ANTICANCER RESEARCH *26*: 2165-2168 (2006)

Comparative Effects of Hypoxia on Normal and Immortalized Human Diploid Fibroblasts

EFTHYMIOS POULIOS[1,2], IOANNIS P. TROUGAKOS[1] and EFSTATHIOS S. GONOS[1]

[1]*Laboratory of Molecular and Cellular Aging, Institute of Biological Research and Biotechnology, National Hellenic Research Foundation, 48 Vas. Constantinou Ave., Athens 11635;*
[2]*Laboratory of Biochemistry, School of Medicine, University of Thessaly, 22 Papakyriazi St., Larissa 41222, Greece*

Abstract. *Hypoxia, a condition of reduced oxygen concentration, is observed in many physiological and pathophysiological states. However, there is rather limited information regarding hypoxic effects in the processes of immortalization and senescence of human cells. Here, the effects of hypoxia induced by either 1.5% O_2, or by a hypoxia-mimetic agent, $CoCl_2$, on the protein expression of normal human diploid fibroblasts (HDFs) undergoing replicative senescence, and in their Simian Virus 40 (SV40) T antigen immortalized counterparts are described. The data demonstrated that, in all cell types assayed, either hypoxia or $CoCl_2$ induced the main regulator of transcriptional responses to reduced oxygen tension, namely the hypoxia inducible factor-1α (HIF-1α), in a dose- and time-dependent manner. In the immortalized HDFs, the transcriptional activity of HIF-1α was also evident by the accumulation of its main downstream gene targets, namely erythropoietin (EPO) and the vascular endothelial growth factor (VEGF). Interestingly, the immortalized HDFs were found to exhibit higher HIF-1α endogenous levels and induction, following cell exposure to hypoxic conditions, as compared to either young or senescent cells. Subsequent analysis of the expression levels of two pro-survival proteins, bcl-2 and clusterin/apolipoprotein J (CLU), in cells exposed to hypoxic conditions, revealed that although bcl-2 was up-regulated independently of the cell type, CLU was induced only in the $CoCl_2$-treated immortalized HDFs. These findings indicate that the distinct cellular contexts of normal and immortalized HDFs may induce differential responses to hypoxia.*

Hypoxia is a condition of reduced oxygen levels which is observed in many pathological states, including cardiovascular ischaemia, pulmonary hypertension, pregnancy abnormalities and cancer (1). Hypoxia can be mimicked by the transition metals, such as cobalt, which is incorporated into heme, thereby lowering its binding affinity to oxygen (2). The hypoxic microenvironment results in the differential expressions of a wide variety of genes involved in anaerobic metabolism, glycolysis, erythropoiesis, respiration, angiogenesis, vasodilation and cell survival (3, 4). The main transcription factor activated in hypoxic conditions is hypoxia inducible factor-1 (HIF-1). HIF-1 is a heterodimer composed of two subunits belonging to the basic helix-loop-helix Per/Arnt/Sim (bHLH-PAS) family, namely the aryl hydrocarbon receptor nuclear translocator (ARNT) or HIF-1β, which is constitutively expressed in the nucleus and HIF-1α, which is specifically regulated by hypoxia (3).

Although it is well documented that hypoxia correlates to a variety of pathophysiological states, such as cancer (1), there is limited information regarding the impact of hypoxia during the immortalization and senescence of normal human diploid fibroblasts (HDFs). It has been shown previously that senescent HDFs exhibited a decreased response to hypoxia, since many hypoxia-responsive genes involved in angiogenesis, defense against oxidative stress and transcription regulation were found to be impaired in these cells (5). Moreover, a comparative study in adult lung fibroblasts and amelanotic melanoma cells revealed significant differences in the regulation of the genes involved in antioxidant cellular defence following cell exposure to ambient oxygen tension (6). Similarly, the analysis of fibroblasts derived from control or diabetic mice revealed several dysfunctions in the diabetic fibroblasts, including an impaired response to hypoxic conditions (7). These studies demonstrated that the cellular state appears to interfere with the cell response to hypoxia. Here, these observations were extended and the differential molecular effects of hypoxic conditions, induced either by lower oxygen concentration or by a hypoxia-mimetic agent, in normal HDFs undergoing replicative senescence, as well as in the corresponding SV40 T antigen immortalized cells, are reported.

Correspondence to: Efstathios S. Gonos, Laboratory of Molecular and Cellular Aging, Institute of Biological Research and Biotechnology, National Hellenic Research Foundation, 48 Vas. Constantinou Ave., Athens 11635, Greece. Fax: +30 210-7273677, e-mail: sgonos@eie.gr

Key Words: Bcl-2, clusterin/apolipoprotein J, human fibroblasts, hypoxia, immortalization, replicative senescence, SV40 T antigen.

0250-7005/2006 $2.00+.40

Materials and Methods

Cell lines and culture conditions. Human embryonic fibroblasts WI-38 and the SV40 T antigen WI-38 immortalized cells (WI-38/T) were obtained from the European Collection of Cell Cultures and were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco Life Technologies) supplemented with 10% fetal bovine serum (v/v), 2 mM glutamine and 1% non-essential amino acids. Early passage young WI-38 cells at cumulative population doubling (CPD) <25 were sub-cultured at a split ratio 1:2 when they had reached confluence, until they entered senescence at CPD >40.

$CoCl_2$ and low oxygen treatments. Young and senescent WI-38 cells, as well as WI-38/T cells, were treated with either 150 μM $CoCl_2$ for 4 h or with 450 μM $CoCl_2$ for 4 to 8 h. Cell culture in reduced oxygen concentration was achieved by growing the cells in a hypoxic incubator (Modular Incubator Chamber; Thermo Electron Corporation) adjusted to an atmosphere of 1.5% O_2, for various periods of time (30 min, 2 h, 4 h, 8 h and 24 h).

Protein extraction and immunoblotting analysis. The cell monolayers were directly lysed in extraction buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 20 μg/ml PMSF in 50 mM Tris-HCl, pH 8.0) and adjusted to contain equal protein by the Bradford (Bio-Rad) assay. Loading was further confirmed by the use of an actin (Santa Cruz; sc-1616) antibody. SDS/PAGE protein fractionation and immunoblotting analysis were carried out as previously described (8). The antibodies against CLU (sc-6419), bcl-2 (sc-509), EPO (sc-7956) and VEGF (sc-507) were purchased from Santa Cruz; the HIF-1α antibody (BD 610959) was from BD Transduction Lab.

Results and Discussion

HIF-1α activation in normal and immortalized HDFs following treatment with $CoCl_2$ or exposure to low oxygen concentration. To investigate whether the hypoxia-mimetic agent $CoCl_2$ alters the expression levels of HIF-1α and its downstream target genes, WI-38/T immortalized and WI-38 early passage (young) normal HDFs were treated with 150 μM $CoCl_2$ for 4 h or with 450 μM $CoCl_2$ for 4-8h. Subsequent immunoblotting analysis revealed an intense up-regulation of the HIF-1α protein levels in the WI-38/T cells in a dose-dependent manner (Figure 1A). Maximum HIF-1α expression levels were recorded in both the immortalized and normal HDFs after cell treatment with 450 μM $CoCl_2$ for 4 h (Figure 1). In addition, HIF-1α induction was moderate in the WI-38 normal cells as compared to their immortalized counterparts (Figure 1B). To verify that HIF-1α up-regulation relates to enhancement of its transcriptional activity, the expression levels of two of its downstream target genes, namely EPO, an erythropoiesis promoting factor and VEGF, a regulator of angiogenesis (9, 10), were assayed in the WI-38/T cells. As shown in Figure 1A, both these proteins were found to accumulate in a pattern similar to HIF-1α induction, thus indicating the formation of the transcriptionally-active HIF-1 heterodimer, as previously suggested (9, 10). Moreover, the expression levels of the pro-survival proteins bcl-2 and CLU were monitored. As shown in Figure 1, cell exposure to $CoCl_2$ induced bcl-2 up-regulation in both the WI-38/T and WI-38 normal cells, while CLU was up-regulated only in the WI-38/T cells.

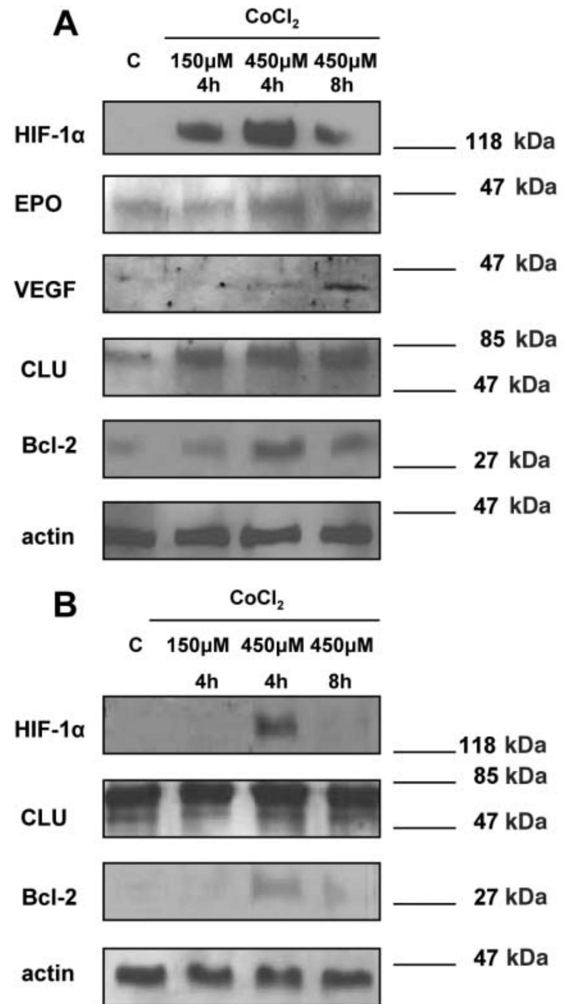

Figure 1. *Effects of $CoCl_2$-mediated hypoxia on the protein expression in immortalized and normal human diploid fibroblasts (HDF). WI-38/T immortalized (A) and WI-38 normal (B) HDFs were treated with 150 μM $CoCl_2$ for 4 h or with 450 μM $CoCl_2$ for 4 to 8 h and protein extracts were analyzed for the expression of HIF-1α, EPO, VEGF, CLU and bcl-2. Maximum HIF-1α induction was observed in cells exposed to 450 μM $CoCl_2$ for 4 h (A, B) in both cell lines. The induction of HIF-1α downstream target genes, such as EPO and VEGF, is evident in the WI-38/T treated cells (A). Bcl-2 was induced in both cell lines (A, B), while CLU was mainly up-regulated in the WI-38/T cells (A). Actin expression was used as a reference for protein loading; C, untreated control cells. Molecular weight markers are indicated on the right of each blot.*

The HDFs were then exposed to a low oxygen concentration for up to 24 h and the expression levels of HIF-1α, bcl-2 and CLU were analyzed. As evident from Figure 2, HDF exposure to low oxygen levels resulted in a time-dependent up-regulation of HIF-1α. In the WI-38/T cells, the HIF-1α

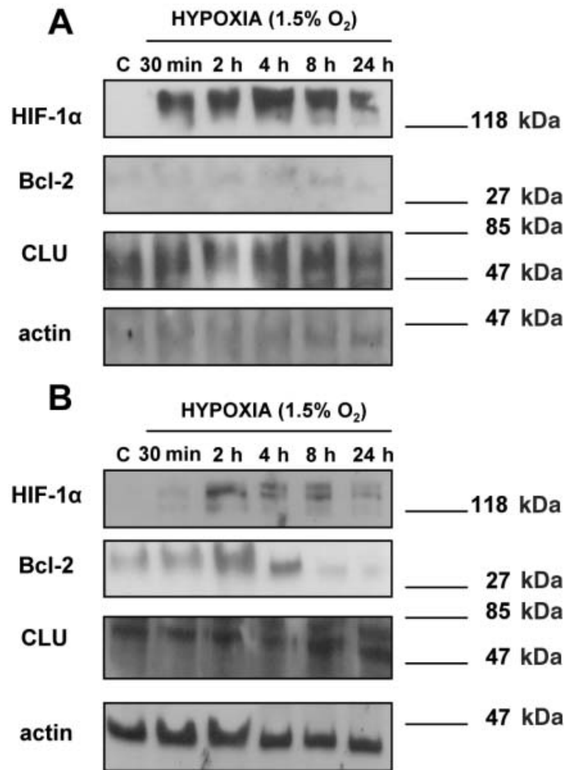

Figure 2. *Effects of low oxygen concentration (1.5%* O_2*) on protein expression in immortalized and normal human diploid fibroblasts (HDF). WI-38/T immortalized (A) and WI-38 normal (B) HDFs were exposed to hypoxic conditions for the indicated time-points and protein extracts were analyzed for the expression of HIF-1α, bcl-2 and CLU. Although HIF-1α was induced in both cell lines in a time-dependent manner, its induction occured earlier and lasted longer in the WI-38/T cells as compared to the corresponding normal cells (A, B). Bcl-2 was only slightly induced, while CLU levels remained constant in HDFs exposed to hypoxia. C, untreated control cells; actin was used as a reference for protein loading. Molecular weight markers are indicated on the right of each blot.*

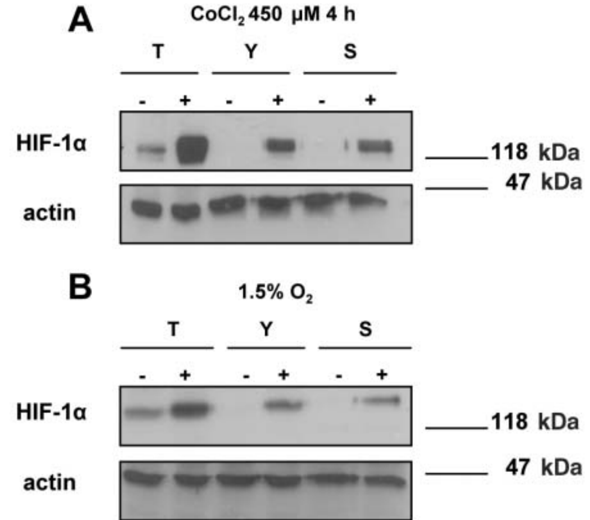

Figure 3. *HIF-1α levels in T antigen immortalized (T), young (Y) and senescent (S) WI-38 cells following treatment with either 450 μM* $CoCl_2$ *for 4 h (A) or exposure to 1.5 %* O_2 *for 2 h (B). HIF-1α expression levels are significantly higher in the immortalized WI-38/T cells as compared to normal cells in both treatments. HIF-1α induction is similar in young and senescent WI-38 cells (A, B). Actin was used for loading normalization; molecular weight markers are indicated on the right of each blot.*

induction was initiated 30 min after the cells had been submitted to hypoxic conditions and lasted for up to 24 h (Figure 2A), whereas in the early passage WI-38 cells, HIF-1α induction was moderate (Figure 2B). Interestingly, bcl-2 was only slightly up-regulated in both cell lines, while the CLU levels remained unchanged.

CLU has been previously implicated in the cell response to various types of stress (11) and is induced in the rodent brain after transient ischemia (12), or following hypoxic-ischemic injury (13). Moreover, studies on $CLU^{-/-}$ mice revealed that CLU is actively involved in the hypoxia-ischemia response (14, 15). Nevertheless, our finding that CLU was up-regulated only in the WI-38/T immortalized HDFs treated with $CoCl_2$ does not suggest a direct involvement of CLU in the cellular response to a hypoxic microenvironment. In contrast, the induction of bcl-2 most probably relates to the absence of apoptosis observed in HDFs exposed to either $CoCl_2$ or to hypoxia (not shown). Similarly, another pro-survival gene, Bcl-XL, was overexpressed in hypoxia-selected cells resistant to apoptosis (16). Moreover, moderate hypoxia was reported to protect cells against apoptosis induced by serum deprivation (17), while $CoCl_2$ was shown to inhibit apoptotic cell death induced in HepG2 cells by either serum deprivation or by tert-butylhydroperoxide (t-BHP)-induced oxidative stress (18).

In conclusion, both treatment with $CoCl_2$ and exposure to 1.5% O_2 resulted in HIF-1α induction in young normal and immortalized HDFs, albeit with different HIF-1α kinetics, whereas $CoCl_2$, apart from mimicking hypoxia, may also induce additional molecular events.

Immortalized HDFs exhibit higher HIF-1α endogenous levels and induction following exposure to hypoxic conditions, as compared to young and senescent cells. Whether replicative senescence affects the cell response to hypoxia was examined. To this end, the WI-38 cells were grown until they had entered senescence. The HIF-1α levels in young and senescent WI-38 cells, as well as in immortalized WI-38/T cells, were comparatively analyzed following exposure to either 450 μM $CoCl_2$ for 4 h or to 1.5% O_2 for 2 h. HIF-1α was up-regulated to a similar extent in both young and senescent WI-38 cells (Figure 3), indicating that the cellular machinery involved in the HIF-1α response to either $CoCl_2$ or to 1.5% O_2 was not impaired during replicative senescence. These findings are in accordance with a previous study, where HIF-1α protein levels were found to be similar in the liver of young and old animals exposed to low oxygen concentrations (19).

As shown in Figures 3A and 3B, the endogenous expression level of HIF-1α was also significantly higher in the immortalized WI-38/T fibroblasts, as compared to the normal cells. In addition, cell exposure to either $CoCl_2$ or 1.5% O_2 induced a more intense up-regulation of HIF-1α in the WI-38/T cells, as compared to either young or senescent cells (Figure 3). The apparent effect of oncogenes (*e.g.,* the SV40 T antigen in the case of WI-38/T cells) to HIF-1α endogenous levels and the intensity of up-regulation in hypoxic conditions were further confirmed after similar studies in IMR90 cells, after transfection with the E6, E7 or both E6 and E7 oncogenes of the human papillomavirus 16 (HPV16) (data not shown).

SV40 T antigen inactivates, among others, the p53 and pRB tumor suppressor gene products (20). The more intense induction of HIF-1α protein in the T antigen immortalized HDFs under hypoxic conditions could be explained by the interplay between HIF-1α and p53. More specifically, during hypoxia, HIF-1α has been reported to protect p53 from mdm-2-dependent degradation, thus inducing p53 activation (21) and also promoting mdm-2 dependent HIF-1 degradation (22). According to this model, inactivation of p53 by the SV40 T antigen in WI-38/T cells may result in the inhibition of HIF-1α degradation by mdm-2, thus resulting in HIF-1α accumulation.

Overall, it is proposed that the distinct cellular contexts of normal and immortalized HDFs may induce differential responses to hypoxia. Current studies aim at revealing the molecular basis of these differences, as well as whether hypoxia alters the *in vitro* lifespan of normal human cells.

Acknowledgements

This work was mainly supported by a European Union full-cost "Proteomage" grant (LSH/FP-6/IP/518230) to E.S.G. E.P. is a recipient of a Ph.D. fellowship awarded from a General Secretariat of Research and Technology (PENED 01EΔ121) grant (coordinator Prof. S. Bonanou, University of Thessaly, Greece).

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Received January 30, 2006
Revised March 24, 2006
Accepted March 30, 2006