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The flavonoid quercetin induces hypoxia-inducible factor-1α (HIF-1α) and inhibits cell proliferation by depleting intracellular iron

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Abstract
Quercetin, a flavonoid with anti-oxidant, metal chelating, kinase modulating and anti-proliferative properties, can induce hypoxia-inducible factor-1α (HIF-1α) in normoxia, but its mechanism of action has not been determined. In this study we characterized the induction of HIF-1α and the inhibition of cell proliferation caused by quercetin in HeLa and ASM (airway smooth muscle) cells and examined the effect of iron on these processes. Furthermore, we investigated the relevance of the intracellular levels of quercetin to HIF-1α expression and cell proliferation. Our data demonstrate that quercetin depletes intracellular calcein–chelatable iron and that supplying additional iron from extracellular or intracellular pools abrogates the induction of HIF-1α by quercetin. Moreover, addition of iron reverses the quercetin-induced inhibition of DNA synthesis, cell proliferation and cycle progression, but to different extents, depending on cell type. We propose that quercetin stabilises HIF-1α and inhibits cell proliferation predominantly by decreasing the concentration of intracellular iron through chelation.

Keywords: *Hypoxia-inducible factor-1, HIF-1α, quercetin, iron, ascorbate, ferric citrate*

Abbreviations: *DFO, desferrioxamine; HRE, hypoxia-response-element; HIF, hypoxia-inducible factor; MAPK, mitogen activated protein kinase;* N*-acetyl-*L*-cysteine, NAC; PHD, prolyl hydoxylase domain protein; PI-3K, phosphatidylinositol-3 kinase; ROS, reactive oxygen species*

Introduction

The hypoxia-inducible transcription factor 1 (HIF-1) is the key mediator of the cellular response to tissue hypoxia (reduced availability of oxygen) [1,2]. HIF-1 induces the expression of over 70 genes, the products of which facilitate anaerobic metabolism, erythropoiesis, angiogenesis and other processes promoting tissue survival at low oxygen tension. Since adaptation to hypoxia is crucial in many pathological conditions, including ischaemic heart disease and solid tumor growth, the control of expression of HIF-1 is the object of intense medical interest [3].

HIF-1 is a heterodimer of two protein subunits, HIF-1α, which is induced by hypoxia and HIF-1β or ARNT, which is constitutively expressed [4]. Under normoxic conditions (21% oxygen), hydroxylation of HIF-1α by O_2-, Fe^{2+}- and oxoglutarate-dependent proline hydroxylases (PHDs: prolyl hydroxylase domain proteins) leads to its recognition by the von Hippel Lindau tumor suppressor protein (pVHL), a

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constituent of an E3 ubiquitin ligase, which targets it for proteosomal degradation [5–8]. Furthermore, in normoxia, hydroxylation of HIF-1α by an asparagine hydroxylase, called factor-inhibiting HIF-1 (FIH-1), leads to HIF-1α transcriptional inactivation [9]. In hypoxia, hydroxylation by the PHDs and FIH-1 is inhibited and, as a consequence, HIF-1α accumulates in a transcriptionally active form. Full activation of HIF-1 requires phosphorylation and nuclear translocation of HIF-1α, heterodimerisation with ARNT, binding to the hypoxia-response-elements (HREs) of target genes and interaction with the coactivator p300/CBP and other proteins [1–3,10,11].

HIF-1α can also be induced in normoxia by a number of chemical and biological agents, by mechanisms that include stabilization from proteolytic degradation or increased transcription and translation [2,12,13]. One of the more commonly employed inducers of HIF-1α is the iron chelator desferrioxamine (DFO) [14], which is thought to lead to HIF-1α accumulation by preventing its hydroxylation and proteolysis, through depletion of the iron required for enzymatic activity by the PHDs [15]. Recently, induction of transcriptionally active HIF-1α by the flavonoid quercetin has also been reported [16], but the mechanism was not investigated.

Flavonoids are widely encountered plant polyphenols, obtained in human diet from fruits, vegetables, tea and red wine and found in our tissues in the low μM range [17–19]. Numerous studies have attributed to flavonoids various health-promoting effects, such as prevention of inflammation, heart disease, angiogenesis and cancer [18–21]. Quercetin (3, 3′, 4′, 5, 7-pentahydroxyflavone) is generally known as an antioxidant [22], but it also possesses a remarkable number of other properties, including metal chelation, inhibition of numerous enzymic activities and inhibition of growth of many neoplastic cells [18]. Quercetin, through its 3′, 4′-hydroxy substitutions, can bind both ferrous and ferric ions and, depending on the pH and the stoichiometry of quercetin to iron, can form mono-, di- or tri-coordinated complexes, the stability constants of which increase the higher the oxidation state of iron and the ligand to metal ratio [23]. Quercetin has been shown to form especially stable 1:1 complexes with Fe (III) *in vitro*, with a $\log K$ of 20 [24], which is at least ten orders of magnitude higher than the stability constants of Fe (III) with its presumed physiological cellular ligands [25]. Chelation of iron by flavonoids contributes to their antioxidant activity [26] and has been shown to protect against nuclear DNA damage in cells exposed to hydrogen peroxide [27]. However, the effect of the iron-complexing ability of quercetin on HIF-1α expression and cell proliferation has not been assessed.

In this study we investigated the effect of quercetin on the normoxic induction of HIF-1α and on cell survival, proliferation and cell cycle progression in the human epithelial cancer cell line HeLa and related them to intracellular concentrations of quercetin and to changes in the labile iron pool. Furthermore, we examined the effect of adding excess ferric citrate or ascorbate, a limiting cofactor for PHD activity [15], on the above processes. Lastly, we examined whether our major findings were also observed in primary cell cultures of rabbit airway smooth muscle cells (ASMC). Our results show that the effects of quercetin on HIF-1α expression are mediated by iron chelation in both cell types studied, but the dependence of its anti-proliferative effects on iron is cell-type specific.

Materials and methods

Reagents

Ferric citrate, quercetin, DFO, *N*-acetyl-L-cysteine (NAC), ascorbate, cycloheximide, actinomycin D, calcein AM and other reagents were purchased from Sigma-Aldrich (St Louis, MO, USA), unless otherwise stated. The kinase inhibitors LY 294002 and PD98059 were from Cell Signaling (Beverly, MA, USA).

Cell cultures

HeLa cells were maintained in Dulbecco's modified Eagle medium (DMEM, High Glucose) (Gibco BRL), supplemented with 10% fetal bovine serum (Biochrom KG Seromed), antibiotic–antimycotic solution (Gibco BRL) and 2 mM L-glutamine (Biochrom KG Seromed). ASMC were prepared and cultured as previously described [28]. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Quercetin stock solution (10 mM) was prepared in dimethylsulphoxide and was appropriately diluted in culture medium. Where present, ferric citrate and other reagents were added at the times and concentrations stated in the legends to Figures. All experiments were performed before cells reached confluence and were repeated at least three times.

Western blot analysis of cellular proteins

Cells were lysed in 20 mM Tris–Cl, 50 mM NaCl, 10% Glycerol, 1% Triton-X100, 0.5% β-mercaptoethanol, 6 mM $MgCl_2$ in the presence of a cocktail of protease inhibitors (pefabloc 1 mM, leupeptin 1 μM and pepstatin 1 μM). Protein extracts (40 μg) were resolved by 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to nitrocellulose membranes according to standard protocols. Western blots were analysed with anti-HIF-1α mouse monoclonal antibody (BD Transduction Laboratories, Franklin Lakes, NJ, USA), or

anti-actin mouse monoclonal antibody (Serotec, Kidlington, Oxford, UK), or anti-transferrin receptor 1 (TfR1) mouse monoclonal antibody (Zymed Laboratories, San Franscisco, CA, USA). Membranes were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (BioRad, Hercules, CA, USA) and proteins were detected by enhanced chemiluminescence (ECL, Amersham-Pharmacia, Piscataway, NJ, USA). All experiments were performed at least three times and representative blots are shown. For quantitation, the ratio of the intensity of the HIF-1α band to the actin band was calculated from image scans of western blots, using a Photoshop program.

DNA synthesis and cell proliferation assays

DNA synthesis was assessed in triplicate wells by the incorporation of [^{3}H]-thymidine. Cells were seeded in 22-mm wells at a density of 10^5 cells/well and were incubated for the indicated periods of time. Four hs before the end of the incubation period, 0.25 μCi [^{3}H]-thymidine (44 Ci/mmol, Amersham) was added to each well and the radioactivity incorporated into trichloracetic acid-insoluble material was measured in a β liquid scintillation counter. Cell proliferation was determined in triplicate 22-mm wells. At various times of incubation, non-confluent cells were recovered with trypsin–EDTA (Biochrom KG) and counted in a Neubauer plate after trypan blue coloration. For determination of the IC50 for quercetin, HeLa cells were cultured with various concentrations of quercetin (0–200 μM), the viable cells were counted after 24 h and the quercetin concentration which produced a 50% decrease in the proliferation of the control cells (number of cells at 24 h − number of cells at 0 h) was established.

Flow cytometric analysis of cellular DNA content

HeLa cells were cultured for 24 h with 100 μM quercetin, in the presence or absence of 0.5 mM ferric citrate. Cells were then detached by trypsinization, counted in a Neubauer haemocytometer and fixed overnight in 70% ice-cold ethanol. After washing with PBS, cells were resuspended at a density of 10^6 cells/ml in staining solution (10 pg/ml propidium iodide, 100 pg/ml RNase A) for 45 min in the dark and then immediately analyzed on an EPICS flow cytometer (Beckman Coulter).

Immunofluorescence microscopy of HIF-1α subcellular localization

HeLa cells growing on glass slides were fixed with 3% formaldehyde and permeabilized with 1% Triton X-100. After blocking nonspecific binding with 3% bovine serum albumin in PBS–0.1% Tween 20 overnight, the cells were incubated with an anti-HIF-1α antibody (BD Transduction Laboratories, 1:200 dilution). Primary antibody was detected by incubation with a fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse antibody (BioRad, 1:50 dilution). The cells were visualized and photographed utilizing a fluorescence Optiphot-2 microscope and UFX-DX camera system (Nikon).

Fluorescence microscopy of calcein–chelatable iron

The method used was adapted from Devireddy et al. [29] and the information supplied in a manufacturer's protocol (Trevigen Inc., MD, USA). HeLa cells growing on glass slides were cultured with no addition or 100 μM quercetin or 150 μM DFO, in the absence or presence of 0.5 mM ferric citrate or 100 μM ascorbic acid. After 4 h, 1 μM calcein AM was added in DMEM without fetal bovine serum and cells were incubated for 30 min and fixed with 3% formaldehyde. Slides were then washed with PBS and cells were counterstained with DAPI (4′,6-diamino-2-phenylindole dihydrochloride), visualized and photographed utilizing a fluorescence microscope as described above. Similar results were obtained when fixation with formaldehyde was omitted (results not shown).

Reporter gene assays

HeLa cells were transiently co-transfected with a hypoxia-responsive luciferase reporter gene and a constitutive β-gal expression vector, using the TransPass D2 DNA Transfection Reagent according to the manufacturers instructions (New England Biolabs, Beverly, MA, USA). Cells were transfected with 1 μg pGL3-5HRE-VEGF plasmid (containing the luciferase reporter gene under the control of five tandemly repeated HRE motifs from the VEGF gene, driven by the SV40 promoter), kindly provided by Dr A. J. Giaccia (Stanford University, CA, USA), or 1 μg pGL3-promoter plasmid (same as the previous plasmid, but without the HREs) and 1 μg CMV-lacZ plasmid, both kindly provided by Dr A. Kretsovali (IMBB, FORTH, Heraklion, Greece). The transfected cells were cultured for 24 h and were subsequently incubated for 4 h with 100 μM quercetin, in the presence or absence of 0.5 mM ferric citrate. Luciferase activity was determined by an assay kit (Promega, Madison, Wis, USA) with a luminometer (TD20/20, Turner Designs) and was normalized to β-galactosidase activity.

HPLC quantification of quercetin in cell lysates and growth medium

HeLa cells (1×10^6 cells/100 mm plate) were incubated in Dulbecco's modified Eagle medium in

the presence of quercetin (100 μM initial concentration), without or with the addition of ferric citrate, for 1–24 h. At the indicated periods of time samples of growth medium were taken and cells were washed with PBS buffer and lysed in 200 μl of lysis buffer (total volume of cell lysate 300 μl). Subsequently, all cell lysate and growth medium samples were submitted to HPLC analysis. Samples were diluted 1:1 with methanol containing 0.1% HCl, vortexed, centrifuged at 2000*g* for 10 min and the supernatants were directly injected into the HPLC system. HPLC analysis was accomplished by the use of a low pressure gradient system comprised of SSI 222D HPLC pump, SSI 232 gradient controller, SSI 500 UV–Vis detector and equipped with RP LC-18-DB (Sigma-Aldrich Co) HPLC column. Chromatographic separation was achieved by binary gradient elution with solvent A containing methanol: water: acetic acid at 10:88:2 v/v and solvent B containing methanol: water: acetic acid at 90:8:2 v/v. The 20 min linear gradient program was applied with the following solvent A content: 100% (initial), 85% (5 min), 30% (10 min), 20% (20 min). The chromatographic data were acquired and processed by the use of Caliber™ data acquisition software (Polymer Laboratories, UK).

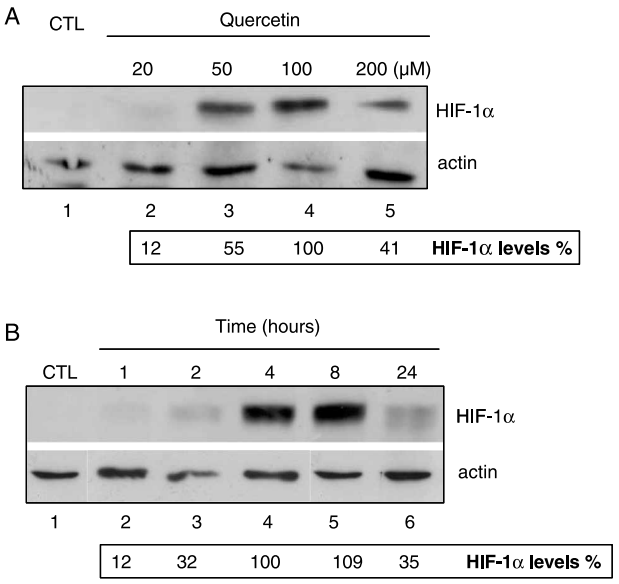

Figure 1. Western blot analysis showing: (A) dependence of HIF-1α induction on quercetin concentration; and (B) kinetics of HIF-1α induction by 100 μM quercetin. HeLa cells were cultured as indicated and cell lysates were prepared and analysed for HIF-1α protein expression, using actin as loading control. Percentage of changes in HIF-1α levels were calculated, as described in Materials and Methods. CTL: Control untreated cells, cultured at normoxic conditions. Each panel is representative of three independent experiments.

Statistical analysis

Graph Pad Instat Statistical package for Windows was used. Data are expressed as mean ± standard deviation (SD). The one way analysis of variance (ANOVA) with Bonferroni posttest was used for the comparison of data and the statistical significance limit was set to $p < 0.05$.

Results

Concentration- and time-dependence of induction of HIF-1a by quercetin in HeLa cells

HeLa cells do not constitutively express HIF-1α at normoxic conditions. Initial experiments with various concentrations of quercetin determined that, after 4 h culturing, HIF-1α protein was barely detectable with 20 μM quercetin, was strongly expressed with 100 μM, but its level declined at higher concentrations (Figure 1(A)). 100 μM quercetin, a concentration not toxic to cells after 4 h culturing, was therefore chosen for all subsequent experimentation. The time course of induction by 100μM quercetin showed that expression of HIF-1α was first detectable at 1 h, was greatest at 4–8 h, but levels declined sharply by 24 h (Figure 1(B)). Reblotting the membranes with anti-actin antibody confirmed equal protein loading and that this transient expression was specific for HIF-1α.

Correlation between the intracellular concentration of quercetin and the expression of HIF-1 α

The kinetics of HIF-1α induction observed in Figure1(B) made us wonder how the levels of HIF-1α protein expressed correlate with the intracellular concentrations of quercetin. We therefore, proceeded in quantitating the amount of quercetin present intracellularly at various time points.

Quercetin taken up by HeLa cells and quercetin remaining in the growth medium were monitored following culturing cells for 1–24 h at an initial concentration of 100μM quercetin. Reversed-phase HPLC with UV detection at 370 nm revealed the presence of quercetin ($t_R = 17.13$ min) in cell lysates and also a new peak ($t_R = 16{,}62$ min), which can be considered as a putative quercetin metabolite (Figure 2(A)). The overall changes in quercetin concentration in growth medium and cells are illustrated in Figure 2(B). Intracellular quercetin achieved a maximum concentration of 40 μM at 4 h of exposure and fell to 8 μM at 24 h, while the concentration of quercetin in the growth medium fell from an initial 100μM to 2μm within 24 h. In contrast, the concentration of the putative quercetin metabolite increased with time both in the intracellular and extracellular compartments (results not shown). These data demonstrate that there is a good correlation (with a few hs time lag) between the levels

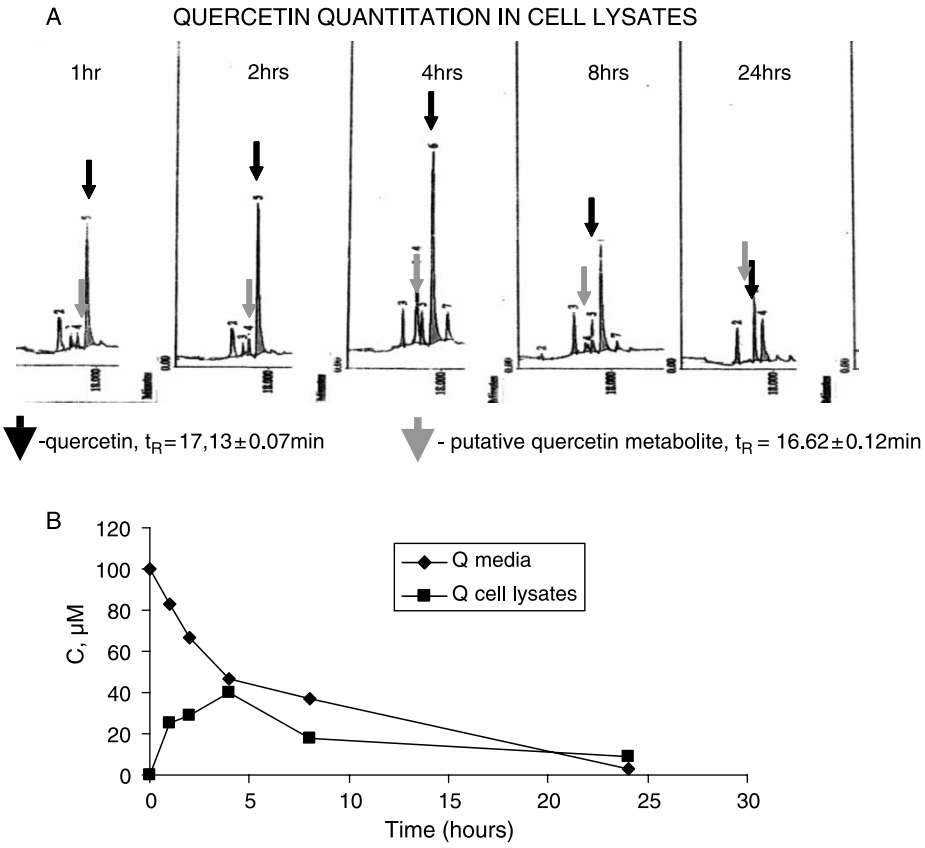

Figure 2. Quantitation of quercetin uptake by HeLa cells. (A) 100 μM quercetin was added to HeLa cells and quercetin and its metabolites were determined at 1, 2, 4, 8, and 24 h of culture in cell lysates by HPLC, as described in Materials and Methods. (B) Kinetics of changes in quercetin concentration in growth medium and cell lysates. One of two independent experiments is shown.

of HIF-1α protein expressed and the levels of quercetin observed intracellularly (compare Figure 1(B) with Figure 2(B)). Decrease in the intracellular concentration of quercetin, evidently as a result of oxidation and biotransformations [30], results in reduced HIF-1α protein levels.

Induction of HIF-1α by quercetin occurs post-transcriptionally, involves protein stabilization and is PI-3K and MAPK-independent

The level of any protein reflects the balance between its synthesis and degradation. Experiments with the inhibitor of transcription actinomycin D showed that the induction of HIF-1α by quercetin was not dependent on new RNA synthesis (Figure 3(A)). To check the role of protein turnover, HIF-1α was induced by quercetin or DFO, an agent known to stabilize HIF-1α by inhibiting its proteolysis, and then new protein synthesis was inhibited by the addition of cycloheximide. In the presence of both quercetin and cycloheximide, HIF-1α protein levels were gradually reduced and became almost undetectable in 1 h (Figure 3(B)). A very similar decline in HIF-1α protein levels was also observed when DFO was used instead of quercetin. Taking into account that the half-life of HIF-1α under normoxic conditions is in the range of few minutes [1], which is less than what we observe, our data are consistent with the interpretation that quercetin, like DFO, stabilizes HIF-1α. However, in both cases the stabilization is not complete and ongoing translation is required for the maintenance of HIF-1α levels.

The phosphatidylinositol-3 kinase (PI-3K) and mitogen activated protein kinase (MAPK) pathways are involved in the regulation of HIF-1α levels or activity by various inducers [2,12,28,31]. Experiments with the specific PI-3K inhibitor, LY 294002 and the MEK (MAPKK) inhibitor, PD98059, at concentrations that completely blocked the activities of both kinases in HeLa cells (results not shown), showed that, as previously reported for HIF-1α induction by hypoxia or DFO [31], the induction of HIF-1α by quercetin, was not significantly dependent on the operation of these kinase signaling cascades (Figure 3(C)).

The data of Figure 3 demonstrate that induction of HIF-1α by quercetin occurs post-transcriptionally and is due to protein stabilization.

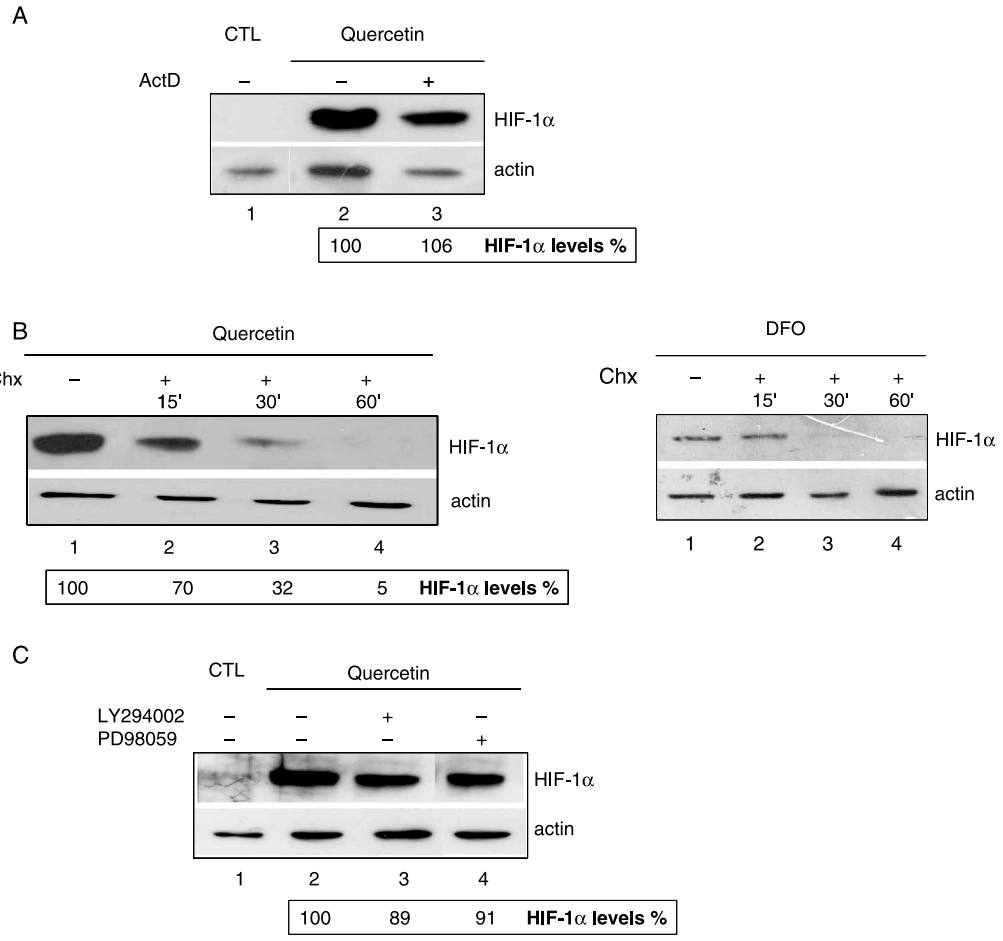

Figure 3. Characterization of HIF-1α induction by quercetin in HeLa cells by Western blot. Effects of inhibiting transcription, translation or PI3K and MAPK signaling. (A) Actinomycin D (Act D, 10 mg/ml) was added (lane 3) or not (lane 2) 15 min before culturing cells for 4 h with 100 μM quercetin. (B) HIF-1α was induced by culturing cells for 4 h with 100 μM quercetin or 150 μM DFO, as indicated (lane 1). Cycloheximide (Chx, 10 μg/ml) was subsequently added and incubation was continued for 15, 30 or 60 min. (lanes 2–4) (C) 50 μM LY294002 (lane 3) or PD98059 (lane 4) was added or not (lane 2) 15 min before culturing cells for 4 h with100 μM quercetin. Cells lysates were processed and results are expressed as in Figure 1. Each panel is representative of three independent experiments.

The pro-oxidant properties of quercetin are not implicated in HIF-1α induction

It is known that under some circumstances, quercetin can act as a pro-oxidant and contribute to the generation of reactive oxygen species (ROS) [32], which in turn can upregulate HIF-1α protein levels [33]. To investigate the possibility that quercetin induces HIF-1α by this mechanism, experiments were conducted in the presence of known radical scavengers, such as NAC, glutathione, Trolox, or ascorbic acid. Abrogation of HIF-1α expression by these antioxidants would implicate the involvement of ROS, and thereby the pro-oxidant properties of quercetin, in HIF-1α induction.

Addition of NAC or glutathione or Trolox, a water-soluble analogue of vitamin E, did not affect the expression of HIF-1α by quercetin (Figure 4 and results not shown). We recently, reported that these compounds similarly failed to affect the induction of HIF-1α by the iron–chelator DFO, whereas they decreased its induction by cobalt, a known ROS producer [31]. However, incubation in the presence of the reducing agent ascorbate (vitamin C), abolished the quercetin-induced expression of HIF-1α. Significantly, ascorbate has also been shown to abolish the induction of HIF-1α by DFO [34], something we confirmed in the present work.

We reasoned that this discrepancy between ascorbate and other antioxidants, could be the result of another, not radical scavenging, property of ascorbate, namely its ability to reduce Fe (III) to Fe (II). Thus, ascorbate may promote HIF-1α degradation by ensuring that the iron present in the active center of the PHDs is maintained in the Fe (II) state [15]. Additionally, since complexes of quercetin [23] or DFO [35] with Fe (II) have lower stability constants than with Fe (III), the presence of ascorbate may prevent effective iron chelation by these agents. Furthermore, by reducing Fe (III) to Fe (II), ascorbate can cause the release of iron from

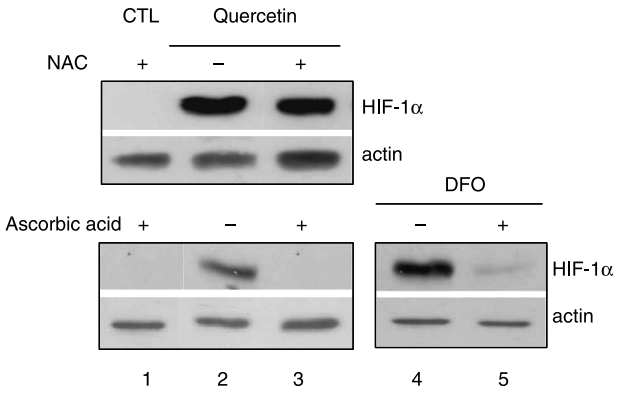

Figure 4. Characterization of HIF-1α induction by quercetin in HeLa cells by Western blot. Effects of antioxidants. NAC (5 mM) or L-ascorbate (100 μM) was added (lanes 1, 3) or not (lanes 2), 2 h before (NAC) or at the same time as (ascorbic acid) 100 μM quercetin (lanes 2, 3) and cells were cultured for 4 h. The effect of ascorbate was also examined in cells cultured with 150 μM DFO for 4 h. Cells lysates were processed and results are expressed as in Figure 1. Each panel is representative of three independent experiments.

ferritin [36], thereby increasing the availability of intracellular iron.

Taken together, the data of Figure 4 implicate not the pro-oxidant, but the iron-binding properties of quercetin in HIF-1α induction.

Ferric citrate abrogates the expression of HIF-1a and decreases the transcriptional activity of HIF-1 induced by quercetin in HeLa cells

To investigate the involvement of iron in the induction of HIF-1α by quercetin, we performed the experiment shown in Figure 5(A). Addition of excess ferric citrate (500 μM), completely abolished the expression of HIF-1α observed after 4 h incubation with 100 μM quercetin and this effect was observed when the excess of iron was added either 1 h before or after the inducer. A similar result was obtained when equimolar amounts (100 μM) of ferric citrate and quercetin were employed. However, in the presence of sub-equimolar amounts of ferric citrate (50 μM), abrogation of HIF-1α expression was only observed when iron was added prior to quercetin.

The effect of iron on the expression of HIF-1α was examined further by immunofluorescence miscroscopy (Figure 5(B)). HeLa cells cultured for 4 h with quercetin gave a strong signal for HIF-1α, demonstrating that quercetin-induced HIF-1α can enter the nucleus. When cells were similarly cultured in the presence of ferric citrate, HIF-1α was no longer detectable, in accord with our Western blot finding (Figure 5(A)).

Next, we examined the effect of iron on the transcriptional activity of the quercetin-induced HIF-1α using a reporter gene assay. Although, quercetin appeared to decrease transcription in general (results not shown), when the data obtained with the HRE-containing reporter plasmid (pGL3-5HRE-VEGF) were normalized with respect to the basal parental plasmid (pGL3-promoter), quercetin was found to produce a 2.6 fold increase in the transcriptional activity of HIF-1, which was reduced to 1.6 fold in the presence of 500μM ferric citrate (Figure 4(C)). These results show that HIF-1α induced by quercetin is indeed transcriptionally active. Furthermore, this transcriptional activity, like the activity of HIF-1 induced by DFO [31], is decreased by iron.

In order to verify that in these experiments the added ferric citrate did not prevent the cellular uptake of quercetin, we performed the experiment shown in Figure 5(D). HPLC analysis confirmed that addition to the growth medium of equimolar amounts (100μM) of quercetin and Fe (III), i.e. a concentration of iron that abolished the quercetin-induced expression of HIF-1α (Figure 5(A)), did not prevent the transport of quercetin from the extracellular to the intracellular compartment. This finding was observed whether iron was added 1 h before or after quercetin. However, the addition of iron affected the kinetics of quercetin uptake and the maximal concentration of intracellular quercetin (40μM) was observed earlier (at 1 h compared to 4 h in the absence of iron).

These results demonstrate that ferric citrate abolishes the induction of HIF-1α by quercetin suggesting that quercetin depletes iron and thereby inhibits an iron-dependent step required for HIF-1α induction. Moreover, together with the results of Figure 4, they suggest that the iron-chelating ability of quercetin can be saturated by an excess of iron provided either from intracellular stores or from extracellular supplementation.

Quercetin decreases the levels of calcein–chelatable iron and affects the expression of iron-regulated genes

In order to demonstrate directly that quercetin affects the levels of intracellular iron, we measured the iron present in HeLa cells by fluorescence microscopy, using calcein AM, a cell-permeable iron chelator that fluoresces when in the free, not complexed with Fe (II) or Fe (III), form. The advantage of using fluorescence microscopy is that one can also observe the localization of calcein and verify that the quenching of its fluorescence is caused by intracellular iron. Figure 6(A) shows that in control HeLa cells the fluorescence is very faint, indicating that the level of calcein–chelatable iron, i.e. the "free" or loosely bound iron present in the "labile iron pool" [37], the fraction of metabolically active iron, is high. In contrast, calcein fluorescence is very intense in cells cultured with quercetin for 4 h, demonstrating that the levels of iron present in the labile iron pool are

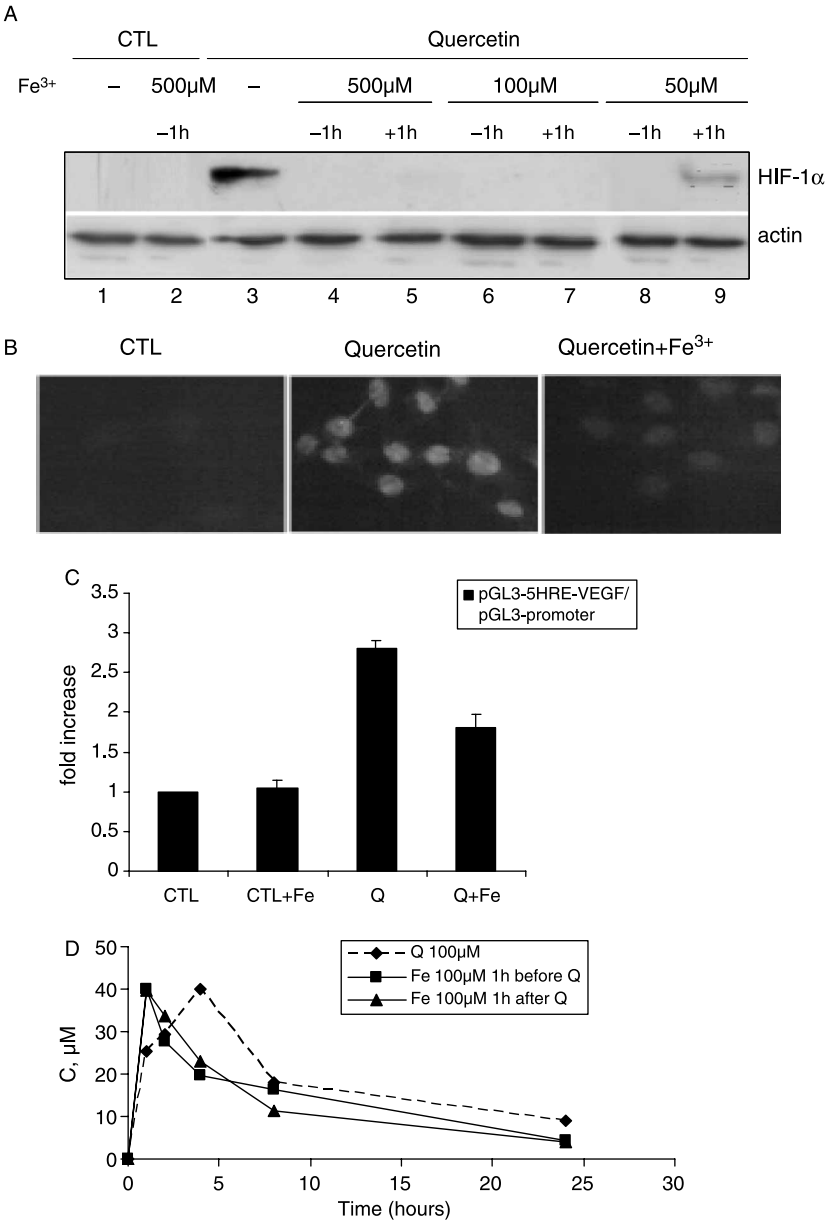

Figure 5. Effect of ferric citrate on HIF-1α expression in HeLa cells cultured with quercetin. (A) Analysis of HIF-1α expression by Western blot of cells cultured for 4 h with 100 μM quercetin, in the presence of 500, 100 or 50 μM ferric citrate (Fe^{3+}), added 1 h before (−1 h) or after (+1 h) the addition of quercetin. CTL: control cells, cultured without quercetin. (B) Detection of HIF-1α by immunofluorescence microscopy in cells cultured for 4 h without (control:CTL) or with 100 μM quercetin, in the absence or presence of 500 μM ferric citrate (Fe^{3+}). (C) Reporter gene (luciferase) assay of the transcriptional activity of HIF-1 upon induction by quercetin in transiently transfected HeLa cells. Results are expressed as fold increase of the luciferase activity (normalized to β-galactosidase activity) obtained from the pGL3-5HRE-VEGF versus the pGL3-promoter plasmids and are the means (± SD) of three separate experiments, performed in triplicate. (D) The effect of ferric citrate on quercetin uptake. HeLa cells were cultured with 100 μM quercetin, in the absence or presence of 100 μM ferric citrate (Fe), added 1 h before or 1 h after the start of the experiment and intracellular levels of quercetin were determined by HPLC. One of two independent experiments is shown.

decreased. As expected, high fluorescence was also observed in cells treated with the iron chelator DFO. Significantly, addition of excess ferric citrate or ascorbate to the quercetin-treated cells decreased calcein fluorescence to control levels, confirming that iron added either exogenously or provided from intracellular stores abrogates the iron depletion caused by quercetin. Similar results were obtained when, as a control, ferric citrate or ascorbate was added to DFO-treated cells. These data demonstrate that, like DFO, quercetin decreases the concentration of intracellular iron present in the labile iron pool, presumably by iron chelation.

To test the downstream biological effect of the quercetin-induced iron depletion, we examined the expression of TfR1, a protein the levels of which are

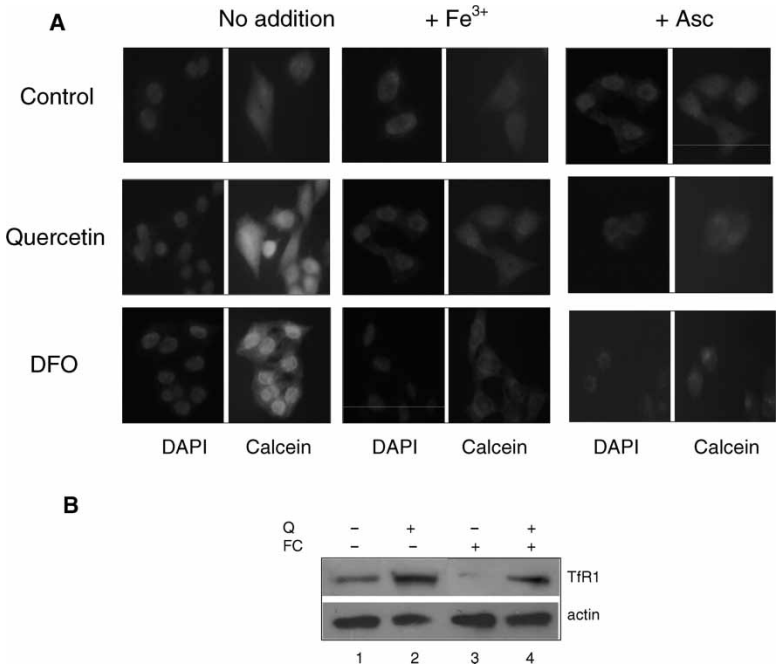

Figure 6. (A) Effect of ferric citrate and ascorbate on calcein-chelatable iron in quercetin-treated HeLa cells. Cells were cultured for 4 h without (control), or with 100 μM quercetin or 150 μM DFO, in the absence (no addition) or presence of 500 μM ferric citrate (+ Fe^{3+}) or 100 μM ascorbate (+ Asc). They were then incubated with calcein AM and DAPI and visualized as described in Materials and Methods. (B) Effect of quercetin and ferric citrate on the expression of TfR1. HeLa cells were cultured for 8 h with no addition or with 100 μM quercetin (Q), in the absence or presence of 500 μM ferric citrate (FC) and cell lysates were analysed for TfR1 expression, using actin as loading control.

post-transcriptionally controlled and inversely related to iron levels [38]. Figure 6(B) confirms that, as in the case of other iron chelators [39], the expression of TfR1 is increased after 8 h treatment with quercetin and that addition of excess ferric citrate decreases the levels of TfR1.

Ferric citrate reverses the quercetin-induced inhibition of HeLa cell proliferation

There is no consensus in the literature about the effects of quercetin on cell survival or proliferation (for reviews see Refs. [18,19]). Since we have shown that at least one of the biological effects of quercetin, namely the induction of HIF-1α, is caused by iron depletion, we examined whether the effects of quercetin on HeLa cell proliferation were also iron-dependent.

In initial experiments we determined that, although culturing HeLa cells with 100 μM quercetin for 4 h has no effect on cell numbers, 24 h incubation with quercetin inhibits cell proliferation with an IC50 of approximately 20 μM (results not shown). Addition of quercetin led to a prompt and concentration-dependent decrease in DNA synthesis, measured as incorporation of tritiated thymidine (Figure 7(A), left panel), which was more pronounced at 4 than at 24 h, presumably due to the higher intracellular concentration of quercetin at the earlier time (Figure 2(B)). Addition of 100 or 500 μM ferric citrate to cells cultured with 100 μM quercetin reversed this decrease in thymidine incorporation (Figure 7(A), right panel). DNA synthesis was again higher at 24 than at 4 h and, in the presence of excess ferric citrate, complete restoration was achieved.

To follow cell proliferation in the presence of 100 μM quercetin, cell numbers were recorded in parallel with thymidine incorporation for 48 h. Although, a progressive partial recovery of DNA synthesis was observed, this was not accompanied by a corresponding increase in cell numbers (Figure 6(B), bottom panels). Indeed, culturing with quercetin resulted in a decrease in cell numbers by 24 h. Nevertheless, when cells cultured with quercetin for up to 24 h were placed in fresh medium without quercetin, their rate of proliferation was similar to the control untreated cultures, indicating that the surviving cells had suffered no irreparable damage (results not shown). Moreover, when 500 μM ferric citrate was added to the cells during culturing with quercetin, complete recovery of DNA synthesis and very substantial restoration of cell numbers were observed (Figure 7(B), bottom panels). By comparison and for control purposes, addition of excess iron to the control cultures in the absence of quercetin had no effect on cell numbers, but produced a small increase in DNA synthesis at 24 h (Figure 7(B), top panels).

These data demonstrate that in HeLa cells the cytotoxic and anti-proliferative effects of quercetin are mainly due to iron depletion, since the decrease

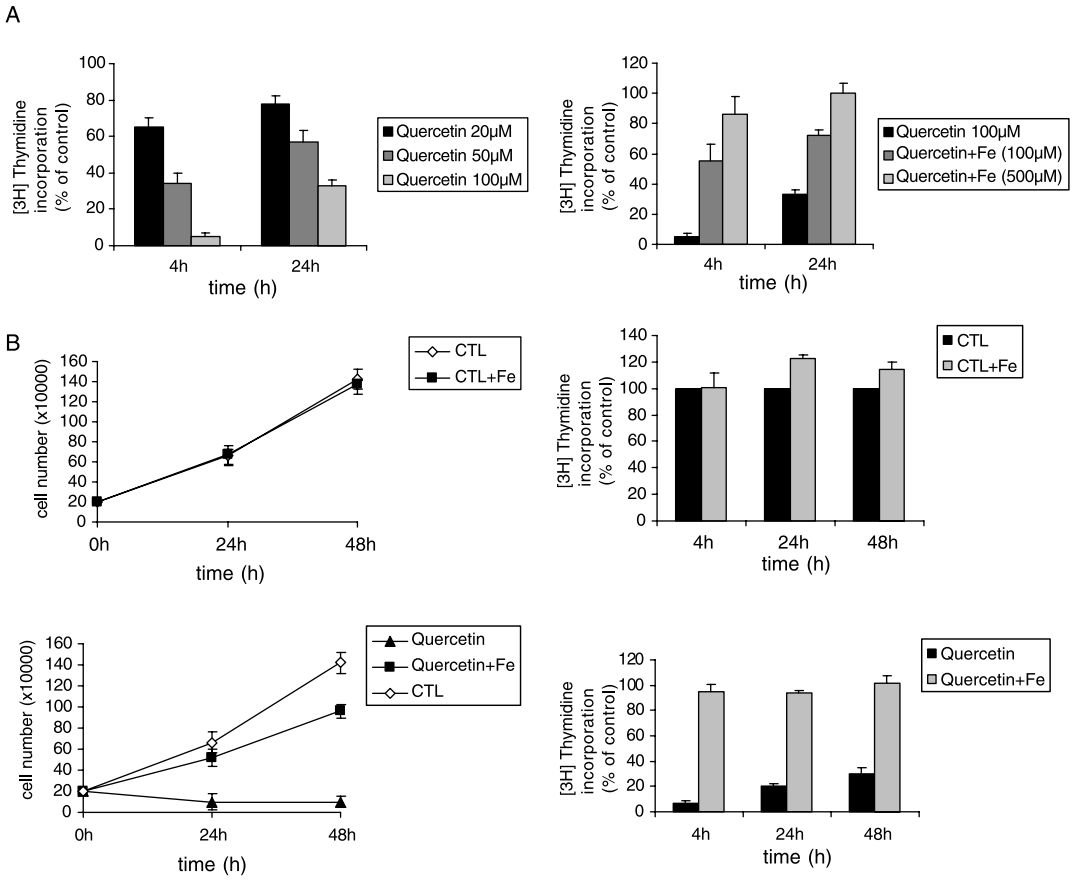

Figure 7. Effect of quercetin and ferric citrate on HeLa cell survival and proliferation. (A) (Left panel). Cells were cultured in the presence of 20, 50 or 100 μM quercetin for 4 or 24 h and the incorporation of [^{3}H]-thymidine was determined. (Right panel). Cells were cultured in the presence of 100 μM quercetin for 4 or 24 h without or with 100 or 500 μM ferric citrate, added 1 h before the start of the experiment, and the incorporation of [^{3}H]-thymidine was determined. (B) Cells were cultured without (CTL, top panels) or with 100 μM quercetin (bottom panels), in the absence or presence of 500 μM ferric citrate (Fe), added 1 h before the start of the experiment, and cell numbers (left panels) and [^{3}H]-thymidine incorporation (right panels) were assayed at 4, 24 and 48 h of culture. All results are the mean (±SD) of three independent experiments.

in both cell viability and proliferation are largely prevented by the addition of iron.

Ferric citrate restores the progression of the cell cycle in quercetin-treated HeLa cells

Iron depletion is known to affect cell growth and proliferation and to result in cell cycle arrest at the G1 phase [40,41]. To elucidate the effects of quercetin and iron on the progression of the HeLa cell cycle, we performed a propidium iodide cell cycle analysis (Figure 8). When cultured for 24 h with quercetin, a significantly greater percentage of viable cells were present in the G1 phase of the cell cycle (approximately 83% compared with 64% in the control cultures), with a corresponding decrease in the percentage of cells present in the S and G2/M phases. However, when 500 μM ferric citrate was added to the cells during culture with quercetin, the distribution of cells in the cycle resembled that of the untreated cultures, with a significant number of cells moving from the G1 to the S and G2/M phases. Addition of excess iron to the control cells also enhanced the progression of the cycle, producing a small decrease in the number of cells present in G1 and a corresponding increase in the cells present in the S and G2/M phases.

These results demonstrate that in HeLa cells addition of iron reverses the quercetin-induced cell cycle arrest at the G1 phase, restoring cycle progression.

Ferric citrate abrogates the expression of HIF-1α and reduces the inhibition of proliferation produced by quercetin in ASM cells

It was of interest to investigate to what extent our key findings with the epithelial cancer cell line HeLa could be duplicated with other cells. For this purpose we used primary cultures of ASMC, which, as previously shown in our laboratory [28], express HIF-1α in response to hypoxia or cobalt.

Addition of 100 μM quercetin to ASMC resulted in strong induction of HIF-α, which was observed by 1 h, reached a peak by 4 h and remained high at 24 h

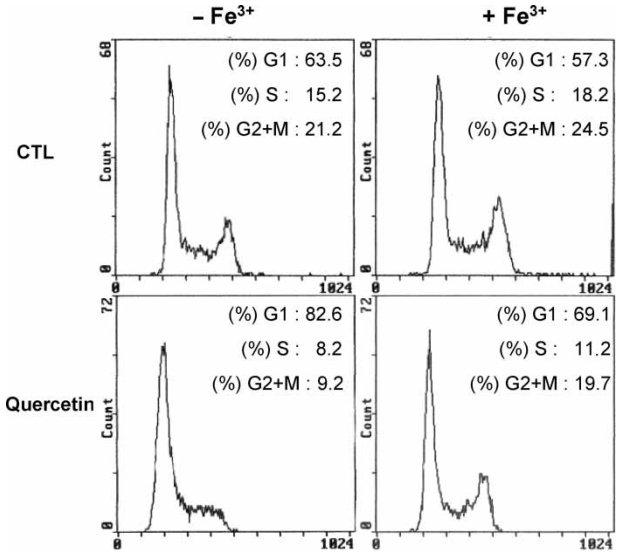

Figure 8. Flow cytometric analysis of the cell cycle in HeLa cells cultured with quercetin, with or without ferric citrate. Cells were cultured for 24 h without (CTL) or with 100 μM quercetin, in the absence or presence of 500 μM ferric citrate (Fe^{3+}), added 1 h before the start of the experiment. Cells were processed, stained with propidium iodide and analyzed, as described in Materials and Methods. The results are representative of three independent experiments.

(Figure 9(A)). The induction of HIF-1α by 4 h treatment with quercetin was completely abolished in the presence of excess ferric citrate (Figure 9(B)), in agreement with our findings in HeLa cells. Addition of quercetin to ASMC led to cessation of proliferation, with a small decline in cell numbers, and to a virtually complete stop of DNA synthesis within 4 h (Figure 9(C), bottom panels), but in contrast to HeLa cells (Figure 7), this inhibition of DNA synthesis was maintained throughout the 48 h period studied. Addition of 500 μM ferric citrate to quercetin-treated ASMC, reduced the inhibition of DNA synthesis caused by quercetin. The inhibition was almost completely reversed at 4 h, but the effect of ferric citrate was only partial (by approximately 25%) at later times. Nevertheless, addition of ferric citrate in the quercetin-treated cells resulted in a net increase in cell numbers by 48 h of culture (Figure 9(C), bottom panels). It should be noted that addition of excess iron to ASMC cultured in the absence of quercetin (Figure 9(C), top panels) led to a 25% decrease in DNA synthesis by 48 h, suggesting that these cells are adversely affected by iron overload. Thus, concerning the effect of iron on quercetin action, our results with ASMC are similar to those obtained with HeLa cells with respect to HIF-1α induction, but differ with respect to cell proliferation, since iron only partly reverses the inhibition caused by quercetin during prolonged (24–48 h) incubation.

Discussion

In recent years quercetin and other flavonoids have attracted a lot of interest, due to their putative health promoting activities, which have been attributed to various effects [17–21]. In attempting to investigate the mechanism by which quercetin induces HIF-1α [16], we hypothesized that many biological effects of quercetin may be mediated by iron-chelation. Our results demonstrate for the first time that two diverse responses to quercetin, induction of the transcription factor HIF-1α and inhibition of DNA synthesis and cell proliferation, are due to its iron-chelating properties, and show that both responses can be abrogated, albeit to different extents, by the addition of excess iron.

Our experiments were performed with 100 μM quercetin, a concentration which results in optimal induction of HIF-1α (Figure 1(A)) and is within the range (10–200 μM) reported in studies with quercetin in the literature [16,42–44]. The characteristics of this induction in HeLa cells, especially the abolition of the quercetin-induced expression of HIF-1α by ferric citrate or ascorbate (Figures 4 and 5), together with the direct demonstration that quercetin decreases the intracellular levels of calcein–chelatable iron and affects the expression of the iron-regulated protein TfR1 (Figure 6), are similar to the characteristics of induction of HIF-1α by DFO [31] and support the model that quercetin stabilizes HIF-1α from degradation due to iron depletion and consequent inhibition of an Fe^{2+}-dependent step. The binding of Fe (III) by quercetin is very tight ($\log K = 20$ [24]) and, although less strong than the binding of Fe (III) by DFO ($\log K = 32.5$ [25]), is orders of magnitude stronger than the binding of Fe (III) by anions, amino acids and other substances ($\log K$ about 10 [25]), thought to chelate the iron present in the "labile iron pool" physiologically. We therefore propose that cellular uptake of quercetin leads to chelation of iron in the labile iron pool, the concentration of which is reported to be less than 1 μM [37], thereby inhibiting the Fe^{2+}-dependent hydroxylation of HIF-1α by the PHDs [15]. Indeed, quercetin has been shown to inhibit two other O_2-, Fe^{2+}- and oxoglutarate-dependent enzymes: FIH-1, the asparagine hydroxylase affecting HIF-1α transcriptional activity, through a specific interaction observed in the presence of excess iron [45] and AlkB, the DNA repair enzyme involved in the adaptive response to DNA alkylation damage, through non-specific iron chelation [46]. Furthermore, another polyphenol, epigallocatechin gallate, was recently demonstrated to increase HIF-1α expression during normoxia through an iron-dependent step, by inhibiting its PHD-dependent degradation [47]. Lastly, the induction of HIF-1α by a related compound, epicatechin gallate, was also abrogated by iron or ascorbate [48]. It therefore

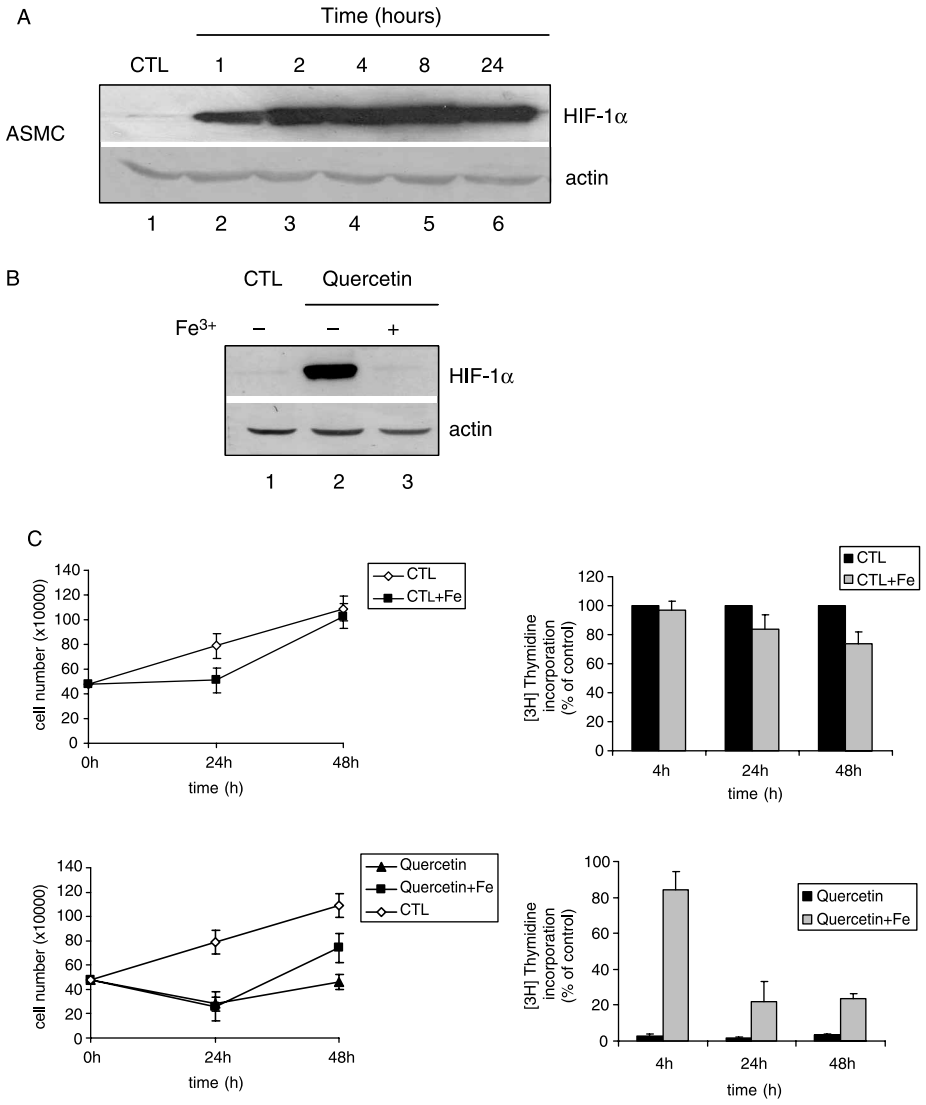

Figure 9. The effect of quercetin and ferric citrate on HIF-1α expression and cell survival and proliferation in ASMC. (A) Kinetics of HIF-1α expression. Cells were cultured in the presence of 100 μM quercetin for the indicated times (1–24 h) and lysates were prepared and analysed for HIF-1α protein expression by Western blot. CTL: control untreated cells. (B) Effect of ferric citrate on HIF-1α expression. Cells were cultured for 4 h with 100 μM quercetin (lanes 2, 3), without (lane 2) or with (lane 3) 0.5 mM ferric citrate (Fe^{3+}), added 1 h before the start of the experiment and HIF-1α expression was analysed by Western blot. CTL: control cells, cultured at normoxic conditions. (C) The effect of quercetin and ferric citrate on ASMC cell survival and proliferation. Cells were cultured, in the absence or presence of 0.5 mM ferric citrate (Fe), added 1 h before the start of the experiment, without (control) or with 100 μM quercetin, and cell numbers (left panels) and [^{3}H]-thymidine incorporation (right panels) were assayed at 4, 24 and 48 h of culture. All results are the mean (±SD) of three independent experiments.

appears that the predominant mechanism of HIF-1α induction by quercetin and other related chemical compounds involves the depletion of intracellular iron. However, certain questions are raised by the transient kinetics of HIF-1α expression (Figure 1(B)) together with the observed changes in intracellular concentrations of quercetin in HeLa cells (Figure 2). These effects may be due to the loss of the iron-binding properties of quercetin (due, for example, to its oxidation or other biotransformations), to alteration of the kinetics of its uptake or its bioavailability in the presence of ferric citrate (Figure 5(D)) or to the fact that its mechanism of action is not confined to metal chelation.

Quercetin has many properties besides metal chelation, including being a proton donor [22], a proteasome [49] and RNA polymerase [50] inhibitor and an ATP mimetic, inhibiting numerous kinases [18,51]. Several of these properties might also affect overall HIF-1α expression or activity, irrespective of, or as well as, iron chelation. For example, quercetin is a potent reducing agent [52], which like ascorbate, may release ferritin-bound iron [53] as Fe (II), which forms less stable complexes with quercetin than Fe

(III) [23]. Especially, in the presence of high levels of quercetin, this could result in an eventual increase in intracellular iron, which may down-regulate, in a negative feed back loop, the level of the HIF-1α protein. This scenario would explain the decrease in HIF-1α expression observed with 200 μM quercetin (Figure 1(A)). Alternatively, during prolonged cell culturing, quercetin may act as a pro-oxidant and contribute to the generation of ROS [32], which have various effects on HIF-1α expression [33]. The relative contribution of these possible mechanisms is unknown at present, but could be both quercetin concentration- and time-dependent.

Quercetin, at concentrations similar to the ones employed in the present study, has been reported either to promote or inhibit cell proliferation [18,43,44,54]. In both cell systems we tried quercetin severely affected growth, particularly at earlier times, when besides cessation of DNA synthesis cell death was noted, especially in the faster growing HeLa cells (compare Figures 7(B) and 9(C)). This growth inhibition by quercetin was dose-dependent, with an IC50 of about 20 μM and coincided with HIF-1α upregulation (Figures1(B) and 9(A)). Thus, a negative correlation appeared to exist between quercetin-induced HIF-1α protein levels and DNA synthesis, as previously reported for DFO [31]. The reported anti-proliferative activities of quercetin on some cells have been variously attributed to interference with DNA replication due to DNA damage [55], altered kinase signaling [51,54], cytoskeletal disorganization [56], interaction with nuclear estrogen receptors [57] and other effects [18]. Recently, it has been proposed that the beneficial or toxic cellular actions of quercetin and other flavonoids are due to their ability to modulate various protein and lipid kinase signaling cascades [51] and other signal transduction pathways [54], by competing with ATP binding. Here we show that a key explanation for the cytotoxic and anti-proliferative effects of quercetin is iron depletion which, as in the case of DFO and other iron-chelators [40,41], delays the progression of cells past the G1 phase of the cycle (Figure 8). This leads to inhibition of cell growth, but can be reversed by the addition of excess iron. However, the ability of added ferric citrate to restore cell proliferation varied: Fe (III) completely restored cell numbers, DNA synthesis and cell cycle progression in HeLa cells incubated with quercetin for up to 48 h (Figure 7(B)), but only partially in ASMC (Figure 9(C)). Thus, whereas the effects of quercetin on HIF-1α induction and inhibition of cell proliferation are in general similar in both cell types, the kinetics of HIF-1α expression and the ability of iron to restore DNA synthesis and cell growth are cell-type specific.

Our results do not address whether iron chelation, rather than inhibition of kinase signaling, is the primary event in the growth inhibition observed with quercetin or if induction of HIF-1α occurs *in vivo*. A key question is whether the relatively high concentrations of quercetin employed in our cell culturing experiments and in similar studies are likely to be present in humans. Quercetin levels of 50–100 μM are indeed difficult to achieve from conventional nutritional sources. Nevertheless, nowadays food supplements highly enriched in flavonoids, and particularly in quercetin, are emerging on the market and the possible misuse of such "health drugs" could lead to high quercetin concentrations in biological fluids and tissues. The net effect of the subsequent induction of HIF-1α and possible cell-type specific inhibition of growth, is difficult to predict, but might well be detrimental in some cases (e.g. cancer treatment).

The role of iron in cells is complex. Iron is essential for the function of numerous proteins and enzymes involved in energy metabolism, DNA synthesis and the progression of the cell cycle [40], but it can also generate, through the Fenton reaction, ROS [38], which have been implicated, besides oxidation of macromolecules, in cell signaling [58]. The different responses obtained with HeLa and ASM cells probably reflect differences in quercetin metabolism, iron requirements and iron and redox-sensitive signaling cascades, between these particular neoplastic and primary cells employed. Indeed, the sensitivity of cells to iron varies and rapidly dividing cells, like HeLa, may be both more dependent on iron and more capable of dealing with an iron overload than slowly proliferating cells, like ASMC. Furthermore, it is known that sustained iron depletion, by interfering with the function of ribonucleotide reductase and various cyclins, p53 and cyclin-dependent kinases or inhibitors, causes cell cycle arrest and can lead to irreversible inhibition of cell growth and induction of apoptosis [40]. Interestingly, in rat aortic smooth muscle cells quercetin was shown to have both proliferative and anti-proliferative effects, depending on the concentration employed [44]. Thus, the reported cytoprotective or cytotoxic effects of quercetin may be dose-dependent and reflect the delicate balance between the beneficial and adverse effects of iron, which has a different set point in different cell types.

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