ANTIOXIDANTS & REDOX SIGNALING
Volume 13, Number 4, 2010

DOI: 10.1089/ars.2009.3000

The Antioxidant Quercetin Inhibits Cellular Proliferation *via* HIF-1-Dependent Induction of p21WAF

Aline Bach,[1] Julia Bender–Sigel,[1] Dieter Schrenk,[2] Daniela Flügel,[1,3] and Thomas Kietzmann[1,3]

Abstract

Flavonoids are dietary antioxidants that may play a role as adjunct nutritional supplements in cancer or during inflammatory disorders. Hypoxia and the transcription factor hypoxia-inducible factor-1α also appear to play a key role in many human cancers. In this study, we investigated the role of quercetin in the hypoxia-dependent HIF-1α induction. It was shown that quercetin induced HIF-1α expression and HIF-1 activity under normoxia and hypoxia in human HepG2 hepatoma cells. By using actinomycin D and cycloheximide, we showed that quercetin acted post-transcriptionally by prolonging the HIF-1α protein half-life. Thereby quercetin interfered with the proline hydroxylation-dependent HIF-1α protein destabilization in the N-terminal HIF-1α transactivation domain. Experiments with quercetin analogues revealed that a flavonol structure and the presence of hydroxyl groups at position 3′ and 4′ are a prerequisite for the HIF-1α stabilizing effects. Further, quercetin inhibited cell proliferation and induced expression of the cell cycle inhibitor p21WAF and knocking-down HIF-1α disrupted these effects. These results provide evidence that quercetin inhibits the cell cycle and that induction of the HIF-system contributes to these effects of quercetin. *Antioxid. Redox Signal.* 13, 437–448.

Introduction

Flavonoids are a large group of polyphenols that occur naturally in nutrients of plant origin. They possess a common phenylbenzopyrone structure (C6–C3–C6), and are categorized according to the saturation level and opening of the central pyran ring, mainly into flavones, flavanols, isoflavones, flavonols, flavanones, and flavanonols (12). Flavonoids possess a variety of biological activities at nontoxic concentrations in organisms, and especially the anti-inflammatory and anticancer actions are of major interest (12, 47). The flavonol quercetin is the most common flavonoid in nature, and the average human intake has been estimated to be ~20 mg/day (12, 47). Although it was suggested that quercetin acts due to its antioxidant and free-radical scavenging properties, the molecular details of quercetin effects associated with antithrombotic, antihypertensive, anti-inflammatory, and anticarcinogenic actions (5) are not yet fully elucidated.

Hypoxia is a major feature of a number of diseases; almost all solid tumors contain hypoxic regions in which O_2 concentrations are greatly reduced compared to the surrounding normal tissue. It is well known that these hypoxic conditions influence the expression of various proteins involved in proliferation and cell cycle progression (38). Thereby, the transcription factor hypoxia-inducible factor-1 (HIF-1) appears to be of special importance by inducing more than 100 genes (30), among them those encoding proteins that are involved in regulating key aspects of the tumor cell phenotype, including cell immortalization and de-differentiation, stem cell maintenance, genetic instability, glucose uptake and metabolism, pH regulation, autocrine growth/survival, angiogenesis, invasion/metastasis, and resistance to chemotherapy. In addition, a number of studies from various tumor entities showed that increased levels of hypoxia-inducible transcription factors (HIFs), especially HIF-1α are associated with a poor prognosis.

HIF-1 is a heterodimeric protein, consisting of two subunits, HIF-1α and HIF-1β, also known as arylhydrocarbon receptor nuclear translocator protein (ARNT). Whereas HIF-1β is expressed constitutively, HIF-1α is increased under hypoxia. Under normoxia HIF-1α is hydroxylated by the action of a family of prolyl hydroxylases (PHDs) and an asparagine hydroxylase (for review, see Ref. 17). The proline hydroxylation at P402 and P564 of human HIF-1α promotes binding of the von Hippel–Lindau (VHL) tumor suppressor protein and degradation *via* a proteasomal pathway (for review, see Refs. 17 and 37). Asparagine hydroxylation at N803 prevents recruitment of the coactivator p300 (26). Under hypoxia, hydroxylase activity is impaired and hydroxylation of HIF-1α is decreased. This leads to its accumulation, dimerization with HIF-1β, and transcriptional activation of

Departments of [1]Chemistry/Biochemistry and [2]Food Chemistry and Toxicology, University of Kaiserslautern, Kaiserslautern, Germany.
[3]Department of Biochemistry, University of Oulu, Oulu, Finland.

target genes *via* binding to hypoxia responsive elements (HREs) (43). Within the last decade a number of investigations, including our own, have shown that HIF-1α is also responsive to different stimuli including growth and prothrombotic factors, inflammatory cytokines, as well as reactive oxygen species (ROS) under nonhypoxic conditions (for review, see Ref. 19). Thus, it seems conceivable that a number of anti-tumorigenic effects of nutrients or drugs may be mediated by their antioxidant potential which subsequently may lower HIF-1 activity.

Despite the promising actions of quercetin and its antitumor activities, the molecular mechanisms underlying theses effects are generally unknown. Since it was previously shown that quercetin may have a negative (10, 26) as well as a positive (15, 39) effect on HIF-1α in different cell types, it was the aim of the present study to investigate whether quercetin has an impact on HIF-1α in HepG2 hepatoma cells and to elucidate the effect of quercetin and other flavonoids on the hypoxia-dependent HIF-1α regulation and on cell proliferation under normoxia and hypoxia.

Materials and Methods

All biochemicals and enzymes were of analytical grade and from commercial suppliers.

Cell culture

HepG2, HT29, Colo320, and DLD1 cells were cultured in a normoxic atmosphere of 16% O_2, 79% N_2, and 5% CO_2 (by vol.) in MEM supplemented with 10% FCS for 24 h. At 24 h, medium was changed and culture was continued at normoxia 16% O_2 or at hypoxia 8% O_2 (87% N_2, 5% CO_2 (by vol.)).

Measurement of ROS production

The generation of ROS was measured using the fluoroprobe 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA, Molecular Probes, Göttingen, Germany) as described (9).

Plasmid constructs

The reporter plasmids, pGl3-Epo-HRE-Luc, pXRE-Luc, pNFkB-Luc (Stratagene, Heidelberg, Germany), pATF6-Luc, and pARE-Luc contain multiple transcription factor binding elements; the pGl3-EpoHRE-Luc contains three hypoxia responsive elements (HRE) from the erythropoietin gene binding the hypoxia-inducible factor (HIF) (18), pXRE-Luc (34) contains three dioxin receptor (AHR/ARNT) binding elements, the pATF6-Luc three binding sites for the transcription factor ATF-6 (41), pNFkB-Luc contains NFkB binding sites, and pARE-Luc contains the antioxidative response element binding the transcription factor Nrf2 (8). The plasmid p21-Luc, containing the human p21 promoter (36) and short hairpin RNA encoding for 19mer shRNA against HIF-1α and for control shRNA (1) were described and used with the siSTRIKE U6 Hairpin Cloning System (Promega, Heidelberg, Germany).

The constructs for pG_5E1B-LUC, pSG424, and for Gal4-HIF1α-TADN, Gal4-HIF1α-TADNM, Gal4-HIF1α-TADC, and Gal4-HIF1α-TADCM (27) were already described. In Gal4-HIF1α-TADNM and Gal4-HIF1α-TADCM proline P564 or asparagine N803 was mutated to alanine, respectively.

RNA preparation and Northern blot analysis

Isolation of total RNA and Northern blot analysis were performed as described (20). Digoxigenin (DIG)-labeled antisense RNAs served as hybridization probes; they were generated by *in vitro* transcription from pCRII-HIF1α and pBS-Actin using T7 polymerase. Blots were quantified with a videodensitometer (Biotech Fischer, Reiskirchen, Germany).

Western blot analysis and determination of HIF-1α protein half-life

Western blot analysis was carried out as described (20). For half-life studies, HepG2 cells were cultured as above and after 24 h cycloheximide (100 μg/ml; Sigma, Deisenhofen, Germany) and/or quercetin was added to the media. At indicated time points thereafter, cells were removed and protein abundance was measured by immunoblot analysis. In brief, media or lysates from HepG2 cells were collected and 100 μg of protein were loaded onto a 10% or 7.5% SDS-polyacrylamide gel and after electrophoresis and electroblotting to a methanol-activated PVDF membrane, proteins were detected with a mouse monoclonal antibody against human HIF-1α (1:2000, BD Biosciences, Heidelberg, Germany), Golgi membrane (GM) (1:10000, Biosciences Göttingen, Germany) or human p21 (1:1000, Santa Cruz, Heidelberg, Germany). The secondary antibody was an anti-mouse IgG HRP (1:5000, Sigma). The ECL system (GE Healthcare, Freiburg, Germany) was used for detection.

Cell transfection and luciferase assay

About 4×10^5 HepG2 cells per 60 mm dish were transfected as described (20). In brief, 2.5 μg of Luc reporter plasmids were transfected. Transfection efficiency was controlled by cotransfection of 0.25 μg *Renilla* luciferase expression vector (pRLSV40) (Promega). The detection of luciferase activity was performed with the Luciferase Assay Kit (Berthold, Pforzheim, Germany). To investigate HIF-1α transactivation, 2 μg reporter construct pG5-E1B-Luc were cotransfected with 500 ng of the Gal4-HIF1αTADN, Gal4-HIF1αTADC, or respective mutant constructs (27). After 5 h, medium was changed and cells were cultured under normoxia for 12 h, then stimulated with quercetin or quercetin analogues and further cultured for 24 h under normoxia or hypoxia.

For shRNA experiments, cells were transfected with 10 or 20 μg psiSTRIKE U6-HIF-1alpha. After transfection, cells were cultured for the next 24 h under normoxia before conducting the experiment.

Proliferation assay

Proliferative activity was evaluated by 5-bromo-2′deoxyuridine (BrdU) labeling (Roche Diagnostics, Roche, Mannheim, Germany) according to the manufacturer's instructions. The absorbance values obtained in the ELISA with the cells under normoxia were set to 100%; values measured under hypoxia were then normalized to the values obtained under hypoxia.

Statistical analysis

Each experiment was performed at least three times and representative data are shown. Data in bar graphs are given as

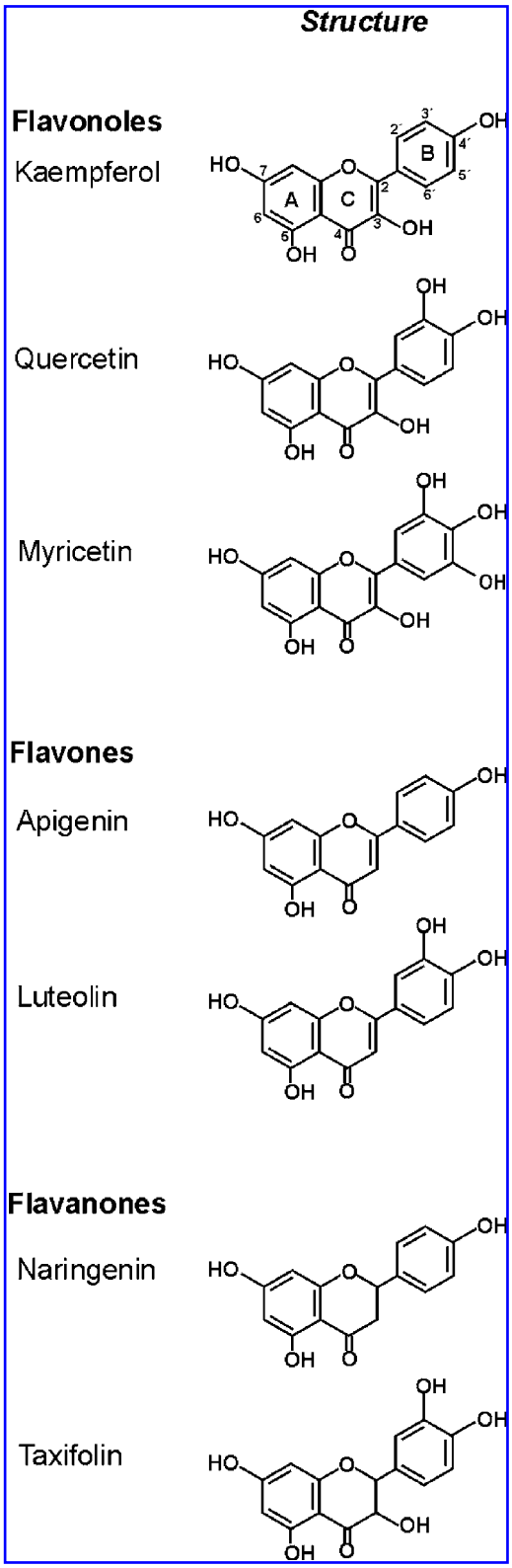


FIG. 1. Chemical structure of different flavonoids. Flavonoids possess a common 2-phenyl-1,4-benzopyrone structure with two aromatic rings (A and B), and can be categorized according to the saturation level and opening of the central pyran ring (C) and the substituent at position 3. The position 7 on the A ring and positions 2′,3′,4′, on the B ring are frequently occupied by various substituents, mostly hydroxyl (OH) groups.

mean values ± standard error of the mean (s.e.m.). Statistical differences were calculated by using the Student *t*-test with error probabilities of $p < 0.05$ to be significant.

Results

Reduction of ROS levels by flavonols, flavones, and flavanones

To investigate whether quercetin and other flavonoids have the potential to reduce ROS levels in HepG2 cells, we compared the effect of hypoxia with that of quercetin and other flavonols, flavones, and flavanones. The additional flavonols used were kaempferol and myricetin, the flavones were apigenin and luteolin and the flavanones were naringenin and taxifolin (Fig. 1). In line with previous reports, hypoxia decreased ROS levels by about 30% and as expected, quercetin as well as all other tested flavonols, flavones and flavanones, when added at a concentration of 100 μM, reduced ROS levels by about 20% (Fig. 2).

Different induction of HIF-1α protein levels and HIF-1 activity by quercetin, myricetin, and luteolin

In the next experiments we tested whether quercetin and all other used flavonols, flavones, and flavanones have an impact on the hypoxia-dependent induction of HIF-1α. To do this, cultured HepG2 cells were treated as above with 100 μM quercetin, kaempferol, myricetin, apigenin, luteolin, naringenin, and taxifolin under normoxia (16% O_2) or hypoxia (5% O_2), respectively, and HIF-1α protein levels were analyzed by Western blotting. Surprisingly, when HepG2 cells were treated with quercetin, myricetin, and luteolin for 4 h, HIF-1α protein levels were induced under normoxia and hypoxia, although the inducible effect under hypoxia was less dominant

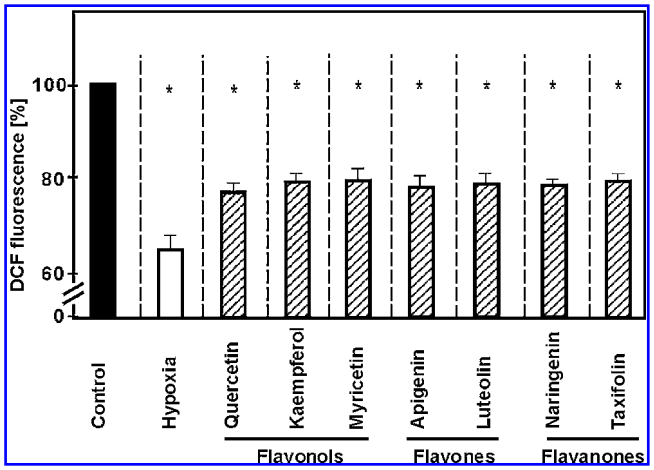
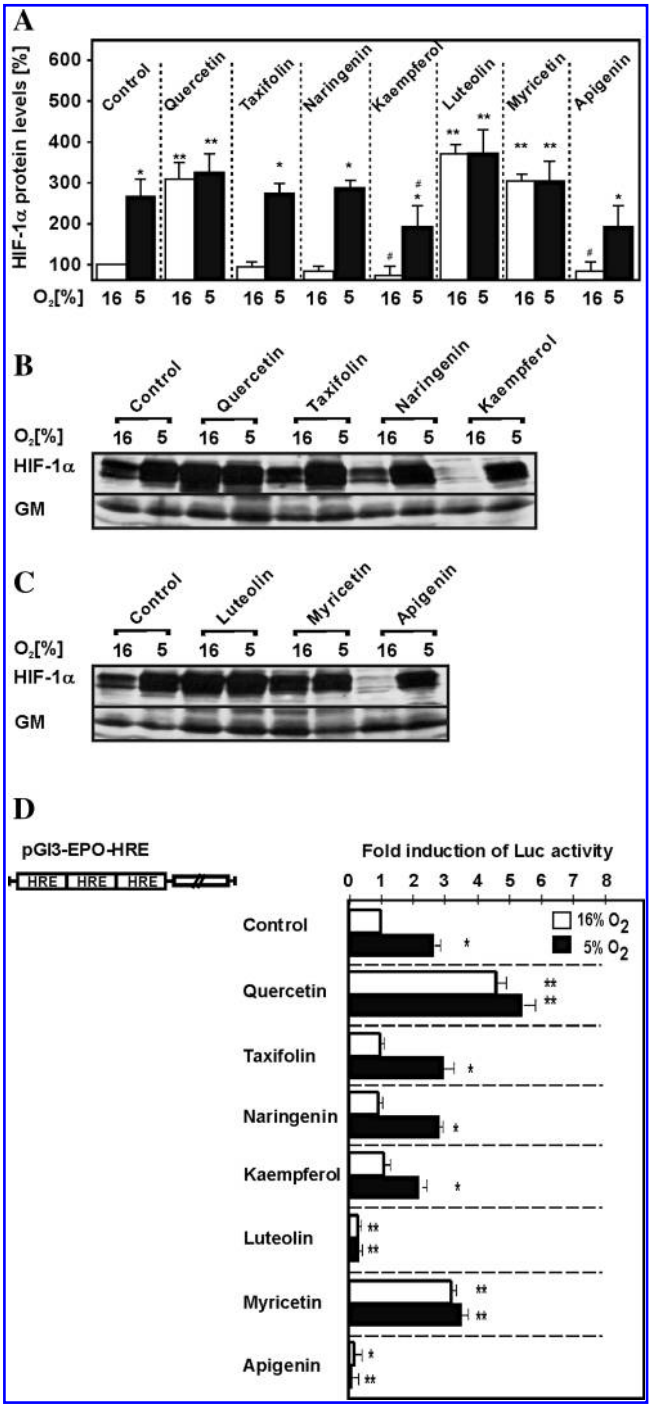


FIG. 2. Reduction of ROS levels by hypoxia and various flavonoids. Cultured HepG2 cells were exposed either to hypoxia (5% O_2) or treated under normoxia (16% O_2) with the flavonols quercetin, kaempferol, myricetin, the flavones apigenin and luteolin, as well as the flavanones naringenine and taxifolin (100 μM) for 30 min. ROS generation was determined by DCF fluorescence. In each experiment DCF fluorescence at 16% O_2 was set to 100%. Values are means ± SEM of three independent culture experiments; *significant difference 16% O_2 vs. 5% O_2 or vs. flavonoid treatment.

than that under normoxia. The flavonol kaempferol, the flavone apigenine and the flavanones naringenin and taxifolin had no effects (Figs. 3A–3C).

To find out whether the induction of HIF-1α mediated by quercetin, myricetin, and luteolin also resulted in enhanced HIF-1 activity, reporter gene analyses were performed with a luciferase construct (pGL3-EPO-HRE) that contains three HIF-binding hypoxia responsive elements (HRE) as enhancer elements in front of the promoter. In HepG2 cells transfected with the plasmid pGL3-EPO-HRE, luciferase (Luc) activity was induced by about 3-fold under hypoxia (Fig. 3D). The treatment of transfected HepG2 cells with quercetin increased Luc activity by about 5-fold under normoxia or hypoxia, respectively. Similarly, myricetin also enhanced Luc activity by about 3.5-fold under normoxia and under hypoxia (Fig. 3D). Taxifolin, naringenin, and kaempferol did not affect Luc activity under normoxia or the hypoxia-mediated induction of Luc activity. Although luteolin enhanced HIF-1α protein levels, surprisingly it decreased Luc activity under normoxia and hypoxia. Further, apigenin decreased Luc activity under both normoxia and hypoxia and abolished thereby the induction of Luc activity by hypoxia (Fig. 3D). Together, these data indicate that a flavonol structure and presence of hydroxyl groups in the 3′ and 4′ position as in quercetin and myricetin induce HIF-1α protein levels and HIF-1 activity. By contrast, the only presence of the hydroxyl groups in the 3′ and 4′ position together with a flavone structure as in luteolin only allows HIF-1α protein induction whereas a flavanone structure like in naringenin and taxifolin has no effects on HIF-1α irrespective of the presence of the hydroxyl groups in the 3′ and 4′ position.



Quercetin induces HIF-1α protein levels via a post-transcriptional mechanism

Given the striking and stable induction of HIF-1α after treatment with quercetin, we hypothesized that quercetin may act in a manner like hypoxia. To gain further insight into this regulation, we compared the induction by quercetin by that mediated by hypoxia. We found that the enhancement of HIF-1α by quercetin under normoxia proceeded in a manner almost similar to that mediated by hypoxia. The hypoxia-dependent induction of HIF-1α was clearly visible after 1 h and reached maximal values after 4 h. Then, HIF-1α levels remained stable until 12 h, where they started to decline (Figs. 4A and 4B). These data are in line with the idea that quercetin may influence HIF-1α levels *via* a similar mechanism as hypoxia.

FIG. 3. Different inductions of HIF-1α protein and HIF-1 activity by the flavonols, flavones, and flavanones. (**A**) HepG2 cells were treated with 100 μmol of each quercetin, kaempferol, myricetin, apigenin, luteolin, naringenine, and taxifolin and further cultured under normoxia (16% O_2) or hypoxia (5% O_2) for 4 h. The HIF-1α protein level measured under normoxia was set to 100%. Values are means ± SEM of three independent culture experiments; *significant difference 16% O_2 vs. 5% O_2; **significant difference 16% O_2 or 5% O_2 vs. flavonoid treatment. (**B, C**) Representative Western blot. 100 μg of protein from the HepG2 cells were subjected to Western analysis with an antibody against HIF-1α or Golgi membrane (GM). Autoradiographic signals were obtained by chemiluminescence and scanned by videodensitometry. (**D**) HepG2 cells were transfected with a Luc gene construct containing three copies of the EPO HRE element in front of the SV40 promoter (pGl3EPO-HRE). The transfected cells were treated with 100 μmol of each quercetin, kaempferol, myricetin, apigenin, luteolin, naringenine, and taxifolin, and further cultured for 24 h under normoxia (16% O_2) or hypoxia (5% O_2). In each experiment the LUC activity of pGL3-EPO-HRE at 16% O_2 was set to 1, respectively. Values are means ± SEM of three independent culture experiments; *significant difference 16% O_2 vs. 5% O_2; **significant difference 16% O_2 or 5% O_2 vs. flavonoid treatment; #significant difference 16% O_2 or 5% O_2 vs. flavonoid treatment.

We next aimed to investigate whether this enhancement of HIF-1α levels was due to transcriptional or post-transcriptional regulation. We have previously reported that the redox sensitive transcription factor NFkB can mediate a transcriptional activation of HIF-1α (1). Therefore, we tested whether the effects of quercetin may affect also the activity of NFkB and other transcription factors. Since HIF-1 is a dimer consisting of HIF-1α and ARNT, and quercetin was also found to be bound by the AHR (34), we included a construct regulated by the xenobiotica responsive element that could be bound by the dioxin receptor consisting of AHR and ARNT. In addition, we have reported that generation of ROS at the endoplasmic reticulum (ER) affects HIF-1α stability (27), and to verify whether quercetin induces effects on the ER like ER stress, we included a construct that is regulated by the ER stress responsive transcription factor ATF-6. In line with our above findings that quercetin reduces ROS levels, we also intended to include a construct regulated by the antioxidant responsive element (ARE) binding the factor Nrf2.

By using these constructs in luciferase reporter gene assays, we found that in addition to the HRE-Luc construct, the NFkB construct was upregulated by hypoxia in line with previous findings (1, 23). Although quercetin again induced HRE-Luc activity by about 5-fold, it reduced the pNFkB-Luc activity in line with the idea that a reduction of ROS levels decreases NFkB activity. Further, quercetin increased the Luc activity from the pXRE-Luc construct by about 2.5-fold under both normoxia and hypoxia. In addition, quercetin had no influence on the ATF-6 regulated Luc activity whereas it induced Luc activity from the pARE-Luc construct by about 3-fold under normoxia and by about 4-fold under hypoxia (Fig. 4C). These data suggest that quercetin does not influence HIF-1α levels *via* transcriptional induction of NFkB, ATF-6, or Nrf2.

To find out whether post-transcriptional mechanisms are involved in the quercetin-dependent HIF-1α induction, HepG2 cells were pretreated with the transcription inhibitor actinomycin D (Act D) or translation inhibitor cycloheximide (CHX) for 30 min prior to stimulation with quercetin. While CHX completely abolished the hypoxia-dependent HIF-1α protein induction, Act D only reduced it by about 50%. It was then found that pretreatment with CHX inhibited HIF-1α induction mediated by both hypoxia and quercetin. In contrast, quercetin could still induce HIF-1α protein levels in the presence of Act D (Figs. 5A and 5B). These results indicated that the induction of HIF-1α by quercetin occurred post-transcriptionally and needed *de novo* synthesis of HIF-1α protein in the cells. To further confirm this, HIF-1α mRNA expression was detected by Northern blot analysis. In line with previous studies (28, 44), we found that hypoxia upregulated HIF-1α mRNA abundance by about 2-fold. By contrast, treatment with quercetin did not show significant increases in HIF-1α mRNA abundance under both normoxia and hypoxia (Figs. 5C and

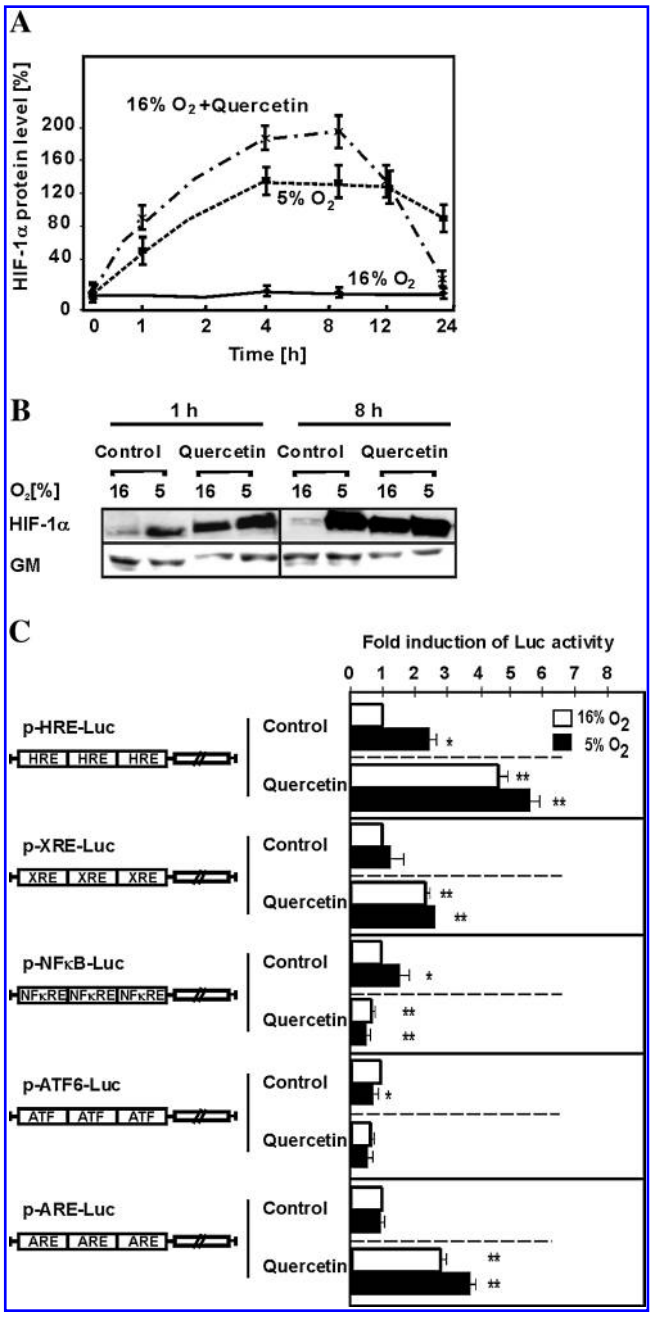


FIG. 4. Modulation of quercetin-dependent HIF-1α induction by actinomycin D (Act D) and cycloheximide (CHX). HepG2 cells were pre-treated with Act D (5 μg/ml) or CHX (10 μg/ml) for 30 min, then stimulated with quercetin (100 μM) under normoxia (16% O_2) or hypoxia (8% O_2) for 4 h. HIF-1α protein was detected by Western blot analysis and HIF-1α mRNA was detected by Northern blot analysis, respectively. (**A**) The expression of HIF-1α protein under normoxia was set to 100%. Values are means ± SEM of three independent culture experiments; *significant difference 16% O_2 vs. 5% O_2; **significant difference 16% O_2 or 5% O_2 vs. quercetin; + significant difference 16% O_2 or 5% O_2 with actinomycin vs. 16% O_2 or 5% O_2 with actinomycin and quercetin. (**B**) Representative Western blot. 100 μg of protein from the whole-cell extract were subjected to Western analysis with an antibody against HIF-1α or Golgi membrane (GM). Autoradiographic signals were obtained by chemiluminescence and scanned by videodensitometry. (**C**) The expression of HIF-1α mRNA under normoxia was set to 100%. Values are means ± SEM of three independent culture experiments; *significant difference 16% O_2 vs. 5% O_2. (**D**) Representative Northern blot analysis. 20 μg of total RNA of each sample were subjected to Northern blot analysis with HIF-1α or β-actin antisense RNA probes. Autoradiographic signals were obtained by chemiluminescence and scanned by videodensitometry.

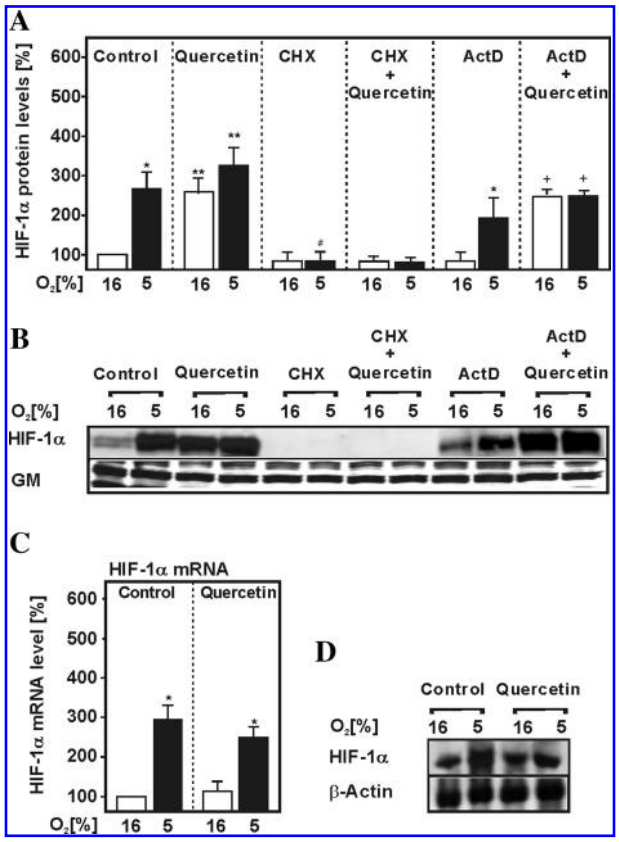


FIG. 5. Time-dependent modulation of HIF-1α protein expression by A23187 and BAPTA-AM. (**A**) HepG2 cells were incubated under normoxia (16% O_2) or hypoxia (5% O_2) or treated with quercetin (100 μM) under normoxia (16% O_2) and then harvested at different time points. HIF-1α protein was detected by Western blot analysis. The expression of HIF-1α under normoxia at 0 h was set to 1. Values are means ± SEM of three independent culture experiments. (**B**) Western blot analysis. 100 μg of protein from HepG2 cells treated with quercetin (100 μM) for 4 h were subjected to Western analysis with an antibody against HIF-1α or Golgi membrane (GM). Autoradiographic signals were obtained by chemiluminescence and scanned by videodensitometry. (**C**) HepG2 cells were transfected with Luc gene constructs containing either three copies of the hypoxia-inducible factor (HIF) binding HRE element (pHRE-Luc), the dioxin receptor (AHR/ARNT) binding xenobiotica response element (pXRE-Luc), the ER stress responsive ATF6 binding element (pATF6-Luc), copies of the nuclear factor kB response element (pNFkB-Luc), and the antioxidative response element binding the transcription factor Nrf2 in front of the promoter. The transfected cells were treated with 100 μmol quercetin and further cultured for 24 h under normoxia (16% O_2) or hypoxia (5% O_2). In each experiment the LUC activity of the respective control at 16% O_2 was set to 1. Values are means ± SEM of three independent culture experiments; *significant difference 16% O_2 vs. 5% O_2; **significant difference 16% O_2 or 5% O_2 vs. quercetin treatment in the same group; #significant difference 5% O_2 vs. 5% O_2 with quercetion treatment; + significant differences 16% O_2 or 5% O_2 with actinomycin vs. 16% O_2 or 5% O_2 with actinomycin and quercetin (D) Representative Northern blot analysis. 20 mg of total RNA of each sample were subjected to Northern blot analysis with HIF-1a or b-actin antisense RNA probes. Autoradiographic signals were obtained by chemiluminescence and scanned by videodensitometry.

5D). These results show that quercetin induced HIF-1α levels mainly post-transcriptionally.

Quercetin enhances HIF-1α protein stability via inhibition of proline hydroxylases

In order to gain further support that quercetin affects HIF-1α protein stability, we determined the half-life of HIF-1α in response to quercetin. To do this, we transfected HepG2 cells with vectors encoding full-length wild-type V5-tagged HIF-1α and thereafter the cells were either treated only with cycloheximide to block ongoing protein synthesis or both, cycloheximide and quercetin. Then, the HIF-1α protein levels were determined by Western analysis with the antibody against the V5-tag. Our data show that, compared to the controls treated only with cycloheximide, quercetin induced the half-life of HIF-1α by about 2-fold (Figs. 6A and 6B).

These results and the data from our experiments with Act D and cycloheximide led to the hypothesis that quercetin can interfere directly with the proline and asparagine hydroxylase mechanisms which influence HIF-1α stability or transactivation. There are two transactivation domains (TADs) present in HIF-1α referred to as amino-terminal (TADN) and carboxy-terminal (TADC) TAD. To investigate whether quercetin may interfere with HIF-1α transactivation, HepG2 cells were cotransfected with the luciferase reporter construct pG5-E1B-Luc that contains 5 copies of a Gal4 response element and vectors allowing expression of fusion proteins consisting of the Gal4-DNA binding domain (Gal4) and either HIF-1α TADN or TADC.

We found that HIF-1α TADN and TADC transactivity could be significantly induced by hypoxia in line with previous studies (3, 16, 27, 35). Treatment with quercetin under normoxia also increased HIF-1α TADN activity by about 2-fold whereas quercetin had no effect on TADC transactivity (Fig. 6C). It has been well demonstrated that the key amino acid proline 564 in the TADN plays a crucial role in HIF-1α stabilization (37). It can be hydroxylated by HIF prolyl hydroxylases (PHDs) under normoxia, thereby mediating the interaction of HIF-1α and VHL which then targets HIF-1α for proteasomal degradation. The mutation of the critical amino acid proline (P 564) in the construct Gal4-HIF1αTADNM, which led to a hydroxylation-resistant fusion protein, caused an increase in transactivity under normoxia and a loss of the response to hypoxia and quercetin (Fig. 6C). Furthermore, asparagine (N 803) in HIF-1α TADC can also be hydroxylated by another hydroxylase named FIH and thus block the interaction of HIF-1α and p300/CBP (11, 25, 29). While this mutation of asparagine 803 to alanine enhanced transactivation, it also abolished hypoxic induction but again, quercetin had no effect on TADCM activity (Fig. 6C). The induction of HIF-1α TADN transactivity by quercetin indicated that it might accumulate HIF-1α through protein stabilization. To test this we measured the Gal4TADN and -TADC protein levels. In line with the transfection assays, hypoxia and quercetin enhanced Gal4-HIF-1αTADN levels while the levels of the degradation resistant Gal4-HIF1αTADNM (P564A) were enhanced and no longer upregulated by hypoxia or quercetin. In contrast, the Gal4-HIF1αTADC and Gal4-HIF1αTADCM (N803A) protein levels were not regulated by hypoxia in line with other studies (6, 16) and quercetin (Fig. 6D). Together, these data along with the transfection assays

indicate that quercetin appears to influence HIF-1α protein stability *via* inhibition of HIF-proline hydroxylation.

Quercetin inhibits cell proliferation via *HIF-1α-dependent induction of p21WAF*

Since quercetin appears to have a negative impact on cellular proliferation, we next tested whether this role may involve HIF-1α. To do this, HepG2 cells were transfected with a vector allowing expression of shRNA against HIF-1α, and BrdU incorporation was measured either in the presence or absence of quercetin under normoxia and hypoxia. For comparison cells were also treated with apigenin which had a negative effect on HIF-1α (Fig. 3). Evaluation of BrdU incorporation showed that hypoxia slightly increased proliferation by about 1.2-fold. By contrast, quercetin and apigenin reduced proliferation by about 50% under both normoxia and hypoxia. While the quercetin effect was completely abrogated by the shRNA depleting HIF-1α, the apigenin effect remained unaffected from expression of the HIF-1α shRNA (Figs. 7A and 7B). This indicates that quercetin is involved in controlling HepG2 cell proliferation *via* HIF-1α.

In order to investigate whether these effects of quercetin are affecting the expression of a cell cycle inhibitor, we aimed to test the expression of the cyclin-dependent kinase inhibitor p21 (waf1/cip1). We demonstrate that, in line with previous reports (2, 4, 7, 14, 36) hypoxia induced p21WAF promoter activity and protein expression by about 2.5-fold. In addition, quercetin induced p21WAF by about 4.5- and 6-fold under normoxia and hypoxia, respectively (Figs. 8A and 8B). Knocking down HIF-1α with shRNA decreased p21WAF promoter activity by about 50% under normoxia but completely abolished the induction of p21WAF expression by hypoxia. In addition, the positive effects of quercetin on p21WAF expression were disrupted upon knockdown of HIF-1α (Figs. 6A and 6B). Together, these results provide evidence that quercetin inhibits the cell cycle *via* HIF-1-dependent induction of the cyclin-dependent kinase inhibitor p21WAF.

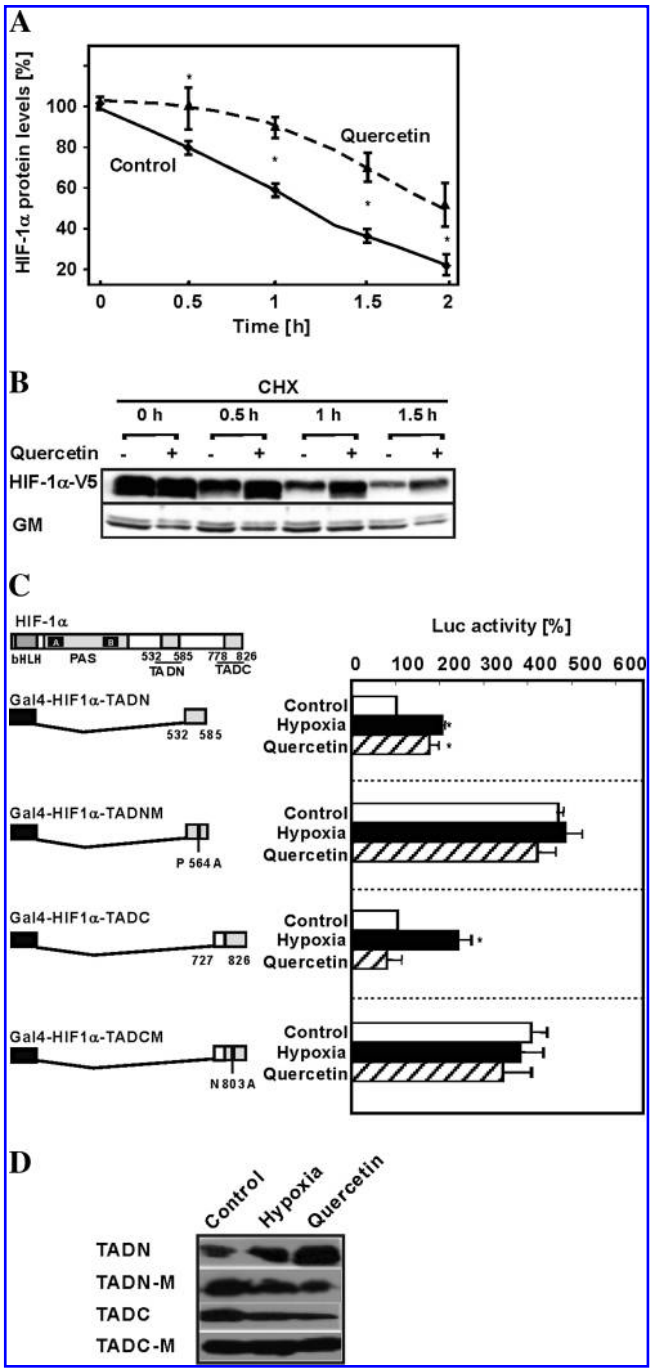


FIG. 6. Enhancement of HIF-1α protein stability and induction of HIF-1α transactivity by quercetin *via* inhibition of prolyl hydroxylase activity. (**A**) Determination of HIF-1α protein half-life. HepG2 cells were transfected with a vector for V5-tagged full-length wild-type HIF-1α and thereafter the cells were either treated only with cycloheximide (100 μg/ml) or both cycloheximide and quercetin (100 μmol). The HIF-1α protein levels were determined at the indicated timepoints by Western analysis with an antibody against the V5-tag. Values are means ± SEM of three independent culture experiments; *significant difference control vs. quercetin. (**B**) Western blot analysis. 100 μg of protein from HepG2 cells treated as above were subjected to Western analysis with an antibody against HIF-1α or Golgi membrane (GM). (**C**) HepG2 cells were cotransfected with a luciferase reporter construct pG5-E1B-LUC and different fusion gene constructs in which the Gal4 DNA binding domain was fused to either the HIF-1α region from amino acid 532–585 containing TADN or 727–826 containing TADC, respectively, as shown on the *left*. The mutations in the constructs are indicated in *italics*. After 24 h, the transfected cells were exposed to hypoxia (5% O_2) or treated with 100 μmol quercetin under normoxia (16% O_2) for 4 h. Values are means ± SEM of six independent culture experiments; *significant differences hypoxia or quercetin vs. normoxia in the same group. (**D**) Western Blot analysis. 50 μg of protein from HepG2 cells transfected with wild-type and mutated Gal4-HIF1α TADN,and Gal4-HIF1αTADC constructs were treated with quercetin (100 μ*M*) for 4 h and then subjected to Western analysis with an antibody against Gal4DBD. Autoradiographic signals were obtained by chemiluminescence.

Discussion

In this study, we investigated the regulation of HIF-1α in response to different flavonoids under normoxia and hypoxia. Our results demonstrate several new findings with respect to HIF-1α regulation and cell proliferation. First, it was found that a flavonol structure and the presence of hydroxyl

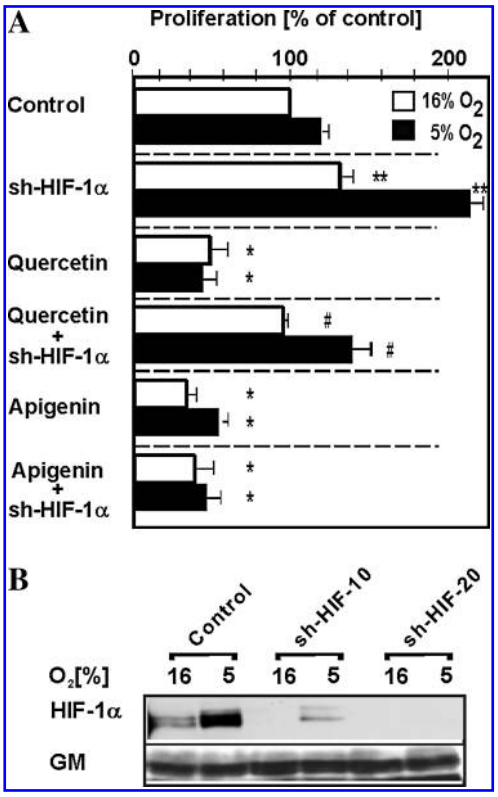


FIG. 7. Knockdown of HIF-1α disrupts quercetin-mediated inhibition of cell proliferation. (**A**) HepG2 cells were transfected with 20 μg of vectors allowing expression of unspecfic control shRNA or shRNA against HIF-1α and cultured under normoxia (16% O_2) and hypoxia (5% O_2) for 24 h. Thereafter cells were treated for 12 h with quercetin (100 μmol) or apigenin (100 μmol). Proliferative activity was assessed by BrdU incorporation and the values under 16% O_2 were set to 100%. Values represent means of three different culture experiments; *significant difference controls vs. quercetin or apigenin in the same group, **significant difference controls versus shHIF-1α in the same group; #significant difference quercetin vs. shHIF-1α with quercetin; (**B**) Representative Western blot. Proteins from the cells transfected in the control with 20 μg of an expression vector for unspecfic control shRNA or 10 μg and 20 μg of an HIF-1α shRNA vector were isolated and detected by Western blot analysis with the HIF-1α and Golgi membrane (GM) antibodies. Autoradiographic signals were obtained by chemiluminescence.

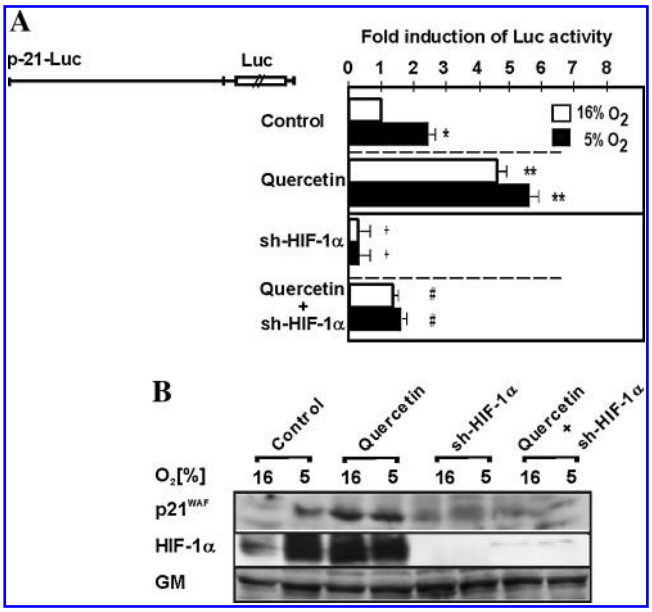


FIG. 8. Quercetin and hypoxia induce p21WAF expression *via* HIF-1α. (**A**) HepG2 cells were cotransfected with a luciferase reporter construct containing 2200 bp of the wild-type p21WAF promoter and in controls with the vectors for unspecific control shRNA or the vectors for shRNA against HIF-1α. The transfected cells were then cultured for the next 34 h under normoxia. Thereafter they were treated with 100 μmol quercetin and further cultured for 24 h under normoxia (16% O_2) or hypoxia (5% O_2). In each experiment the LUC activity of the respective control at 16% O_2 was set to 1. Values are means ± SEM of three independent culture experiments; *significant difference 16% O_2 vs. 5% O_2; **significant difference 16% O_2 or 5% O_2 vs. quercetin treatment in the same group; + significant difference 16% O_2 or 5% O_2 control vs. shHIF-1α in the same group; #significant difference quercetin vs. quercetin with shHIF-1α in the same group. (**B**) Representative Western blot. 100 μg of proteins from the cells transfected as above were isolated and analyzed by Western blotting with the p21, HIF-1α, and Golgi membrane (GM) antibodies. Autoradiographic signals were obtained by chemiluminescence.

groups in the 3′ and 4′ position as in quercetin is necessary for the induction of HIF-1α protein levels and HIF-1 activity. Second, we showed that quercetin acted post-transcriptionally by prolonging the HIF-1α protein half-life. Third, quercetin acted by interfering with the proline hydroxylation-dependent HIF-1α protein destabilization in the N-terminal HIF-1α transactivation domain. Fourth, quercetin inhibited cell proliferation *via* HIF-1-dependent induction of the cell cycle inhibitor p21WAF.

Flavonoids and ROS as different modulators of HIF-1α function

Although the primary mode of quercetin action appears to be its role as ROS reducing antioxidant, it has also been shown to block the activation of the transcription factor nuclear factor kB (NFkB) and the protein kinase C (PKC) as well as protein kinase B (PKB/Akt) signal transduction (45). Since the formation of ROS requires molecular oxygen, it has been suggested that ROS may be involved in the response to varying oxygen tensions. Indeed, several groups, including our own, have previously reported that formation of ROS is decreased under hypoxia, whereas others have reported that ROS are increased under hypoxia (19). Earlier findings consistent with the obtained data from the present study showing that hypoxia decreases ROS showed that the addition of quercetin to cells resulted in decreased ROS levels (45). Thus, we hypothesized that flavonoids may act on HIF-1α by reducing ROS. Interestingly, it was previously shown that quercetin may have a negative (10, 26) as well as a positive (39, 46) effect on HIF-1α. Although our studies indicating that quercetin increases the level of HIF-1α in HepG2 cells and in colon carcinoma cells (not shown) are in line with the latter findings, this effect seems not to be entirely due to the ROS scavenging activities of quercetin. We found that quercetin as a flavonol and the other tested flavonols (kaempferol, myricetin), the

flavones (apigenin, luteolin), or flavanones (naringenin, taxifolin) reduced ROS levels to about the same extent. This effect can be attributed to the free radical-scavenging activity of flavonoids for which the catechol group at the B ring present in all used compounds is the essential part. However, a positive effect on HIF-1α protein and HIF-1 activity could only be demonstrated by myricetin and quercetin which had the most potent effect. Thus, the inducible action of quercetin and myricetin appears not to be mediated by a simple reduction of ROS, since all other flavonoids also reduced ROS. The positive action of quercetin and myricetin was strongly dependent on the presence of hydroxyl groups in the 3′ and 4′ position of the B ring since kaempferol, another flavonol without these hydroxyl groups, was without effect on HIF-1α (Fig.3). By contrast, the only presence of the hydroxyl groups in the 3′ and 4′ position together with a flavone structure or a flavanone structure had different effects. Luteolin, a flavone containing the hydroxyl groups in the 3′ and 4′, had an inducible effect on HIF-1α protein levels but rather a repressing effect on HIF-1 activity whereas apigenin lacking the 3′ hydroxyl group was without effect. In addition, the flavanones had no effect whether they contained the 3′ and 4′ hydroxyl groups (Fig.3).

These findings clearly show that specific structural requirements are necessary for the flavonoid effects on HIF-1α which is in line with another study (15). In addition, it seems likely that ROS are not the primary mediators of the quercetin effects, although a reduction in ROS levels could be detected.

Quercetin does not induce HIF-1 activity via *enhanced transcription*

Our study showed that quercetin did not affect HIF-1α mRNA levels, indicating that the enhanced HIF-1α protein levels are the result of a post-transcriptional mechanism. In line addition of actinomycin D could not block the quercetin-dependent induction of HIF-1α protein. However, application of actinomycin D inhibited hypoxia-induced HIF-1α accumulation by about 50%, implicating that hypoxia regulates HIF-1α mRNA transcription. Although HIF-1α mRNA was regarded to be constitutively expressed in cultured cells independent of oxygen tensions (42), we and others (18, 44, 48) found that this appeared not to be the case since an induction of HIF-1α mRNA expression was detected under hypoxia. We have previously reported that the redox-sensitive transcription factor NFkB can mediate a transcriptional activation of HIF-1α (1). By using a NFkB responsive luciferase reporter gene construct, we found that in addition to the HRE-Luc construct, the NFkB construct was upregulated by hypoxia in line with previous findings (1, 23). However, quercetin reduced the pNFkB-Luc activity and simultaneously induced HRE-Luc activity. These data further indicate that a transcriptional mechanism due to a reduction of ROS levels or decreased NFkB activity does not account for the induction of HIF-1α in response to quercetin.

Quercetin enhances HIF-1α protein stability via *interfering with proline hydroxylation*

The inhibition of the quercetin-dependent HIF-1α induction by cycloheximide further pointed to a post-transcriptional mechanism. These results are in line with a study showing that quercetin stabilizes HIF-1α and causes nuclear localization of the protein in a transcriptionally active state (46). Similar to our study, another study found that quercetin induced HIF-1α under normoxia, whereas kaempferol, taxifolin, and rutin were inactive (40). Localization of HIF-1α by immuno- and direct fluorescence microscopy together with *in vitro* phosphorylation assays revealed that flavonoids may inhibit HIF-1 activity by impairing the MAPK-dependent phosphorylation of HIF-1α, thereby decreasing its nuclear accumulation (40). With respect to the present study, such a mechanism may only account for luteoline which was able to increase HIF-1α protein but not HIF-1 activity. However, apigenine, another flavone, did not show that effect. Given the similar inhibition of HRE-Luc activity exerted by luteolin and apigenin, this suggests that luteolin and apigenin may act similarly on MAPK activity but with a different effect on HIF-1α protein levels which points to different molecular mechanisms exerted by flavonoids in the regulation of HIF-1α.

The time course experiments and the increased protein half-life indicated that quercetin enhanced HIF-1α by a mechanism similar to that exerted by hypoxia. Thus we hypothesized that quercetin accumulates HIF-1α through protein stabilization. Oxygen-dependent prolyl hydroxylation and asparginyl hydroxylation have been shown to be of major importance for HIF α-subunit protein stability and coactivator recruitment. Under hypoxia, HIF-1α accumulation is mainly caused by protein stabilization *via* the inhibition of HIF PHDs, thereby blocking VHL-mediated ubiquitylation and proteasomal degradation.

The activity of the PHDs is dependent on O_2, Fe(II), α-ketoglutarate, and requires ascorbate as a cofactor. Thus, under hypoxia, activity of PHDs is decreased, VHL binding and ubiquitylation reduced and HIF-1α accumulates. Our results with the Gal4-HIF-1α transactivation assay revealed that quercetin interfered with the proline hydroxylation since it enhanced HIF-1α-TADN activity and mutation of proline 564 which is critical for HIF-1α protein stability abolished this effect (Fig. 6). Thus, our findings are in line with a study showing that quercetin decreased ubiquitylation of HIF-1α (33).

Our report further demonstrated no regulation of the HIF-1α TADC by quercetin *via* affecting the asparagine hydroxylase FIH. Although hypoxia increased TADC activity which could be abolished upon mutation of N803, quercetin did not have an effect on either the wild-type TADC or the mutant TADCM (Fig. 6). Since FIH also belongs to the α-ketoglutarate dioxygenases having the same cofactor requirements although slightly different kinetics than PHDs (22), it may be similarly affected by quercetin or reduced ROS due to limitation of the availability of ascorbate and Fe(II). Interestingly, FIH has been described to have a higher Km value for ascorbate than PHDs, although the physiological consequences of this observation are not clear to date (22). Our data showing no involvement of FIH together with previous reports demonstrating that the HIF-1α TADC can also be directly regulated *via* a redox-sensitive mechanism *via* redox factor-1 (Ref-1) (13) and the presence of a redox-sensitive cysteine (3, 6) indicate that the quercetin effects on HIF-1α are independent of ROS.

As pointed out, ascorbate was found to be important for ensuring the activity of prolyl hydroxylases, probably by helping to keep iron in a reduced state (21). Previous reports indicated that quercetin and other flavonoids deplete the intracellular chelatable iron pool which would subsequently induce HIF-1α *via* an inhibition of PHDs and FIH (39).

Similarly, angiotensin-II has been described to enhance HIF-1α levels in smooth muscle cells by affecting the availability of ascorbate and Fe(II) (32). Interestingly, one of the previous studies also showed that the transcriptional activity of HIF-1 could be reduced without a decrease in HIF-1α protein levels upon HIF-1α induction by desferrioxamine and subsequent treatment with flavonoids (40). This would indicate that quercetin may interfere with the ROS-dependent oxidation of Fe(II) to Fe(III), possibly *via* a Fenton reaction that we have previously described (27). However, the findings of this study show that the antioxidant action of quercetin did not cause or diminish ER stress since it had no impact on the activation of the transcription factor ATF-6 while at the same time quercetin induced the activity of the antioxidant responsive element (ARE) binding the factor Nrf2 (Fig. 5C).

Together, we propose that the HIF-1α protein stabilization in response to quercetin is due to a novel ROS independent mechanism affecting the functions of the PHDs.

Quercetin acts as inhibitor of the cell cycle in a HIF-1-dependent manner

The present study shows a direct involvement of HIF-1α in quercetin-mediated inhibition of cell proliferation. The finding that quercetin inhibits cell cycle progression and likely tumor growth by inducing the expression of HIF-1α and the cyclin-dependent kinase inhibitor p21WAF, is at a first glance surprising because HIF-1α overexpression is associated with tumor growth and angiogenesis in animal models and with increased patient mortality in clinical studies (49).

The p21WAF expression is frequently induced by DNA damage in cells with wild-type p53 and it then contributes to the arrest of cell growth. However, it was unknown until recently whether p21WAF may be responsive to HIF-1α and whether cooperation between HIF-1α and p53 contributes to the cell cycle arrest. Initial reports examining the role of HIF-1α deficiency found that a number of cell cycle regulating genes like those encoding p53, p21, Bcl-2 are HIF-1α dependent, while others like p27 or GADD153 were HIF-1α independent (2), suggesting indeed that HIF-1α can contribute to cell cycle arrest. In addition, experiments with different cell types containing either a deletable *hif-1α* or *p53* allele as well as double-knockout cells revealed that loss of HIF-1α abolished growth arrest in a p53-independent fashion and caused an increased progression into the S phase (7). In addition, hypophosphorylation of the retinoblastoma protein under hypoxia was HIF-1α dependent, and HIF-1α deficiency again negatively affected expression of the cyclin-dependent kinase inhibitors p21 but also p27 (7). These findings clearly point out that HIF-1 is a major regulator of the cell cycle arrest. Our present findings indicating that hypoxia and quercetin increase p21WAF transcription as revealed by the reporter gene assay and subsequently p21WAF protein expression in a HIF-1α-dependent manner agree with the previous findings and are in line with an earlier report showing that neither the PI3K/Akt nor the p53 signalling pathway is required for quercetin-induced HIF-1α accumulation (33).

Some flavonoids have been tested in clinical trials, and quercetin and its prodrug QC12 have entered already Phase I clinical studies to test their emerging potential as prospective anticancer drug candidates (12). Clinical studies revealed that especially quercetin may reduce the risk of lung cancer (31) and like other flavonoids it belongs to a group of bioactive compounds that are present in plant food. It is widely accepted that a high dietary intake of fruits and vegetables is associated with a reduced risk of common human cancers, including cancers of the lung, breast, prostate, and colon, where it appeared that a diet supplemented with 2% quercetin reduced carcinogen-induced development of aberrant crypt foci (24).

Although largely unknown, it might be tempting to speculate that the beneficial effects of quercetin and other flavonoids are, at least in part, being mediated *via* the HIF-1-dependent induction of p21WAF. Thus, a better understanding of the molecular mode of action of these natural products will contribute to the development of more specific preventive strategies against cancer.

Taken together, the findings of the present study extend our understanding of the mechanisms by which especially quercetin mediates its effects on HIF-1α protein stability and provide insight into the quercetin-mediated regulation of growth arrest *via* HIF-1-dependent induction of p21WAF expression.

Acknowledgment

This study was supported by the programme "Wissen schafft Zukunft" "Nutrition and intestinal health" A4, from the Ministry of Education Rheinland Pfalz, Germany.

Author Disclosure Statement

No competing financial interests exist.

References

1. Bonello S, Zahringer C, BelAiba RS, Djordjevic T, Hess J, Michiels C, Kietzmann T, and Gorlach A. Reactive oxygen species activate the HIF-1alpha promoter *via* a functional NFkappaB site. *Arterioscler Thromb Vasc Biol* 27: 755–761, 2007.
2. Carmeliet P, Dor Y, Herbert JM, Fukumura D, Brusselmans K, Dewerchin M, Neeman M, Bono F, Abramovitch R, Maxwell P, Koch CJ, Ratcliffe P, Moons L, Jain RK, Collen D, Keshert E, and Keshet E. Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature* 394: 485–490, 1998.
3. Carrero P, Okamoto K, Coumailleau P, O'Brien S, Tanaka H, and Poellinger L. Redox-regulated recruitment of the transcriptional coactivators CREB-binding protein and SRC-1 to hypoxia-inducible factor 1alpha. *Mol Cell Biol* 20: 402–415, 2000.
4. Chandel NS, Vander HM, Thompson CB, and Schumacker PT. Redox regulation of p53 during hypoxia. *Oncogene* 19: 3840–3848, 2000.
5. Du G, Lin H, Wang M, Zhang S, Wu X, Lu L, Ji L, and Yu L. Quercetin greatly improved therapeutic index of doxorubicin against 4T1 breast cancer by its opposing effects on HIF-1alpha in tumor and normal cells. *Cancer Chemother Pharmacol* 2009.
6. Ema M, Hirota K, Mimura J, Abe H, Yodoi J, Sogawa K, Poellinger L, and Fujii–Kuriyama Y. Molecular mechanisms of transcription activation by HLF and HIF1alpha in response to hypoxia: Their stabilization and redox signal-induced interaction with CBP/p300. *EMBO J* 18: 1905–1914, 1999.

7. Goda N, Ryan HE, Khadivi B, McNulty W, Rickert RC, and Johnson RS. Hypoxia-inducible factor 1alpha is essential for cell cycle arrest during hypoxia. *Mol Cell Biol* 23: 359–369, 2003.
8. Gong P, Hu B, Stewart D, Ellerbe M, Figueroa YG, Blank V, Beckman BS, and Alam J. Cobalt induces heme oxygenase-1 expression by a hypoxia-inducible factor-independent mechanism in Chinese hamster ovary cells: Regulation by Nrf2 and MafG transcription factors. *JBiolChem* 276: 27018–27025, 2001.
9. Gorlach A and Kietzmann T. Superoxide and derived reactive oxygen species in the regulation of hypoxia-inducible factors. *Methods Enzymol* 35: 421–446, 2007.
10. Hasebe Y, Egawa K, Yamazaki Y, Kunimoto S, Hirai Y, Ida Y, and Nose K. Specific inhibition of hypoxia-inducible factor (HIF)-1 alpha activation and of vascular endothelial growth factor (VEGF) production by flavonoids. *Biol Pharm Bull* 26: 1379–1383, 2003.
11. Hewitson KS, McNeill LA, Riordan MV, Tian YM, Bullock AN, Welford RW, Elkins JM, Oldham NJ, Bhattacharya S, Gleadle JM, Ratcliffe PJ, Pugh CW, and Schofield CJ. Hypoxia-inducible factor (HIF) asparagine hydroxylase is identical to factor inhibiting HIF (FIH) and is related to the cupin structural family. *J Biol Chem* 277: 26351–26355, 2002.
12. Hirpara KV, Aggarwal P, Mukherjee AJ, Joshi N, and Burman AC. Quercetin and its derivatives: Synthesis, pharmacological uses with special emphasis on anti-tumor properties and prodrug with enhanced bio-availability. *Anticancer Agents Med Chem* 9: 138–161, 2009.
13. Huang LE, Arany Z, Livingston DM, and Bunn HF. Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit. *J Biol Chem* 271: 32253–32259, 1996.
14. Iida T, Mine S, Fujimoto H, Suzuki K, Minami Y, and Tanaka Y. Hypoxia-inducible factor-1alpha induces cell cycle arrest of endothelial cells. *Genes Cells* 7: 143–149, 2002.
15. Jeon H, Kim H, Choi D, Kim D, Park SY, Kim YJ, Kim YM, and Jung Y. Quercetin activates an angiogenic pathway, hypoxia inducible factor (HIF)-1-vascular endothelial growth factor, by inhibiting HIF-prolyl hydroxylase: A structural analysis of quercetin for inhibiting HIF-prolyl hydroxylase. *Mol Pharmacol* 71: 1676–1684, 2007.
16. Jiang BH, Zheng JZ, Leung SW, Roe R, and Semenza GL. Transactivation and inhibitory domains of hypoxia-inducible factor 1alpha. Modulation of transcriptional activity by oxygen tension. *J Biol Chem* 272: 19253–19260, 1997.
17. Kaelin WG. Proline hydroxylation and gene expression. *Annu Rev Biochem* 74: 115–128, 2005.
18. Kietzmann T, Cornesse Y, Brechtel K, Modaressi S, and Jungermann K. Perivenous expression of the mRNA of the three hypoxia-inducible factor alpha-subunits, HIF1alpha, HIF2alpha and HIF3alpha, in rat liver. *Biochem J* 354: 531–537, 2001.
19. Kietzmann T and Gorlach A. Reactive oxygen species in the control of hypoxia-inducible factor-mediated gene expression. *Semin Cell Dev Biol* 16: 474–486, 2005.
20. Kietzmann T, Roth U, and Jungermann K. Induction of the plasminogen activator inhibitor-1 gene expression by mild hypoxia *via* a hypoxia response element binding the hypoxia inducible factor-1 in rat hepatocytes. *Blood* 94: 4177–4185, 1999.
21. Knowles HJ, Raval RR, Harris AL, and Ratcliffe PJ. Effect of ascorbate on the activity of hypoxia-inducible factor in cancer cells. *Cancer Res* 63: 1764–1768, 2003.
22. Koivunen P, Hirsila M, Gunzler V, Kivirikko KI, and Myllyharju J. Catalytic properties of the asparaginyl hydroxylase (FIH) in the oxygen sensing pathway are distinct from those of its prolyl 4-hydroxylases. *J Biol Chem* 279: 9899–9904, 2004.
23. Koong AC, Chen EY, and Giaccia AJ. Hypoxia causes the activation of nuclear factor kappa B through the phosphorylation of I kappa B alpha on tyrosine residues. *Cancer Res* 54: 1425–1430, 1994.
24. Kyle JA, Sharp L, Little J, Duthie GG, and McNeill G. Dietary flavonoid intake and colorectal cancer: A case-control study. *Br J Nutr* 1–8, 2009.
25. Lando D, Peet DJ, Whelan DA, Gorman J, and Whitelaw ML. Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch. *Science* 295: 858–861, 2002.
26. Lee DH and Lee YJ. Quercetin suppresses hypoxia-induced accumulation of hypoxia-inducible factor-1alpha (HIF-1alpha) through inhibiting protein synthesis. *J Cell Biochem* 105: 546–553, 2008.
27. Liu Q, Berchner–Pfannschmidt U, Moller U, Brecht M, Wotzlaw C, Acker H, Jungermann K, and Kietzmann T. A Fenton reaction at the endoplasmic reticulum is involved in the redox control of hypoxia-inducible gene expression. *Proc Natl Acad Sci USA* 101: 4302–4307, 2004.
28. Liu Q, Moller U, Flugel D, and Kietzmann T. Induction of plasminogen activator inhibitor I gene expression by intracellular calcium *via* hypoxia-inducible factor-1. *Blood* 104: 3993–4001, 2004.
29. Mahon PC, Hirota K, and Semenza GL. FIH-1: A novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. *Genes Dev* 15: 2675–2686, 2001.
30. Manalo DJ, Rowan A, Lavoie T, Natarajan L, Kelly BD, Ye SQ, Garcia JG, and Semenza GL. Transcriptional regulation of vascular endothelial cell responses to hypoxia by HIF-1. *Blood* 105: 659–669, 2005.
31. Neuhouser ML. Dietary flavonoids and cancer risk: Evidence from human population studies. *Nutr Cancer* 50: 1–7, 2004.
32. Page EL, Chan DA, Giaccia AJ, Levine M, and Richard DE. Hypoxia-inducible factor-1{alpha} stabilization in nonhypoxic conditions: Role of oxidation and intracellular ascorbate depletion. *Mol Biol Cell* 19: 86–94: 2008.
33. Park SS, Bae I, and Lee YJ. Flavonoids-induced accumulation of hypoxia-inducible factor (HIF)-1alpha/2alpha is mediated through chelation of iron. *J Cell Biochem* 103: 1089–1998, 2007.
34. Pohl C, Will F, Dietrich H, and Schrenk D,. Cytochrome P450 1A1 expression and activity in Caco-2 cells: Modulation by apple juice extract and certain apple polyphenols. *J Agric Food Chem* 54: 10262–10268, 2006.
35. Pugh CW, O'Rourke JF, Nagao M, Gleadle JM, and Ratcliffe PJ. Activation of hypoxia-inducible factor-1; Definition of regulatory domains within the alpha subunit. *J Biol Chem* 272: 11205–11214, 1997.
36. Salnikow K, Costa M, Figg WD, and Blagosklonny MV. Hyperinducibility of hypoxia-responsive genes without p53/p21-dependent checkpoint in aggressive prostate cancer. *Cancer Res* 60: 5630–5634, 2000.
37. Schofield CJ and Ratcliffe PJ. Signalling hypoxia by HIF hydroxylases. *Biochem Biophys Res Commun* 338: 617–626, 2005.
38. Semenza GL. HIF-1 and tumor progression: Pathophysiology and therapeutics. *Trends Mol Med* 8: S62–S67, 2002.
39. Triantafyllou A, Liakos P, Tsakalof A, Chachami G, Paraskeva E, Molyvdas PA, Georgatsou E, Simos G, and Bonanou S. The flavonoid quercetin induces hypoxia-inducible factor-1alpha (HIF-1alpha) and inhibits cell proliferation by depleting intracellular iron. *Free Radic Res* 41: 342–356, 2007.

40. Triantafyllou A, Mylonis I, Simos G, Bonanou S, and Tsakalof A. Flavonoids induce HIF-1alpha but impair its nuclear accumulation and activity. *Free Radic Biol Med* 44: 657–670, 2008.
41. Wang Y, Shen J, Arenzana N, Tirasophon W, Kaufman RJ, and Prywes R. Activation of ATF6 and an ATF6 DNA binding site by the endoplasmic reticulum stress response. *J Biol Chem* 275: 27013–27020, 2000.
42. Wenger RH and Gassmann M. Oxygen(es) and the hypoxia-inducible factor-1. *Biol Chem* 378: 609–616, 1997.
43. Wenger RH, Stiehl DP, and Camenisch G. Integration of oxygen signaling at the consensus HRE. *Sci STKE* 2005: re12, 2005.
44. Wiener CM, Booth G, and Semenza GL. *In vivo* expression of mRNAs encoding hypoxia-inducible factor 1. *Biochem Biophys Res Commun* 225: 485–488, 1996.
45. Williams RJ, Spencer JP, and Rice–Evans C. Flavonoids: Antioxidants or signalling molecules? *Free Radic Biol Med* 36: 838–849, 2004.
46. Wilson WJ and Poellinger L. The dietary flavonoid quercetin modulates HIF-1 alpha activity in endothelial cells. *Biochem Biophys Res Commun* 293: 446–450, 2002.
47. Yang CS, Landau JM, Huang MT, and Newmark HL. Inhibition of carcinogenesis by dietary polyphenolic compounds. *Annu Rev Nutr* 21: 381–406, 2001.
48. Yu AY, Frid MG, Shimoda LA, Wiener CM, Stenmark K, and Semenza GL, Temporal, spatial, and oxygen-regulated expression of hypoxia-inducible factor-1 in the lung. *Am J Physiol* 275: L818–L826, 1998.
49. Zagzag D, Zhong H, Scalzitti JM, Laughner E, Simons JW, and Semenza GL, Expression of hypoxia-inducible factor 1alpha in brain tumors: Association with angiogenesis, invasion, and progression. *Cancer* 88: 2606–2618, 2000.

Address correspondence to:
Dr. Thomas Kietzmann
Fachbereich Chemie, Abteilung Biochemie
Universität Kaiserslautern
Erwin Schrödinger Str. 54
D-67663 Kaiserslautern
Germany

E-mail: tkietzm@gwdg.de

Date of first submission to ARS Central, November 16, 2009; date of acceptance, December 2, 2009.

Abbreviations Used

ARE = antioxidant responsive element
ARNT = arylhydrocarbon receptor nuclear translocator
bHLH-PAS = basic helix-loop-helix Per-ARNT-Sim
DIG = digoxigenin
EPO = erythropoietin
GM = Golgi membrane element
HIF = hypoxia-inducible factor
HRE = hypoxia response element
Luc = luciferase
PHD = proline hydroxylase domain containing protein
ROS = reactive oxygen species
TAD = transactivation domain
VHL = von Hippel Lindau tumor repressor protein
XRE = xenobiotica response

This article has been cited by:

1. Federico Dajas. 2012. Life or death: Neuroprotective and anticancer effects of quercetin. *Journal of Ethnopharmacology* **143**:2, 383-396. [CrossRef]
2. Davide Staedler, Elita Idrizi, Blanka Halamoda Kenzaoui, Lucienne Juillerat-Jeanneret. 2011. Drug combinations with quercetin: doxorubicin plus quercetin in human breast cancer cells. *Cancer Chemotherapy and Pharmacology* . [CrossRef]
3. Gabriella Leonarduzzi, Barbara Sottero, Giuseppe Poli. 2010. Targeting tissue oxidative damage by means of cell signaling modulators: The antioxidant concept revisited. *Pharmacology & Therapeutics* **128**:2, 336-374. [CrossRef]
4. Thomas Kietzmann . 2010. Intracellular Redox Compartments: Mechanisms and Significances. *Antioxidants & Redox Signaling* **13**:4, 395-398. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]