetin in FHL-124 cells.

To further investigate activation of the HIF-1 pathway by quercetin, we measured secretion of VEGF by FHL-124 cells following quercetin treatment (Fig. 4). For both quercetin concentrations (10 and 30 μM) and at all time points studied (24–72 h), there was a significant increase in VEGF secretion from quercetin-treated compared to untreated cells. The increased VEGF secretion was dose-dependent, and reached a maximum (3.5-fold) after 72 h incubation with 30 μM quercetin. These data show that there are sustained changes in the cell as a result of exposure to quercetin associated with activation of the HIF-1 pathway.

Quercetin has previously been shown to chelate iron ions (Leopoldini et al., 2006). HIF-1 prolyl hydroxylase (HPH) is an iron dependent enzyme involved in the initial stages of oxygen sensitivity of HIF-1α. When Fe^{2+} is chelated, the degradation of HIF-1α is prevented and the subsequent accumulation of HIF-1α eventually leads to HIF-1 activation. To investigate the role of Fe^{2+} in the induction of HIF-1 by quercetin in FHL-124 cells, experiments were performed in which excess Fe^{2+} was added to the culture medium. We first looked at HIF-1α protein accumulation in cells treated with 30 μM quercetin in the presence or absence of 100 μM $FeSO_4$ (Fig. 5A and B). The large accumulation of HIF-1α observed with quercetin alone was blocked in the presence of $FeSO_4$. $FeSO_4$ alone had no effect on HIF-1α protein levels. The effects of Fe^{2+} on quercetin-induced nuclear translocation of HIF-1α were also investigated (Fig. 5C). Immunocytochemistry showed that nuclear translocation of HIF-1α induced by quercetin was also prevented in cells treated with quercetin together with excess $FeSO_4$. $FeSO_4$ alone did not cause an increase in HIF-1α in the nucleus. These data suggest that the mechanism by which quercetin induces HIF-1 signaling pathway could involve Fe^{2+} chelation and subsequent inhibition of prolyl hydroxylase (HPH) activity.

4. Discussion

In this study we have investigated gene expression changes that occur as a result of exposure to the bioflavonoid quercetin. Using arrays, we found that the majority of genes that were upregulated by quercetin were known to be controlled by the transcription factor HIF-1. HIF-1 is involved in the co-ordination of the cellular

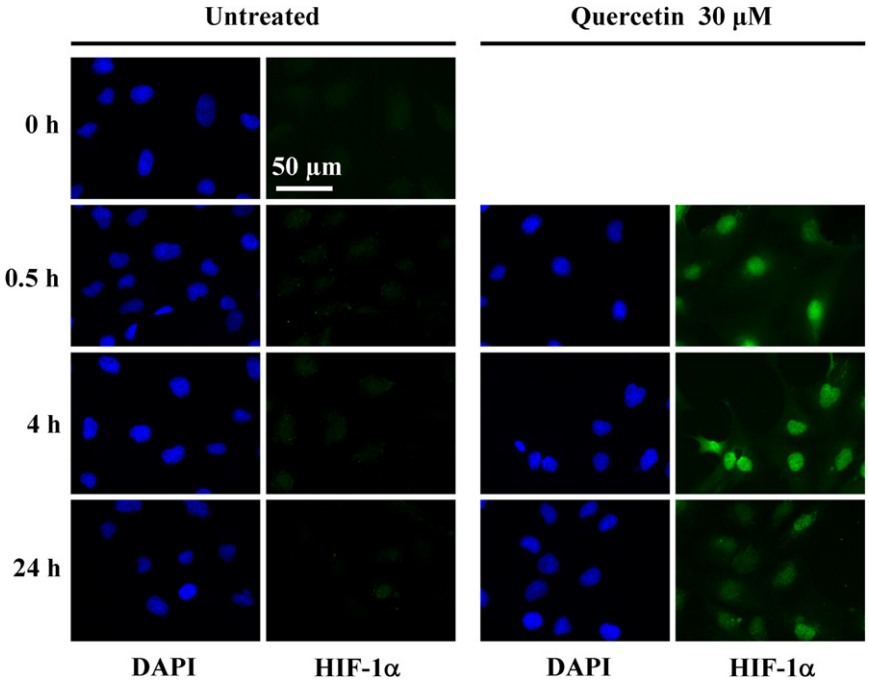

Fig. 2. HIF-1α nuclear translocation in response to quercetin. Fluorescent micrographs showing HIF-1α (green) immunostaining of FHL-124 cells. Nuclei are labelled using DAPI (blue). Cells were exposed to 30 μM quercetin for 0.5, 4 and 24 h. HIF-1α nuclear translocation is observed subsequent to quercetin treatment, at all time points.

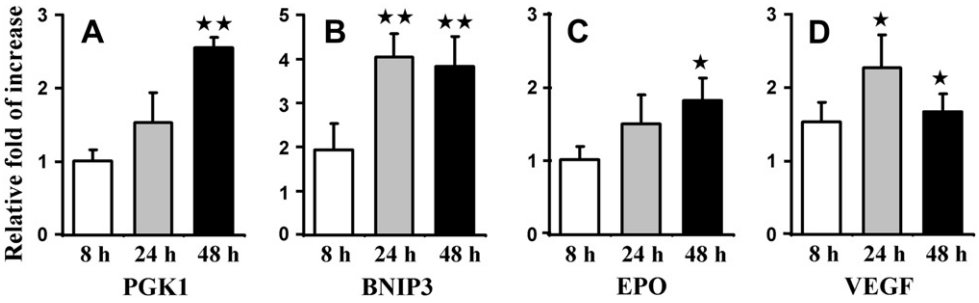

Fig. 3. Quercetin increases *PGK1* (A), *BNIP3* (B), *EPO* (C) and *VEGF* (D) mRNA levels in FHL-124 cells. Cells were treated with 10 μM quercetin for 8 (white bars), 24 (grey bars) and 48 h (black bars). Total RNA was extracted and qRT–PCR was performed. Data is expressed as relative-fold increase of quercetin-treated compared to untreated cells, after normalization to 3 internal controls (see Materials and Methods for details). Each bar represents the mean ± S.E.M. from 5 independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ relative to untreated cells.

response to a lack of oxygen and regulation of oxygen homeostasis (Semenza, 1998). It is a ubiquitously expressed heterodimer composed of the two subunits HIF-1α and HIF-1β. The HIF-1β subunit is constitutively expressed under all O_2 conditions and is located within the nucleus of the cell. The oxygen sensitivity of the system is confered by the properties of HIF-1α (Dery et al., 2005; Semenza, 2002). The processes involved are summarised in Fig. 6. Under normoxic conditions, HIF-1α is hydroxylated on proline residues 402 and 564 by the enzyme prolyl hydroxylase (HPH). HPH requires the presence of iron (Fe^{2+}), O_2 and 2-oxoglutarate as cofactors in order to function effectively (Schofield and Ratcliffe, 2004). Hydroxylation causes the binding of HIF-1α to the von Hippel–Landau tumour suppressor protein (VHL), targetting the HIF-1α for ubiquitination by E3 ubiquitin-protein ligase which in turn leads to degradation via the cytoplasmic proteosome. Under conditions of normal O_2 tension, HIF-1α is constantly synthesized, but as a result of the processes described above, is immediately targeted for degradation, giving it a half-life of approximately 5 min (Wang et al., 1995). This means that if there is an inhibition of this process, then cellular HIF-1α levels can rapidly increase, as occurs under conditions of low O_2 tension. In hypoxia, the lack of availability of O_2 means that hydroxylation cannot occur. HIF-1α no longer binds to VHL and therefore is not targeted for degradation causing a rapid increase in HIF-1α levels. It translocates to the nucleus of the cell where it binds with HIF-1β forming the HIF-1 transcription factor. HIF-1 then binds to the hypoxia-response element (HRE) of target genes, inducing the expression of a suite of genes that are involved in adaption to low oxygen conditions. Over 70 HIF-1 target genes have been identified (Semenza, 2004). Erythropoietin (EPO) and vascular endothelial growth factor (VEGF or VEGF-A), for example, are two well-studied HIF-1 regulated genes, involved in increasing oxygen delivery to tissues via the stimulation of red blood cell production and regulation of angiogenesis respectively (Ferrara, 2000; Ridley et al., 1994). However, as well as upregulating genes which are directly associated with enabling the cell to adapt to low oxygen conditions, HIF regulated

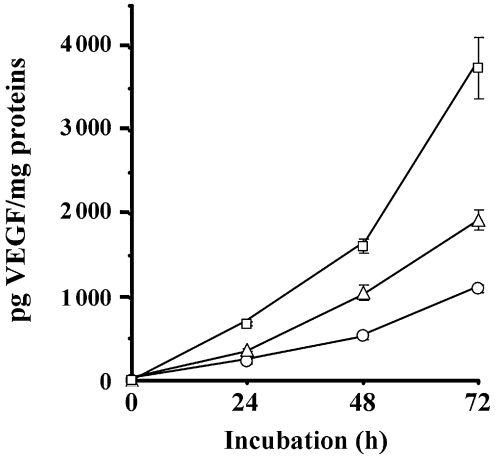

Fig. 4. Quercetin increases the secretion of VEGF into FHL-124 cell media. Cells were treated in absence (○) or presence of quercetin (10, (△) and 30 μM, (□)) for 0–72 h. Secreted VEGF was measured and normalized as described in Materials and Methods. Quercetin shows a dose- and time-dependent increase in VEGF secretion. Each point represents the mean ± S.E.M. of 4 independent experiments.

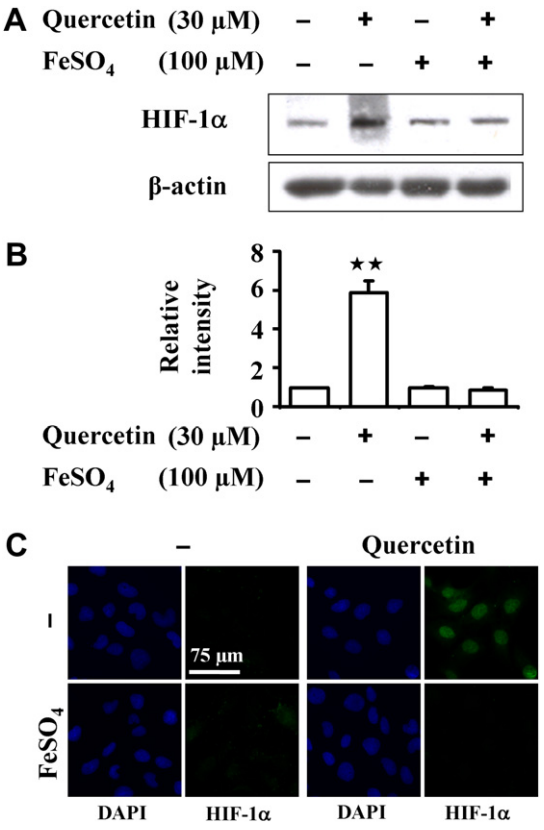

Fig. 5. Effect of $FeSO_4$ on quercetin-induced HIF-1α accumulation in FHL-124 cells. Cells were exposed to 30 μM quercetin in presence or absence of 100 μM $FeSO_4$ for 2 h. (A) Western blot of HIF-1α and β-actin protein. (B) Densitometric analysis of HIF-1α normalized to β-actin. Each bar represents the mean ± S.E.M. (arbitrary units) of 3 independent experiments. $^{**}p < 0.01$ relative to untreated cells. (C) Fluorescent micrographs showing DAPI (blue) and HIF-1α (green) immunostaining of FHL-124 cells.

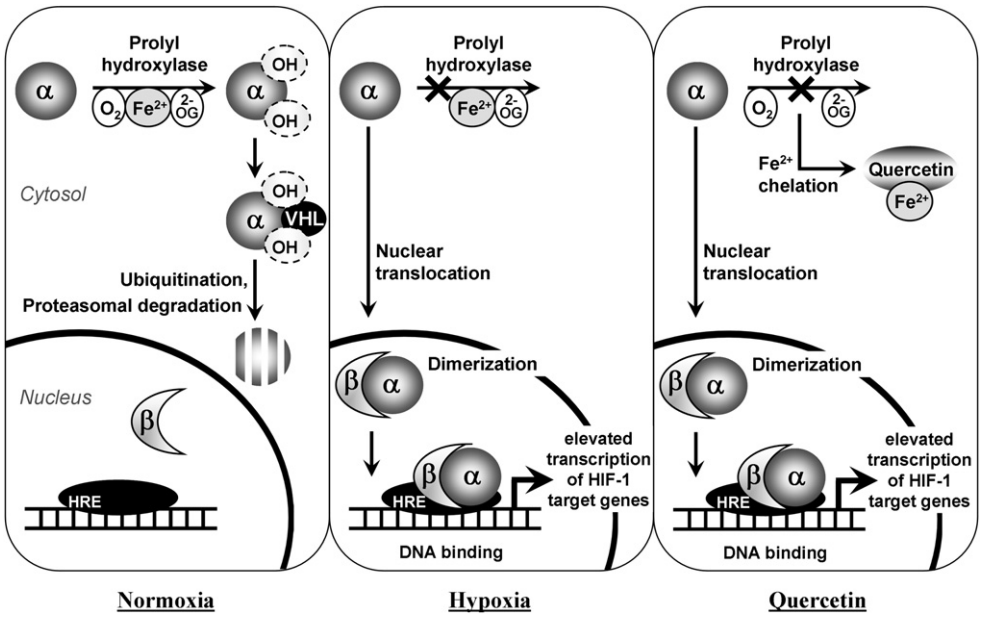

Fig. 6. The HIF-1 signaling pathway, depicting cellular events under normal oxygen tensions and in hypoxia. The possible mechanism of action of quercetin in HIF-1 regulation as an iron chelator is also shown. α = HIF-1α, β = HIF-1β, 2-OG = 2-oxoglutarate, VHL = von Hippel–Landau protein, HRE = hypoxia-response element.

proteins such as EPO and VEGF have also been associated with cytoprotective properties (Castilla et al., 2000; Maiese et al., 2004). It was therefore of interest that the majority of genes upregulated by quercetin were HIF-1 regulated as this may contribute to the cytoprotective properties of this flavonoid. Investigating the effect of quercetin on HIF-1 signaling in greater detail showed that quercetin increased intracellular HIF-1α protein levels and caused nuclear translocation. Four HIF-1 regulated genes were measured by quantitative PCR and expression of each of these genes increased. Moreover, there was a dose-dependent increase in VEGF secretion by quercetin, which was maintained over a 72 h period. Although there have been reports concerned with other cell types showing that quercetin can upregulate genes via the HIF-1 pathway (Triantafyllou et al., 2007; Wilson and Poellinger, 2002), this is the first report showing quercetin treatment has long-lasting effects, sustained over 72 h. This study is also the first to indicate the relative dominance of this pathway in the expression response of quercetin, with more than half the genes upregulated being controlled via the HIF-1 pathway. Interestingly, none of the genes highlighted by the array data are known to be regulated via the antioxidant response element (ARE) and there were no indications that other signaling pathways were involved. This does not preclude a role for quercetin in other pathways in the lens, but in our experiments the overriding regulator of expression seen was HIF-1.

It is important to consider the mechanism by which quercetin acts to stimulate the HIF-1 pathway. Quercetin is known to chelate Fe^{2+} (Leopoldini et al., 2006) and it has been suggested that the increase in HIF-1α is a direct result of this property (Jeon et al., 2007; Park et al., 2007; Triantafyllou et al., 2007). Fe^{2+} is a cofactor for HPH, an enzyme which catalyses the hydroxylation of HIF-1α thus targeting it ultimately for degradation via the proteosome. In the absence of Fe^{2+} this cannot occur and therefore HIF-1α is stabilised and survives such that it can go on to activate HIF-1 regulated genes. Fe^{2+} chelation therefore mimics the effects of hypoxic conditions. Significantly, the iron chelator 1,10-phrenanthroline has been shown to increase HIF-1α levels in the rat lens in vivo (Shui et al., 2008). In our experiments, adding an excess of Fe^{2+} inhibited the effects of quercetin on HIF-1 pathway activation. The presence of excess Fe^{2+} would overcome the effects of quercetin-mediated Fe^{2+} chelation, thus enabling sufficient free Fe^{2+} in the cell to support HPH activity. These data therefore suggest that the major action of quercetin in stimulating the HIF-1 pathway is via Fe^{2+} chelation and consequent inhibition of HPH. However, it should be noted that there are alternative interpretations of these data. It is also possible that when quercetin is bound to Fe^{2+} it is unable to perform its usual actions within the cell, thus an excess of Fe^{2+} would inhibit any effect of quercetin independent of the mechanism involved.

Given the stimulation of the HIF-1 pathway by quercetin shown in the experiments presented, it is interesting to look further at the role of this pathway in the lens. As previously discussed, a major downstream pathway of HIF-1 signaling is that mediated by VEGF, a growth factor that is active in the stimulation of angiogenesis (Ferrara, 2000; Leung et al., 1989). Despite the avascular nature of the lens, adult lens has been shown to express both the VEGF-A protein and the VEGF receptor, VEGFR2, indicating active VEGF signaling in the lens (Gilbert et al., 1998; Shui et al., 2003). Further, VEGF signaling appeared to be sensitive to the levels of oxygen (Shui et al., 2003). These observations led to investigation of the role of the HIF-1 pathway in the lens and an elegant series of experiments have demonstrated an important role for signaling via this pathway in lens growth (Shui et al., 2008; Shui and Beebe, 2008). The hypoxic environment of the lens is sufficient to activate the HIF-1 pathway, which has been shown to be constitutively active in the lens (Shui et al., 2008; Shui and Beebe, 2008). Lens growth is known to decline with age in all species and in rat an approximately 3-fold decrease in lens epithelial cell proliferation occurs between months 1 and 8 after birth (Shui and Beebe, 2008). Increasing the oxygen around the lens in 8 month old rats increased levels of proliferation back to that of a 1 month old animal (Shui and Beebe, 2008). These experiments indicate that the decrease in lens growth with age is caused by the decrease in oxygen (Shui and Beebe, 2008) and that this is mediated by HIF signaling (Shui et al., 2008). Moreover, using a conditional knock-out system, it was shown that expression of HIF-1α is necessary for the normal growth of the lens and survival of fibre cells (Shui et al., 2008). It appears therefore that HIF signaling plays a fundamental role in the lens. It

has been further postulated that the observed HIF-dependent suppression of growth is important in protecting the lens against nuclear cataract (Shui and Beebe, 2008). Epidemiological data has shown that as the size of the lens increases, there is an increased risk of nuclear cataract formation (Klein et al., 1998, 2000). Inappropriate lens epithelial cell proliferation could be a contributing factor in nuclear cataract and agents, such as quercetin, which stimulate the HIF-1 pathway could therefore potentially decrease the risk of cataract formation.

A recent study looking at quercetin and its interaction with the HIF-1 pathway raises important questions to be considered in relation to the lens. Triantafyllou et al. (2008) used HeLa cells to investigate the effects of flavonoids on HIF-1α. It was found that as well as an increase in HIF-1α levels, that quercetin (100 μM) also inhibited translocation of HIF-1α to the nucleus via the p44/42 MAPK pathway. This inhibition became more apparent when the HIF-1 pathway was stimulated either by iron chelation or hypoxia (1% O_2). Our data clearly show intense HIF-1α staining in the nucleus, with little remaining in the cytoplasm of cells treated with quercetin (30 μM) (Fig. 2), suggesting that nuclear translocation of HIF-1α was not significantly inhibited in our experiments. Whether these differences are, for example, due to the concentration of quercetin used, or differences between cell lines remains to be determined, but certainly given that the lens exists under conditions of relative hypoxia (approximately 3% O_2) (Shui et al., 2008; Shui and Beebe, 2008) and that the HIF-1 pathway is constitutively active in lens, it will be very important to investigate signaling in lens epithelial cells under conditions of differing O_2 tension.

In considering quercetin as an agent that could reduce the risk of cataract formation, it is plausible that quercetin could act via multiple pathways. Oxidative stress is a recognised factor in the formation of cataract (Spector, 1995; Taylor et al., 1995; Truscott, 2005) and quercetin, as a potent antioxidant, could directly inhibit oxidative damage. Indeed this has already been suggested and quercetin treatment has been shown to decrease lens opacity in an oxidative model of cataract (Sanderson et al., 1999). As a result of the experiments presented here, it will be important to investigate the role of HIF-1 signaling in cytoprotection as this could also contribute to any potential anti-cataract activity. It will also be of key importance to investigate the effects of quercetin on HIF-1 activation under conditions of reduced oxygen in order to specifically relate to the conditions experienced by the lens in vivo. In addition, the bioavailability of quercetin to the lens requires consideration. Peak plasma concentrations of total quercetin (largely as quercetin metabolites) are reported in the low micromolar range (up to 7.6 μM) (Manach et al., 2005) in bioavailability studies following intake of a quercetin-rich food (onion). Whether quercetin reaches the lens and in what form is an important question for future research.

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