ACADEMIC PRESS

Biochemical and Biophysical Research Communications 293 (2002) 446–450

BBRC

www.academicpress.com

# The dietary flavonoid quercetin modulates HIF-1α activity in endothelial cells

William J. Wilson and Lorenz Poellinger*

*Department of Cell and Molecular Biology, Medical Nobel Institute, Karolinska Institutet, S-171 77 Stockholm, Sweden*

Received 27 March 2002

**Abstract**

The mechanism of activation of the transcription factor hypoxia-inducible factor-1 (HIF-1) has been studied extensively. Under normal cellular oxygen conditions protein levels of the alpha subunit (HIF-1α) are kept low due to massive ubiquitination and subsequent proteosomal degradation. However, during hypoxia ubiquitination is inhibited, causing stabilisation of the HIF-1α protein. HIF-1α can then translocate to the nucleus and facilitate transcription of numerous target genes, the majority of which are involved in glycolysis and angiogenesis via heterodimerisation with the beta subunit (HIF-1β/ARNT). Until now hypoxia has been the only naturally occurring signal shown to activate this transcription factor. We report here that the dietary flavonoid quercetin also activates HIF-1α in all steps of its activation pathway, in a manner similar to hypoxia. We found that quercetin, an inhibitor of Ser/Thr kinases, stabilises HIF-1α and causes nuclear localisation of the protein in a transcriptionally active state. Taken together these results strongly indicate that the dietary flavonoid quercetin regulates HIF-1 function at normal oxygen concentrations. 

*Keywords:* HIF-1α; bHLH-PAS; Hypoxia; Quercetin; Flavonoid; Kinase inhibitor; Endothelial cells; Protein regulation; Green fluorescent protein; Signal transduction

The bHLH-PAS family of proteins are signal-inducible transcription factors. One member of this family, hypoxia-inducible factor-1α (HIF-1α), is involved in activation of gene transcription during exposure to hypoxia.

Knockout studies in mice have shown that HIF-1α is essential for angiogenesis/vasculogenesis as is its heterodimeric partner protein ARNT [1–3]. This phenotype is explained by the fact that HIF-1α is a key transcription factor in the induction of the vascular endothelial growth factor (VEGF) gene, a major angiogenic factor required for vessel formation. In addition, HIF-1α regulates many other genes required for glucose and hypoxia homeostases (reviewed in [4]). We have previously reported that HIF-1α is rapidly degradedvia the 26S proteasome after ubiquitination under normoxic conditions [5]. More recently it has been shown that HIF-1α is the target for a family of prolyl hydroxylase proteins [6–8]. Hydroxylation of HIF-1α facilitates recruitment of an E3 ubiquitin ligase, the von Hippel–Lindau tumour suppressor protein (VHL), which targets HIF-1α for degradation under normoxia.

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) and other related flavonoids are dietary compounds that have been attributed many characteristics such as anti-oxidant and anti-cancer properties, so much so that it has appeared as a health food supplement in recent years. It is also present in extracts from *Ginko biloba* and St. Johns Wort, both popular health supplements (for review see [9]). Although quercetin has been reported to inhibit many Ser/Thr kinases [10], it is specific to several kinases at low concentrations, including, casein kinase II (ID50 40 nM) [11,12].

Here we have investigated the effect of quercetin on the activation and function of HIF-1α. Our results show that quercetin can act as a potent activator of HIF-1α which results in the increased transcription of HIF-1 target genes. Understanding the role of HIF-1α in angiogenesis and the molecular mechanisms leading to its activation will hopefully provide us with interesting

* Corresponding author. Fax: +46-8-34-88-19.
*E-mail address:* lorenz.poellinger@cmb.ki.se (L. Poellinger).

0006-291X/02/$ - see front matter 
PII: S0006-291X(02)00244-9

models for treatment of cancers and tumours that perturb the HIF-1α signalling pathway.

## Materials and methods

*Cell culture*. Murine brain endothelial cells (a gift from Yihai Cao, MTC, KI) (MBECs) were cultured in F12 HAMS media and HeLa cells in Dulbecco's modified Eagle's media, supplemented with 10% foetal calf serum, penicillin (50 units/ml), and streptomycin (50 μg/ml) (Life Technologies, UK). Hypoxic conditions were created by maintaining cells in a sealed chamber flooded with 94% nitrogen and 5% $CO_2$ until oxygen was present at 1%. All cells were maintained at 37 °C. Quercetin was purchased from ICN and dissolved in DMSO to a concentration of 10 mM. Further dilutions were performed in cell culture media.

*Isolation of poly(A)$^+$ mRNA and Northern blotting*. Isolation of poly(A)$^+$ mRNA from endothelial cells was performed as previously published [13]. Five micrograms of poly(A)$^+$ RNA from each treatment was electrophoresed, blotted onto Zeta-probe membrane (Bio-Rad Laboratories, USA), and cross-linked. Probes were made with the RediPrime II kit (Amersham Pharmacia Biotech, Sweden), using fragments of cDNA from VEGF, glucose transporter 1 (glut-1), GAPDH, and actin.

*Plasmid constructs*. The plasmids pCMV-HIF-1α, pCMV-HR-E.tk.Luc, pGFP-HIF-1α, pGAL4DBD-HIF-1α, pGAL4DBD-HIF-N-TAD (amino acids 531–584), and pGAL4DBD-HIF-C-TAD (amino acids 776–826) have been described previously [5,14,15].

*Transfections and reporter gene analysis*. Transfections of cells were performed using Fugene6 (Roche, USA) as recommended by the manufacturer. Cells were seeded into six well plates and transfected at approximately 80% confluency. After 14 h they were then treated with either quercetin or hypoxia for the specified amount of time. For luciferase assays cells were washed twice in PBS, lysed in 210 μL Cell Lysis Buffer (Promega, USA) and a 30 μL aliquot was taken for assaying luciferase activity using BioTherma (Sweden) ATP and Luciferin. Luciferase activity was normalised to protein concentration using the Bradford assay. Results are the average of three experiments performed in triplicate.

*Western blotting*. To study protein levels of HIF-1α during quercetin treatment, cells treated with a time course of quercetin (10 μM) were lysed and whole cell extract was prepared as previously described [5]. Forty micrograms of protein was electrophoresed and blotted on to a Hybond-N$^+$ membrane. Western blotting using antibodies against HIF-1α and ECL detection were used to visualise HIF-1α protein. Monoclonal antibodies against HIF-1α were purchased from Novus Biologicals, USA and diluted 1–2000 before use.

## Results

### *HIF-1α contains potential serine/threonine phosphorylation sites*

Phosphorylation has been found in several cases to direct ubiquitination of proteins. A computational analysis was performed of the human HIF-1α sequence using a kinase recognition neural network, provided by the NetPhos 2.0 prediction server at the CBS, Denmark [16]. This revealed the presence of many potential serine/threonine phosphorlyation sites for the kinases casine kinase II, PKA, PKC, and GSK3 (unpublished results). Several of these are conserved within the related proteins EPAS [17] and HIF-3α [18], indicating a possible role in HIF-1α regulation. The relative proximity (between 5 and 40 amino acids) of five of these sites to a conserved penta-amino acid motif (APYIP) conserved in all HIFα proteins, which mediates binding to the ubiquitin ligase VHL [19,20], suggests that these sites may act as signals in mediating HIF-1α degradation.

### *The Ser/Thr kinase inhibitor quercetin induces HRE reporter activity*

Based on our sequence analysis, we decided to examine the effects of the Ser/Thr kinase inhibitor quercetin on the HIF signalling pathway. HeLa cells, which contain very high endogenous hypoxia-inducible levels of HIF-1α protein [21], were transfected with a vector containing three hypoxia-responsive element (HRE) binding sites cloned upstream of a luciferase reporter gene and treated with increasing amounts of quercetin or dipyridyl at 100 μM for 16 h (Fig. 1A). Dipyridyl, in analogy to other iron chelators, inhibits the prolyl hydroxylation of HIF-1α and therefore

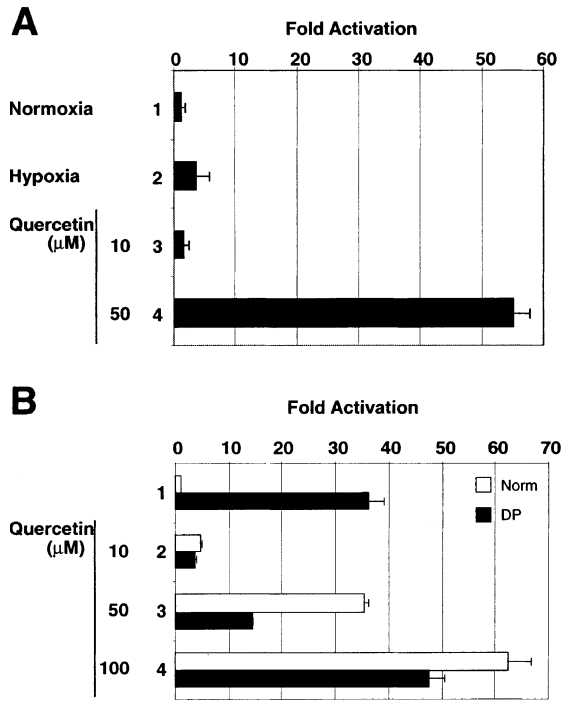


Fig. 1. HeLa cells were transfected with a HRE-luciferase reporter vector (A) and MBECs were transfected with both HRE-luciferase reporter vector and HIF-1α expression vector (B). The cells were treated with increasing concentrations of quercetin (10, 50, or 100 μM) for 16 h, whereupon they were harvested after washing twice in phosphate-buffered saline. Luciferase activity was measured, and after adjusting the values relative to the value at normoxia, the fold activation was plotted. Mean values are shown for three independent experiments, with error bars representing the standard error.

functions as a hypoxia mimic. A substantial increase in activity of the reporter gene was observed in a dose-dependent manner.

To test if the HIF-1α protein was mediating the reporter gene activity observed in HeLa cells, we transfected mouse brain endothelial cells (MBECs) with HRE reporter and HIF-1α expression vectors (Fig. 1B). These cells were used due to the relatively lower levels of endogenous HIF-1α and more importantly the physiological role of these cells in angiogenesis. MBECs, after transfection, were treated with quercetin or hypoxia for 16 h. Again, we observed an increase in reporter activity in response to increasing doses of quercetin. This time the fold activation was much greater with the addition of the HIF-1α expression vector than with endogenous HIF-1α seen in the HeLa cells. The maximal increase (100 μM quercetin) was greater than that observed with dipyridyl, 62-fold induction versus 36-fold, showing that the additional amounts of expressed HIF-1α were made avaliable for quercetin-inducible transcription.

*Quercetin stabilises HIF-1α protein*

The hypoxic response is made possible through the prevention of HIF-1α degradation; therefore we examined the effects of quercetin on HIF-1α protein levels. We hypothesised that HIF-1α protein was increased by quercetin treatment. A Western blot of whole cell extracts from HeLa cells treated with quercetin or hypoxia was detected with anti-HIF-1α antibodies. As seen in Fig. 2, hypoxia induces a characteristic broad band of HIF-1α as observed in many cell types [22] and evident on this blot due to differentially phosphorylated forms of the protein. As expected, we also saw increased levels of HIF-1α after 4 h of treatment with 50 μM quercetin, rising to a maximum after 6 h and continuing over 12 h. In addition to the main HIF-1α band induced after 4 h of quercetin treatment, we observed a less mobile sharp band appearing at 6 h. This may represent a specific form of phosphorylated HIF-1α.

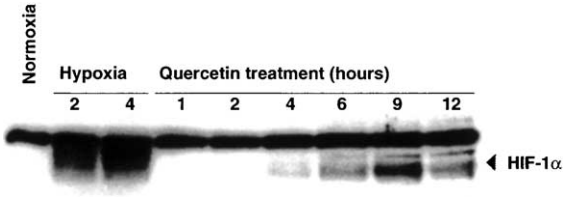


Fig. 2. A Western blot of HIF-1α in HeLa cells treated with either hypoxia for 2 or 4 h (lanes 2 and 3), quercetin (50 μM) for 1, 2, 4, 6, 9, and 12 h (lanes 4–9), and no treatment (lane 1). The prominent band above the marked HIF-1α band is a non-specific signal we see with this antibody.

*Transfection with GAL4 reporter and Gal4-HIF-1α chimeras*

Since endogenous HIF-1α showed potent activation in the presence of quercetin on a HRE reporter vector, we were interested to see if we could observe a similar pattern of activation using the HIF-1α protein fused to a heterologous DNA binding domain, namely the Gal4-DNA binding domain (Gal4-DBD). Gal4-HIF-1α chimeras were transfected into MBEC cells together with a Gal4-luciferase reporter vector. After 12 h of treatment at 5, 10, or 50 μM quercetin the cells were harvested and luciferase activity was measured.

The Gal4-HIF-1α construct activated transcription from the reporter gene (Fig. 3A) in a similar dosage-dependent pattern as seen with the full-length HIF-1α construct upon the HRE reporter. Induction of the Gal4 reporter was observed in normoxia and potentiated in hypoxia. A similar result was observed when only the N- or C-terminal activation domains (C-TAD, N-TAD) of HIF-1α were used (Fig. 3B), with the C-TAD exhibiting poor activation with respect to the N-TAD, and even full-length HIF-1α construct.

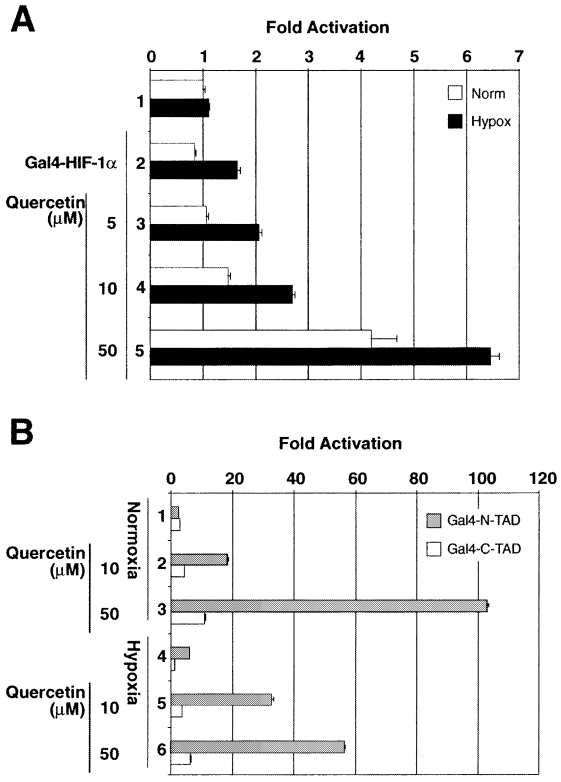


Fig. 3. MBECs were transfected with HRE-luciferase reporter vector together with either Gal4-HIF-1α (A) or Gal4-N-TAD or Gal4-C-TAD constructs (B) and treated with quercetin at the stated concentrations and/or hypoxia for 16 h, before assaying for luciferase activity. Results are displayed as described for Fig. 1.

*Quercetin induces HIF-1α target gene expression*

Poly(A)$^+$ mRNA was extracted from MBECs treated with 10 μM quercetin for 2, 3, 4, 6, 9, or 12 h. A Northern blot prepared from these samples was probed for the expression of HIF-1 target genes (Fig. 4). VEGF mRNA was detected after 3 h, gradually increasing to a peak at 6 h and remaining constant through 12 h. Glut-1 mRNA showed a similar pattern of induction as VEGF. These findings parallel studies of HIF-1α target gene regulation as seen by hypoxia induction [1].

*Quercetin induces nuclear localisation of HIF-1α*

Nuclear translocation of HIF-1α is a hypoxia-dependent step in the signalling pathway. To monitor the cellular localisation of HIF-1α following quercetin treatment a construct consisting of GFP fused to full-length HIF-1α was transfected into MBECs. After treatment with quercetin for 10 h the fusion protein was visualised via fluorescent microscopy (Fig. 5). In untreated cells, HIF-1α was located predominantly in the cytoplasm. Treatment with 50 μM quercetin-induced nuclear localisation of the HIF-1α fusion protein. This nuclear localisation was slightly enhanced upon co-treatment with dipyridyl, which was used instead of hypoxia so that localisation of the fusion protein in living cells could be performed. GFP alone was expressed in both cytoplasm and nucleus and was unaffected by quercetin treatment.

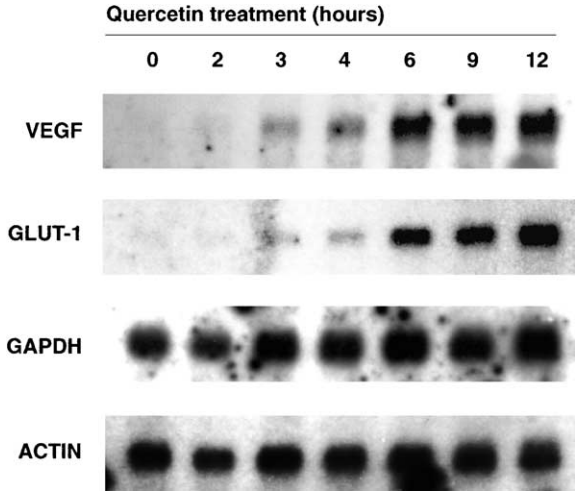


Fig. 4. MBECs were treated in 30 cm$^2$ plates with 10 μM quercetin for various times (0, 2, 3, 4, 6, 9, or 12 h). After extraction and purification of poly(A)$^+$ mRNA, electrophoresis, and blotting, the membrane was probed with $^{32}$P labelled probes to the various genes.

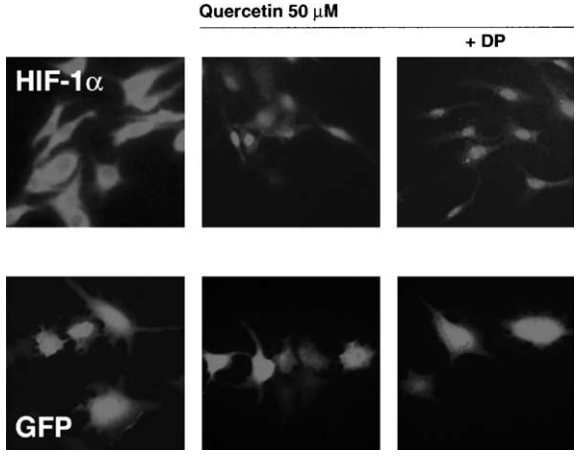


Fig. 5. Vectors expressing GFP and GFP fused to full-length HIF-1α were transfected into MBECs grown on glass coverslips. After treatment with quercetin (50 μM) and/or dipyridyl (100 μM) or vector (DMSO) for 10 h, cells were mounted on glass slides and GFP was visualised using fluorescent microscopy.

## Discussion

Initial transfections with a reporter gene construct containing a DNA binding site for HIF-1 (i.e., a hypoxia-response element) fused upstream of a luciferase reporter gene showed that it was responsive to quercetin treatment in a dose-dependent manner under conditions of normoxia. Upon co-transfection with a HIF-1α expression vector in endothelial cells this respon se was greatly enhanced, indicating that quercetin functions via HIF-1α. In fact, transfection study results, using Gal4-C-TAD and Gal4-N-TAD in endothelial cells, show that both the transactivation domains of HIF-1α are responsive to quercetin treatment. After quercetin treatment of HeLa cells at normoxia HIF-1α was found to be stabilised and migrated when analysed by SDS–PAGE slightly lower than the hypoxia-stabilised HIF-1α. In addition, quercetin-induced nuclear translocation of HIF-1α.

Hypoxia-induced stabilisation of HIF-1α protein levels in HeLa cells results in a broad band, as assessed by SDS–PAGE analysis. After treatment with phosphatase this band is reduced to a lower, more narrow band [22]. Interestingly the mobility of this lower band corresponds to the HIF-1α band induced by quercetin, possibly reflecting a specific state of phosphorylation of HIF-1α. In this context it is interesting to note that Davies et al. [12] have reported that quercetin activates MAPK2/ERK2. In addition there is evidence that the p42 MAPK pathway regulates HIF-1α function since the p42/p44 MAPK (ERK1/2) inhibitor PD98059 inhibits HIF-1α-mediated activation of target genes in certain cells [23].

Quercetin is known to have anti-oxidant properties [9] which may be the cause of activation of the HIF-1α protein. Previous experiments with reducing compounds such as dithiothreitol have shown that there is no effect on HIF-1α activity [24], so it seems unlikely that quercetin exerts its effect on HIF-1α via this mechanism.

It has also been observed that by treating cells with the inhibitors of tyr-kinases genistein and herbimycin A, the Ser/Thr kinase inhibitor 2-aminopurine and the Ser/Thr phosphatase inhibitor sodium fluoride, the induction of HIF-1α is blocked [24]. In contrast, sodium orthovanadate (a tyr-phosphatase inhibitor) increases basal HIF-1α levels and activity, probably through a strongly activating MAPK activity [22].

These experiments with various kinase and phosphatase inhibitors fail to show the total role of a particular kinase inhibitor (or chemical compound) with activation of the HIF-1α pathway. This indicates that quercetin is targeting a more general mechanism in the HIF signalling pathway, similar to hypoxia. It will therefore be interesting to investigate a possible effect of quercitin on the function of prolyl hydroxylases [7,8] which facilitate the interaction with pVHL, leading to the subsequent degradation of the protein.

The implications of a dietary compound having a regulatory effect on a molecule as pivitol as HIF-1α in the process of angiogenesis cannot be overlooked. Recently it has been shown that endothelial cells expressing hypoxia-induced VEGF can organise into networks without any other cell types or exogenous growth factors [25]. Our findings that quercetin can also induce VEGF expression from endothelial cells may have implications for people with diets high in quercetin and even supplemented with 'health drugs' containing this compound. Moreover, with findings that flavonoids and related polyphenol molecules (such as catechin and resveratrol, which exist together with quercetin in green tea and red wine), exert anti-tumourigenic and anti-angiogenesis effects [26] and have cardiovascular protective ability [27], we now have available to us interesting tools for examining the molecular mechanisms by which these molecules modulate signalling pathways.

## References

[1] N.V. Iyer, L.E. Kotch, F. Agani, S.W. Leung, E. Laughner, R.H. Wenger, M. Gassmann, J.D. Gearhart, A.M. Lawler, A.Y. Yu, G.L. Semenza, Genes Dev. 12 (1998) 149–162.
[2] H.E. Ryan, J. Lo, R.S. Johnson, EMBO J. 17 (1998) 3005–3015.
[3] K.R. Kozak, B. Abbott, O. Hankinson, Dev. Biol. 191 (1997) 297–305.
[4] P.J. Ratcliffe, J.F. O'Rourke, P.H. Maxwell, C.W. Pugh, J. Exp. Biol. 201 (1998) 1153–1162.
[5] P.J. Kallio, W.J. Wilson, S. O'Brien, Y. Makino, L. Poellinger, J. Biol. Chem. 274 (1999) 6519–6525.
[6] A.C. Epstein, J.M. Gleadle, L.A. McNeill, K.S. Hewitson, J. O'Rourke, D.R. Mole, M. Mukherji, E. Metzen, M.I. Wilson, A. Dhanda, Y.M. Tian, N. Masson, D.L. Hamilton, P. Jaakkola, R. Barstead, J. Hodgkin, P.H. Maxwell, C.W. Pugh, C.J. Schofield, P.J. Ratcliffe, Cell 107 (2001) 43–54.
[7] M. Ivan, K. Kondo, H. Yang, W. Kim, J. Valiando, M. Ohh, A. Salic, J.M. Asara, W.S. Lane, W.G. Kaelin Jr., Science 292 (2001) 464–468.
[8] P. Jaakkola, D.R. Mole, Y.M. Tian, M.I. Wilson, J. Gielbert, S.J. Gaskell, A. Kriegsheim Av, H.F. Hebestreit, M. Mukherji, C.J. Schofield, P.H. Maxwell, C.W. Pugh, P.J. Ratcliffe, Science 292 (2001) 468–472.
[9] D.W. Lamson, M.S. Brignall, Altern. Med. Rev. 5 (2000) 196–208.
[10] A.K. Srivastava, J.L. Chiasson, Prog. Clin. Biol. Res. 213 (1986) 315–318.
[11] T. Maekawa, S. Kosuge, S. Sakamoto, S. Funayama, K. Komiyama, K. Ohtsuki, Biol. Pharm. Bull. 22 (1999) 667–673.
[12] S.P. Davies, H. Reddy, M. Caivano, P. Cohen, Biochem. J. 351 (2000) 95–105.
[13] C.M. Sadek, B.L. Allen-Hoffmann, J. Biol. Chem. 269 (1994) 16067–16074.
[14] P.J. Kallio, K. Okamoto, S. O'Brien, P. Carrero, Y. Makino, H. Tanaka, L. Poellinger, EMBO J. 17 (1998) 6573–6586.
[15] P. Carrero, K. Okamoto, P. Coumailleau, S. O'Brien, H. Tanaka, L. Poellinger, Mol. Cell. Biol. 20 (2000) 402–415.
[16] N. Blom, S. Gammeltoft, S. Brunak, J. Mol. Biol. 294 (1999) 1351–1362.
[17] H. Tian, S.L. McKnight, D.W. Russell, Genes Dev. 11 (1997) 72–82.
[18] Y.Z. Gu, S.M. Moran, J.B. Hogenesch, L. Wartman, C.A. Bradfield, Gene Expr. 7 (1998) 205–213.
[19] K. Tanimoto, Y. Makino, T. Pereira, L. Poellinger, EMBO J. 19 (2000) 4298–4309.
[20] M.E. Cockman, N. Masson, D.R. Mole, P. Jaakkola, G.W. Chang, S.C. Clifford, E.R. Maher, C.W. Pugh, P.J. Ratcliffe, P.H. Maxwell, J. Biol. Chem. 275 (2000) 25733–25741.
[21] P.J. Kallio, I. Pongratz, K. Gradin, J. McGuire, L. Poellinger, Proc. Natl. Acad. Sci. USA 94 (1997) 5667–5672.
[22] D.E. Richard, E. Berra, E. Gothie, D. Roux, J. Pouyssegur, J. Biol. Chem. 274 (1999) 32631–32637.
[23] E. Minet, T. Arnould, G. Michel, I. Roland, D. Mottet, M. Raes, J. Remacle, C. Michiels, FEBS Lett. 468 (2000) 53–58.
[24] G.L. Wang, B.H. Jiang, G.L. Semenza, Biochem. Biophys. Res. Commun. 212 (1995) 550–556.
[25] G. Helmlinger, M. Endo, N. Ferrara, L. Hlatky, R.K. Jain, Nature 405 (2000) 139–141.
[26] Y.D. Jung, M.S. Kim, B.A. Shin, K.O. Chay, B.W. Ahn, W. Liu, C.D. Bucana, G.E. Gallick, L.M. Ellis, Br. J. Cancer 84 (2001) 844–850.
[27] J.M. Wu, Z.R. Wang, T.C. Hsieh, J.L. Bruder, J.G. Zou, Y.Z. Huang, Int. J. Mol. Med. 8 (2001) 3–17.