Nitric Oxide Is a Factor in the Stabilization of Hypoxia-Inducible Factor-1α in Cancer: Role of Free Radical Formation

Marisol Quintero,[1] Peter A. Brennan,[3] Gareth J. Thomas,[2] and Salvador Moncada[1]

[1]The Wolfson Institute for Biomedical Research, University College London; [2]Tumour Biology Laboratory, Cancer Research UK Clinical Centre, Queen Mary's Medical and Dental School at Bart's and the London, London, United Kingdom; and [3]Department of Oral and Maxillofacial Surgery, Queen Alexandra Hospital, Portsmouth, United Kingdom

Abstract

Widespread expression of the α-subunit of hypoxia-inducible factor (HIF-1α) was observed in samples of human oral squamous cell carcinoma. In all the cases, this was accompanied by a widespread distribution of nitric oxide (NO) synthases (NOS). Furthermore, in three human cell lines derived from human oral squamous cell carcinoma, the accumulation of HIF-1α was prevented either by inhibition of NOS activity with the nonspecific NOS inhibitor N^G-monomethyl-L-arginine or by the antioxidants *N*-acetyl-L-cysteine and ascorbic acid. We suggest that, in certain forms of cancer, NO might be responsible for the accumulation of HIF-1α by a mechanism dependent on free radicals. (Cancer Res 2006; 66(2): 770-4)

Introduction

The dimeric transcription factor hypoxia-inducible factor (HIF-1) is a key regulator of the cellular response to hypoxia (1). Its nuclear translocation follows cytosolic accumulation of its α-subunit (HIF-1α), which results from inhibition of the O_2-sensitive prolyl hydroxylases (PHDs; ref. 2). These enzymes, at physiologic O_2 concentrations, constantly modify HIF-1α and target it for proteasomal degradation (3, 4). Following inhibition of PHDs, HIF-1α dimerizes with the constitutive HIF-1β to form HIF-1, which is responsible for activation of a variety of genes, including those involved in glycolysis, erythropoiesis, angiogenesis, and vascular remodeling (5). Although inhibition of PHDs due to hypoxia is recognized as the main mechanism responsible for the stabilization of HIF-1α, it has become evident that this can also occur by other mechanisms. Some growth factors and cytokines increase the synthesis of HIF-1α (see ref. 6), whereas some compounds that chelate iron seem to stabilize it, probably by acting on the protein itself or by affecting the action of the PHDs (7).

The presence of HIF-1α has been observed in different types of cancer and there is some indication that this is associated with malignancy and lack of response to treatment (8). Originally, the presence of HIF-1α in cancer was attributed to tumor hypoxia (9) but recent evidence suggests that other mechanisms may also be involved (10, 11). Increases in nitric oxide (NO) generation have been observed in certain forms of cancer and there is also evidence for an association between concentrations of NO, malignancy, and resistance to treatment (12). At physiologic concentrations, NO decreases the stabilization of HIF-1α in hypoxia due to its ability to inhibit mitochondrial respiration and redirect O_2 to reactivate the PHDs (13). However, at the high concentrations produced in certain forms of cancer, NO also increases the presence of HIF-1α by an action probably involving a free radical mechanism (14, 15). In those experiments, however, NO was either added exogenously or generated in an overexpressed cell system. In view of this, we decided to investigate whether endogenous NO is involved in the presence of HIF-1α in human oral squamous cell carcinoma, a malignancy in which both HIF-1α and NO are known to occur (10, 16). To do this, we studied the distribution of NO synthase (NOS) and of HIF-1α in samples of human oral carcinoma tissue and in cell lines obtained from human oral squamous cell carcinoma, where we also investigated the effect of free radical production on HIF-1α expression.

Materials and Methods

Immunohistochemistry. Immunohistochemistry for inducible NOS (iNOS), endothelial NOS (eNOS), and HIF-1α proteins was carried out on 29 formalin-fixed specimens of human oral squamous cell carcinoma and 10 samples of normal oral mucosa. Monoclonal antibodies to iNOS, eNOS (BD Biosciences, Oxford, United Kingdom), and HIF-1α (Novus Biologicals, Littleton, CO) were used as previously described (16, 17), and samples were developed using an avidin biotin horseradish peroxidase system (DAKO, Glostrup, Denmark). Positive controls were normal kidney for HIF-1α and an oral squamous cell carcinoma known to have high eNOS expression and activity. Negative controls consisted of omitting the primary antibody to test for nonspecific secondary antibody binding and the use of the irrelevant primary antibodies CD34 and p53, with the same isotypes as the primary antibodies being evaluated in this study, to assess for nonspecific primary antibody. Tumors were graded as showing no expression (negative) or expression (positive) for staining relative to known controls by two researchers using a conference microscope (Axioskop 2, Zeiss, Jena, Germany). Tumors were graded as HIF-1α positive only if both nuclear and cytoplasmic staining was seen (17).

Cells and reagents. Human umbilical venous endothelial cells (HUVEC) were purchased from PromoCell (Heidelberg, Germany). Cells were grown as previously described (18).

A panel of 11 oral squamous cell carcinoma cell lines was initially screened for NOS expression, and three cell lines (H157, H357, and BICR6, generously provided by Prof. S.S. Prime, University of Bristol Dental School, Bristol, United Kingdom and Prof. E.K. Parkinson, Institute of Dentistry, Queen Mary's Medical and Dental School, Bart's and the London, United Kingdom) were used for subsequent work.

Cells were grown in standard keratinocyte growth medium (KGM) as described (19). KGM comprised α-MEM containing 10% FCS (Globepharm, Surrey, United Kingdom) supplemented with 100 IU/L penicillin, 100 μg/L streptomycin, 1.8×10^{-4} mol/L adenine, 5 μg/mL insulin, 1×10^{-10} mol/L cholera toxin, 0.5 μg/mL hydrocortisone, and 10 ng/mL epidermal growth factor (Sigma, Dorset, United Kingdom). All cells were tested routinely for *Mycoplasma*.

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Requests for reprints: Salvador Moncada, The Wolfson Institute for Biomedical Research, University College London, The Cruciform Building, London WC1E 6BT, United Kingdom. Phone: 44-2076796666; E-mail: s.moncada@ucl.ac.uk.

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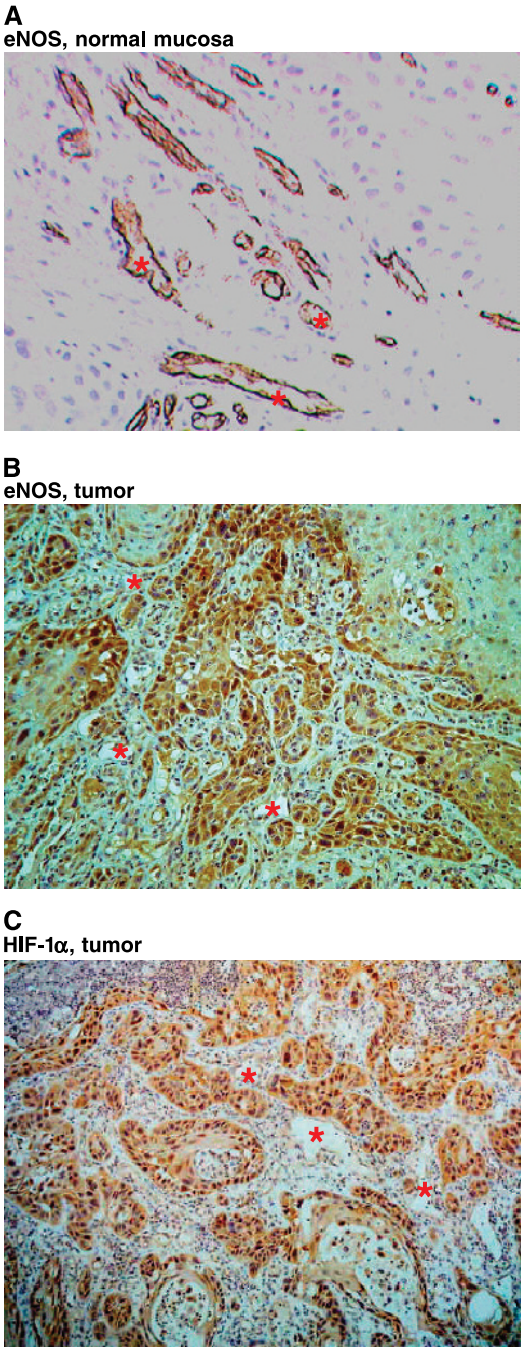

Figure 1. Immunohistochemical staining for eNOS and HIF-1α in human oral samples. *A,* eNOS expression in blood vessels of normal mucosa (magnification, ×60). *B,* eNOS is expressed diffusely throughout the entire tumor tissue (magnification, ×70). *C,* widespread presence of nuclear and cytoplasmic HIF-1α (magnification, ×60). *, prominent blood vessels.

Custom *SMART*pool small interfering RNA (siRNA) reagents targeting HIF-1α or random (nontargeting siRNA) were purchased from Dharmacon RNA Technologies (Chicago, IL) and were used according to the instructions of the manufacturer to generate siRNA knock down HIF-1α for BICR6 cells. Semiquantitative real-time PCR was carried out essentially as described by Medhurst et al. (20) using SYBRGreen as a detection system for the following genes: *HIF-1α, hexokinase II* (*HK-II*), *hemoxygenase* (*HO*), and *matrix metalloproteinase 9* (*MMP9*). *β-Actin* was used as a housekeeping gene to normalize targeted gene expression.

Acc X06985 hHO-F: GCCCCCACTCAACACCC, hHO-R: AGAAAGCTGAGTGTAAGGACCCA; Acc NM_000189 hHKII-F: GCCGGGCTGAGATTCTTCT, hHKII-R: CGTGTCCGGTTGTCCCAC; Acc BC006093 hMMP9-F: GCTCACCTTCACTCGCGTG, hMMP9-R: CGCGACACCAAACTGGATG; Acc BC012527 hHIF-1α F: TGTGAACCCATTCCTCACCCATCA, hHIF-1α R: CAGTTTCTGTGTCGTTGCTGCCAA; Acc NM_001101 hβ-actin-F: ACCATGGATGATGATATCGCC, hβ-actin-R: GCCTTGCACATGCCGG.

Experimental procedures were carried out when the cells were 80% confluent. Hypoxia was achieved by incubation of the cells using an O_2-controlled hypoxic chamber (Coy Laboratory Products, Ann Arbor, MI) at maintained O_2 concentrations and 37°C. *N*-acetyl-L-cysteine (NAC) and L-ascorbic acid were purchased from Sigma; N^G-monomethyl-L-arginine (L-NMMA) was purchased from Alexis (Nottingham, United Kingdom).

Preparation of cytosolic extracts. Cells were scraped off in ice-cold PBS containing phosphatase inhibitors. Cell pellets were homogenized in 50 mmol/L Tris-HCl (pH 7.5) containing 0.1 mmol/L DTT, 0.2 mmol/L EDTA, and 10 μg/mL protease inhibitor cocktail (benzamidine, leupeptin, aprotinin, and antipain). After 30 minutes of incubation on ice, the cells were centrifuged (12,000 × *g*, 30 minutes, 4°C) and the supernatant was retained. Protein concentrations were determined by protein assay (Bio-Rad, Hemel Hempstead, United Kingdom). Two hundred micrograms of protein were transferred to nitrocellulose membranes (Amersham Pharmacia, Little Chalfont, Buckinghamshire, United Kingdom). The membranes were then incubated with shaking in 10% milk in wash buffer (PBS/0.1% Tween 20) for 1 hour at room temperature. The membrane was washed twice (15 minutes per wash) in wash buffer before incubation overnight at 4°C, with gentle shaking, with primary anti-eNOS, anti-iNOS, and anti-neuronal NOS (nNOS; Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1/2,500 in 1% milk in wash buffer. The membrane was washed six times (5 minutes per wash) and then incubated, with gentle shaking for 2 hours at room temperature, with horseradish peroxidase–conjugated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) diluted 1/5,000 in 1% milk in wash buffer.

The membrane was washed as before and proteins were visualized using enhanced chemiluminescence (Amersham, Aylesbury, United Kingdom).

Nitrite measurement. The amount of NO formed was estimated by measuring nitrite levels in the extracellular medium using the Griess reagent kit (Molecular Probes, Leiden, The Netherlands). Cells were incubated overnight in the absence or presence of the NOS inhibitor L-NMMA (1 mmol/L).

Accumulation and activation of HIF-1α. Cells were incubated for 8 hours under different experimental conditions. Nuclear extraction was carried out as previously described (18). Protein content in the nuclear extraction was determined to adjust the amount to 60 μg per well for each sample. Samples of 60 μg of protein were analyzed by Western

Table 1. The immunohistochemical distribution of HIF-1α, eNOS, and iNOS

Cases (*n* = 29)	HIF-1α	eNOS	iNOS
14	+	+	+
10	+	+	–
2	–	–	+
1	–	+	–
2	–	–	–
Control (*n* = 10)	–	+*	–

NOTE: The expression of HIF-1α, eNOS, and iNOS was analyzed in 29 cases of oral squamous cell carcinoma and 10 samples of normal oral mucosa.
*Localized in blood vessels.

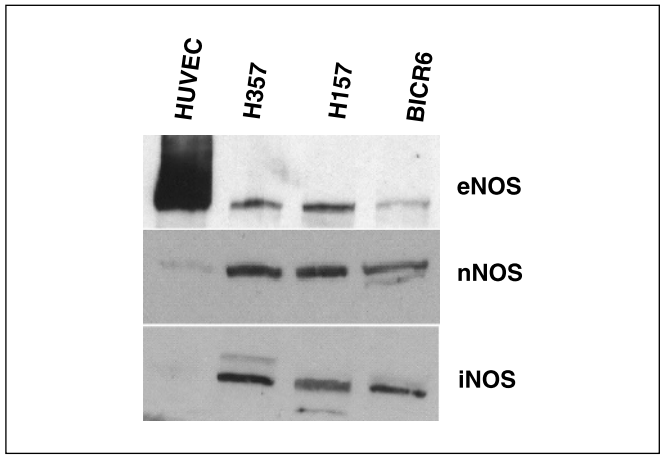

Figure 2. Expression of eNOS, nNOS, and iNOS in oral squamous carcinoma cell lines. Western blot analysis showing the expression of eNOS, nNOS, and iNOS protein in HUVEC, H357, H157, and BICR6 cells. The blot shows the presence of the three isoforms in all three cancer cells, in contrast with HUVEC that only express the endothelial isoform. Representative of results obtained in three separate experiments.

blot using a mouse monoclonal antibody (BD Biosciences) against HIF-1α, followed by an antimouse horseradish peroxidase conjugate (DAKO). The protein band was detected by enhanced chemiluminescence (Amersham Pharmacia). Activation of HIF-1 was quantified in 5 to 10 μg of nuclear extract by specific binding of HIF-1 to an oligonucleotide containing the HRRE for the *Epo* gene by means of the TransAM HIF-1 kit (Active Motif, Rixensart, Belgium) according to the instructions of the manufacturer.

Results

Immunohistochemical expression of HIF-1α and NOS in oral squamous cell carcinoma. A total of 29 carcinoma samples and 10 normal oral mucosal epithelial biopsies were analyzed for this study. In normal oral mucosa, eNOS was localized to blood vessel endothelium in the submucosa in all cases (Fig. 1*A*). iNOS and HIF-1α were not observed either in the epithelium or submucosa in any of the normal samples. The presence of eNOS, HIF-1α, and iNOS was detected in 25 of 29, 24 of 29, and 16 of 29 cancer cases, respectively (see Table 1). All three proteins were found in 14 of 29 cases, with eNOS and HIF-1α both being expressed in a further 10 of 29 cases. The expression of both HIF-1α and eNOS was observed diffusely throughout the entire tumor tissue, and there was no obvious correlation between either eNOS or HIF-1α and the distance from blood vessels (Fig. 1*B* and *C*, respectively). In the carcinoma samples, eNOS staining was seen in tumor cells and in the endothelium of both normal and tumor blood vessels (Fig. 1*B*). HIF-1α staining was also found to be both nuclear and cytoplasmic and was mainly localized to tumor cells (Fig. 1*C*). In no case was HIF-1α present in the absence of eNOS. The presence of iNOS was observed in 16 of 29 cases, being mainly localized to tumor cell cytoplasm, although some staining was also seen in peri-tumoral macrophages (not shown).

NOS in cancer cells. The presence of eNOS, iNOS, and nNOS was verified by Western blot. All three NOS isoforms were present in the cancer cell lines H357, H157, and BICR6; this was unlike the HUVEC in which only eNOS was found (Fig. 2). L-NMMA treatment significantly inhibited the generation of NO in BICR6

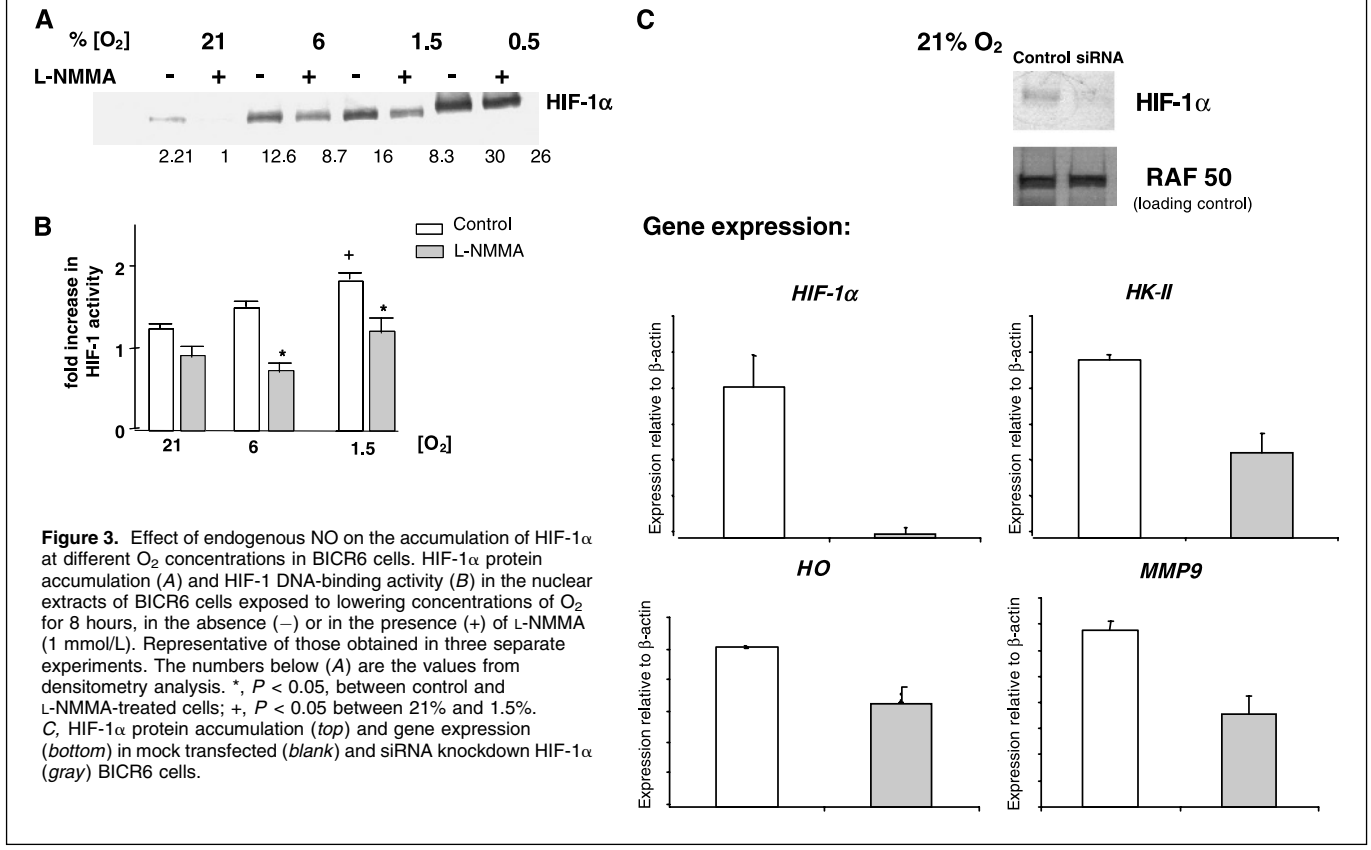

Figure 3. Effect of endogenous NO on the accumulation of HIF-1α at different O_2 concentrations in BICR6 cells. HIF-1α protein accumulation (*A*) and HIF-1 DNA-binding activity (*B*) in the nuclear extracts of BICR6 cells exposed to lowering concentrations of O_2 for 8 hours, in the absence (−) or in the presence (+) of L-NMMA (1 mmol/L). Representative of those obtained in three separate experiments. The numbers below (*A*) are the values from densitometry analysis. *, $P < 0.05$, between control and L-NMMA-treated cells; +, $P < 0.05$ between 21% and 1.5%. *C,* HIF-1α protein accumulation (*top*) and gene expression (*bottom*) in mock transfected (*blank*) and siRNA knockdown HIF-1α (*gray*) BICR6 cells.

cells from 0.3 ± 0.05 (control) to 0.06 ± 0.02 nmol nitrite/ 5×10^6 cells (treated).

NO-dependent stabilization of HIF-1α in cancer cells. The effect of endogenous NO on the stabilization of HIF-1α by lowering the O_2 concentration was analyzed in the three different cancer cell lines. Figure 3*A* shows that in BICR6 cells stabilization of HIF-1α occurs at high O_2 concentrations, so that it could even be observed at 21% O_2. As the O_2 concentration decreased, the stabilization of HIF-1α became more apparent. Figure 3*B* shows increases in nuclear binding of HIF-1 in BICR6 cells at different O_2 concentrations. The stabilization and binding were both reduced in the presence of L-NMMA. The NO donor DETA-NO restored the stabilization of HIF-1α in cells that had been treated with L-NMMA, confirming that the effect of L-NMMA is specifically due to inhibition of NO synthesis (data not shown). Figure 3*C* shows that HIF-1α expression is abolished in siRNA knockdown BICR6 cells. This is accompanied by a reduction in expression of *HK-II, HO,* and *MMP9,* which are downstream targets of HIF-1α. Figure 4*A* shows that treatment with the NOS inhibitor L-NMMA abolished the stabilization of HIF-1α at 3% O_2 in the three cell types assayed. L-NMMA treatment did not, however, affect the stabilization of HIF-1α when the O_2 concentration was lowered to 0.5%, as shown in Fig. 4*B*.

Constitutive NO-dependent stabilization of HIF-1α in tumor cells. To test directly whether the effect of endogenous NO on HIF-1α stabilization was due to its ability to generate free radicals, the three cell lines were incubated at 3% O_2 in the absence or presence of antioxidants and HIF-1α protein concentrations in the nuclear extracts were measured by Western blot. Incubation of the three cell lines with 2.5 mmol/L NAC and 1 mmol/L L-ascorbic acid prevented the stabilization of HIF-1α at 3% O_2 (Fig. 5*A*). When the experiment was carried out at lower O_2 concentrations (0.5%), no significant effect was observed after pretreatment with antioxidants (Fig. 5*B*).

Discussion

We have observed a widespread distribution of HIF-1α in 24 of 29 human samples of oral squamous cell carcinoma. The widespread distribution indicates that HIF-1α stabilization is not exclusively associated with areas of potential low O_2 concentration. This is in agreement with previous observations (10, 11) showing a dual distribution pattern for HIF-1α, one distal from the blood vessels, suggesting that it was probably related to hypoxia, and another diffuse pattern independent of vessel proximity.

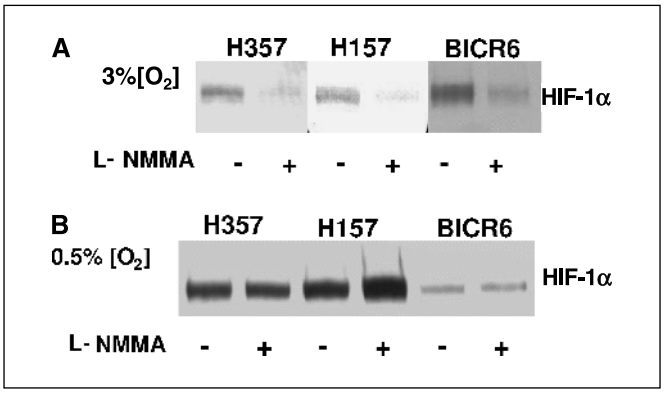

Figure 4. Effect of endogenous NO on the accumulation of HIF-1α in oral squamous carcinoma cells at low O_2 concentrations. *A,* HIF-1α protein accumulation in the nuclear extracts of cells treated with (+) or without (−) 1 mmol/L L-NMMA, exposed to 3% O_2 for up to 8 hours. *B,* as (*A*), but with cells exposed to 0.5% O_2. Representative of results obtained in three separate experiments.

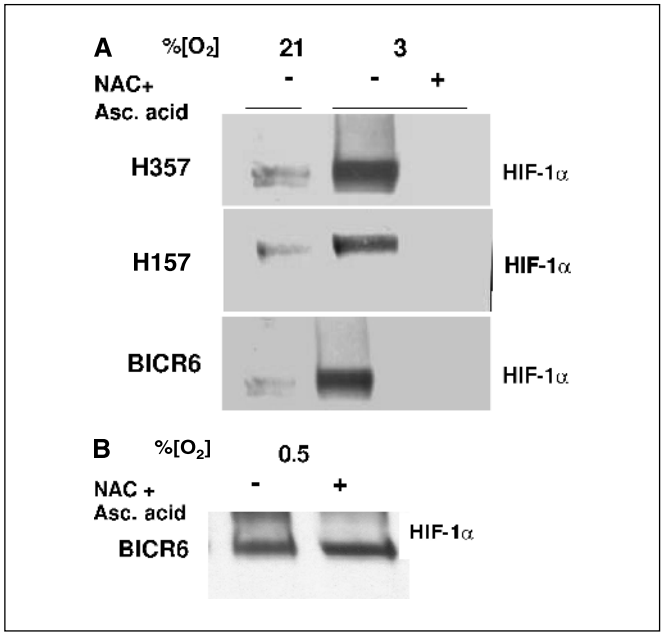

Figure 5. Effect of antioxidants on the accumulation of HIF-1α in oral squamous carcinoma cells. *A,* Western blot analysis showing the accumulation of HIF-1α in nuclear extracts from cells exposed to 3% O_2 for up to 8 hours in the absence (−) or presence (+) of antioxidants (NAC and L-ascorbic acid). *B,* as (*A*), but with BICR6 cells exposed to 0.5% O_2. Representative of those obtained in three separate experiments.

In the three cell lines used, we found stabilization of HIF-1α at O_2 concentrations well above those that would be considered hypoxic and, therefore, independent of PHD inhibition. In one cell line, HIF-1α was observed even at ambient O_2 concentration (21%). The mechanism for this accumulation is not clear; however, our experiments indicate that it is related to NO because inhibition of its generation by the NOS inhibitor L-NMMA prevented HIF-1α stabilization in all three cell lines. In our studies on malignant tissue we found that, of the 24 samples containing HIF-1α, both iNOS and eNOS were widely expressed in 14 cases, whereas the remaining 10 cases expressed eNOS alone. In the cancer cell lines, we found the three isoforms of NOS without any obvious difference in protein concentration. NOS has been widely observed in cancer (21, 22). Originally, iNOS, which is induced in pathophysiologic situations such as inflammation and tissue degeneration, was thought to be associated with cancer (23); however, the involvement of eNOS has also been suggested (24). In our current results, eNOS was present more often than iNOS in the tumor samples examined. It is not currently known why the expression of different NOS isoforms in cancer is not uniform.

The fact that the NO-dependent effect on HIF-1α stabilization in the three cell lines studied could be inhibited by NAC and L-ascorbic acid suggests that the mechanism is dependent on a free radical reaction, secondary to an interaction of NO with O_2 or O_2-derived species. Malignant tissue is known to be rich in free radicals (25), the origin of which has not been completely elucidated (26). Thus, NO generated from any isoform might be released into an environment that favors free radical reactions, leading to, among other things, the formation of peroxynitrite, a highly oxidant species that has also been detected in some cancers (27). Thus, it is

possible that the effects of NO in the cancer phenotype are more dependent on the environment in which it is produced than on its concentration or the specific isoform of NOS by which it is generated.

The stabilization of HIF-1α at a low O_2 concentration (0.5%) was not affected by treatment with L-NMMA, suggesting that hypoxia rather than NO was responsible for this stabilization. This suggestion is supported by the fact that antioxidants also had no effect on the stabilization of HIF-1α at this low O_2 concentration. This is in agreement with previous observations (28). The stabilization of HIF-1α in this condition is most likely to be due to inhibition of PHDs (29).

In view of the above, it is possible that accumulation of HIF-1α in cancer may be the result of a complex interaction of several factors, including hypoxia and NO, the combination of which we have previously shown to be highly synergistic in stabilizing HIF-1α (15). The profile of activities of these different factors may vary not only from cancer to cancer but also from one region of a cancer to another. Traditionally, tumor hypoxia has been associated with poor prognosis and resistance to treatment (30). Because the hypoxia-associated resistance to treatment is probably due, at least in part, not to the hypoxia itself but to the cellular defense mechanisms activated by it, any other factors leading to the same defense mechanisms, such as in this case the effect of NO on HIF-1α stabilization, are likely to reinforce the cancer phenotype. A corollary of our study is that inhibition of NO generation, or a decrease in free radical formation, may be useful adjuncts to other therapies in the management of cancer.

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