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SHORT REPORT

Hypoxia Induces Pro-Fibrotic and Fibrosis Marker Genes in Hepatocellular Carcinoma Cells Independently of Inflammatory Stimulation and the NF-κB Pathway

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Abstract: Hypoxia and its key mediators hypoxia inducible Factors (HIFs) are implicated in the development of liver diseases of diverse etiologies, often in interplay with inflammatory mediators. We investigated the interplay between hypoxia and proinflammatory mediators in the development of liver fibrosis, using human hepatocellular carcinoma Huh7 cells as a model. Treatment of Huh7 with DMOG or under hypoxia, induced HIF-1α protein levels and the expression of genes for pro-fibrotic (TGF-β1, PDGFC, PAI-1) and fibrosis (LOX, P4HA1, P4HB) markers. Knockdown of HIF-1α decreased the induction of PDGFC, LOX and P4HA1, showing the involvement of HIF-1 in their regulation. Interestingly, incubation of Huh7 cells under hypoxia did not cause activation of the NF-κB pathway. In contrast, inflammatory mediators such as tumor necrosis factor α (TNFα) and lipopolysaccharides (LPS) activated the NF-κB pathway, but failed to increase HIF-1α protein levels. Moreover, TNFα had a weaker effect than hypoxia on the induction or did not induce pro-fibrotic and fibrosis markers, respectively, while LPS enhanced only the hypoxic induction of P4HB. In conclusion, the above findings suggest that hypoxia and HIF-1 play an important role in the development of fibrosis in hepatocellular carcinoma, which appears to be independent of the activation of the NF-κB pathway.

Keywords: Huh7, HIF-1, fibrosis, NF-κB, TNFα, LPS

Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver malignancy, and one of the most frequent and lethal cancers worldwide. The vast majority of HCC cases are associated with inflammation, oxidative stress, fibrosis and cirrhosis. Proinflammatory and profibrotic factors play an important role in HCC initiation, promotion and metastasis and their expression is often correlated with poor prognosis.[1] Hypoxia acts as an aggravating factor of cell damage and inflammation, and stimulates angiogenesis and fibrogenesis. Chronic hypoxia is increasingly documented as an important determinant of fibrosis and carcinogenesis in the liver as in other tissues.[2,3] The effects of hypoxia are primarily mediated by the oxygen-sensitive hypoxia-inducible factors (HIFs). HIFs promote cancer development and progression via the induction of target genes that regulate oxygen delivery, metabolism, proliferation and apoptosis. Significant evidence indicates that HIFs play an important role in the pathogenesis and pathophysiology of HCC.[4]

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HIFs are heterodimeric transcription factors, comprising of unique HIFα subunits and a common HIFβ subunit (or aryl hydrocarbon receptor translocator; ARNT). The protein levels of the HIFα subunits are regulated, mainly at the post-transcriptional level. At physiological oxygen levels, HIFα is ubiquitinated and degraded at the proteasome. Under low oxygen concentration, HIFα is stabilized, dimerizes with ARNT and activates the transcription of target genes. The expression and activity of HIF-1α, the most widely expressed and better studied HIFα subunit, are also controlled by oxygen-independent mechanisms. To this end, the NF-κB signaling pathway has been shown to transcriptional upregulate HIF-1α synthesis and activity, mainly in cells of the immune system.[5]

Despite the known involvement of inflammation and hypoxia on HCC development, their exact contribution to the induction of pro-fibrotic and fibrosis marker genes in HCC is poorly characterized.

Materials and Methods

Materials

Tris-Base, Bromophenol Blue, Triton X-100, PMSF, TNFα, LPS were purchased from Sigma-Aldrich (St Louis, MO, USA), SDS, DTT and glycine from AppliChem (Darmstadt, Germany), NaCl and $MgCl_2$ from Merck (Darmstadt, Germany), dimethyloxalyl glycine (DMOG) from Alexis Biochemicals (CA, USA), nitrocellulose membranes from Macherey-Nagel (Düren, Germany), Dulbecco's modified Eagle's medium (DMEM) High Glucose, fetal bovine serum (FBS)-South America Origin and penicillin/streptomycin from Biosera (Nuaille, France) and dimethyl sulfoxide (DMSO) from Jena Bioscience (Jena, Germany).

Cell Culture and Treatment

Human hepatocarcinoma (Huh7) cells, a kind gift from Dr. Martina Muckenthaler, University of Heidelberg (originally purchased from the Japanese Collection of Research Bioresources), were grown in DMEM High Glucose supplemented with 10% FBS, 100 U/mL penicillin/streptomycin at 37°C in a humidified incubator under 5% CO_2. During hypoxic treatment, cells were exposed for the indicated time to 1% O_2, 95% N_2 and 5% CO_2 in an IN VIVO2 200 hypoxia workstation (RUSKINN Life Sciences/Baker Ruskinn, Sanford, Maine, USA). Cells were treated with TNFα, LPS, DMOG or DMSO at the appropriate concentration as solvent control. Before treatment with TNFα, cells were cultivated in FBS-free medium overnight.

siRNA-Mediated Silencing

siRNA HIF-1α (20nM, Qiagen, Venlo, Netherlands), or AllStars siRNA (20nM, Qiagen, Venlo, Netherlands) used as a negative control, was introduced in cells using Viromer Blue (Lipocalyx, Halle-Saale, Germany).

RNA Isolation and Quantitive Real-Time PCR

Total RNA was extracted using NucleoZol Reagent (Macherey-Nagel, Duren, Deutschland). cDNA was synthesized with PrimeScript RT Reagent Kit-Perfect Real-Time (Takara Clontech, Mountain View, CA, USA). Quantitative real-time PCR was performed using the KAPA SYBR FAST qPCR Kit (Kapa Biosystems, Wilmington, MA, USA) and primers (forward/reverse) for mRNAs coding for human TGF-β1 (5′-GAGCCCTGGACACCAACTAT-3′/5′-AAGTTGGCATGGTAGCCCTT-3′), PDGFC (5′-TTATGTGGAAACTACCCTGCGATTC-3′/5′-TCTCTCATGCTGAGGATCTTGT-3′), PAI-1 (5′-GAAAGGCAACATGACCAGGC-3′/5′-CATGCGGGCTGAGACTATGA-3′), LOX (5′-CGACGACCCTTACAACCCCTAC-3′/5′-AAATCTGAGCAGCACCCTGTG-3′), P4HB (5′-AGCTCGACAAAGATGGGGTT-3′/5′-GGCAAGAACAGCAGGATGTG-3′), P4HA1 (5′-AGGGGTTGCTGTGGATTACC-3′/5′-GTCATGTACTGTAGCTCGGC-3′) and β-actin (5′-CCAACCGCGAGAAGATGA-3′/5′-CCAGAGGCGTACAGGGATAG-3′) as internal control. Each measurement was performed in duplicate and relative gene expression was analyzed using the $\Delta\Delta C_t$ method.

Total Cellular Protein Extraction and Western Blot Analysis

Total cellular protein extraction and Western blot analysis were done as previously described,[6] using antibodies against HIF-1α (in house, rabbit polyclonal),[6] NF-κB p65 (D14E12, Cell Signaling), Phospho-NF-κB p65 (Ser536, 93H1, Cell Signaling), β-actin (AC-74, Sigma) and α-tubulin (B-5-1-2, Sigma). Signals were visualized by Enhanced Chemoluminescence using an Uvitec Cambridge Chemoluminescence Imaging System with the help of Alliance Software (ver. 16.06).

Statistical Analysis

Data are presented as mean ± standard deviation (SD). Statistical analysis was performed using the unpaired *t*-test in the SigmaPlot v. 9.0 software (Systat).

Results

The role of hypoxia on the development of fibrosis was investigated by analyzing the mRNA levels of genes coding for fibrosis mediators (TGF-β, PDGFC, PAI-1) and fibrosis markers (LOX, P4HB, P4HA1) in Huh7 cells treated for 24 hrs with the hypoxia mimetic prolylhydroxylase inhibitor DMOG (1mM). Incubation with DMOG resulted in the induction of HIF-1α protein levels (Figure 1A) and, in parallel, to the increased expression of fibrosis mediators (TGF-β and PAI-1) or fibrosis markers (LOX, P4HB, P4HA1) (Figure 1B).

To further examine the induction of fibrosis-related genes under low O_2 concentration and the role of HIF-1, Huh7 cells were treated with HIF-1α specific or control siRNA and incubated under normoxia (21% O_2) or hypoxia (1% O_2) for 24 hrs. The induction of HIF-1α under hypoxia and siRNA-mediated suppression was confirmed by Western blot analysis (Figure 1C). Incubation under hypoxia caused a significant increase of the mRNA levels of TGF-β, PDGFC, PAI-1, LOX, P4HB, and P4HA1 (Figure 1D). Suppression of HIF-1α expression reduced the hypoxic induction of PDGFC, LOX and P4HA1 mRNA levels.

The involvement of inflammation and the NF-κB signaling pathway in the induction of fibrosis-related genes under low oxygen was examined by incubation of Huh7 cells in the presence or absence of inflammatory factors under normoxia or hypoxia. Treatment of Huh7 cells with TNFα (10ng/mL) under normoxia for 4 hrs caused the activation of the NF-κB signaling pathway, as shown by the increased levels of the phosphorylated at Ser536 p65 NF-κB subunit. In contrast, the increase in phospho-p65 levels was not detectable, when Huh7 cells were incubated under hypoxia with or without TNFα (Figure 2A). Moreover, the protein levels of HIF-1α increased after incubation of the cells under low O_2, but were not affected by the presence of TNFα (Figure 2A).

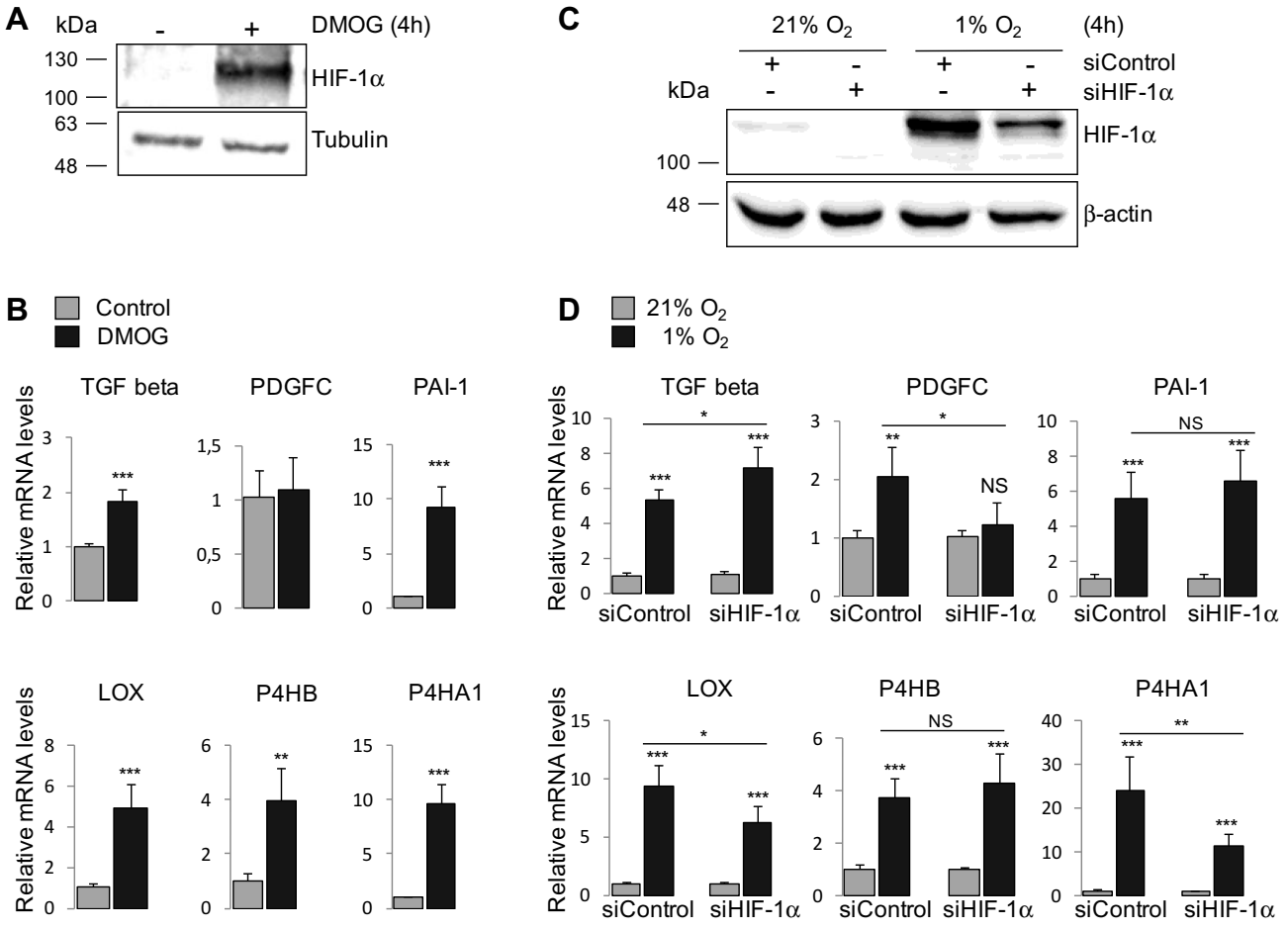

Figure 1 DMOG and hypoxia induce the expression of fibrosis-related genes in Huh7 cells. Cells were incubated with DMOG (1mM) for 4 hrs (**A**) or 24 hrs (**B**), or transfected with HIF-1α or control siRNAs and incubated 24 h post-transfection under 21% O_2 or 1% O_2 for an additional 24 hrs (**C** and **D**). (**A** and **C**) Western analysis of HIF-1 expression with anti-HIF-1α, tubulin or β-actin antibodies. (**B** and **D**) Quantitative real-time PCR determination of TGF-β, PDGFC, PAI-1, LOX, P4HB, and P4HA1 mRNA levels. Values represent the mean ± SD of 4 experiments performed in duplicate. Statistical analysis was done by *t*-test (*P<0.05, **P<0.01, ***P<0.001).

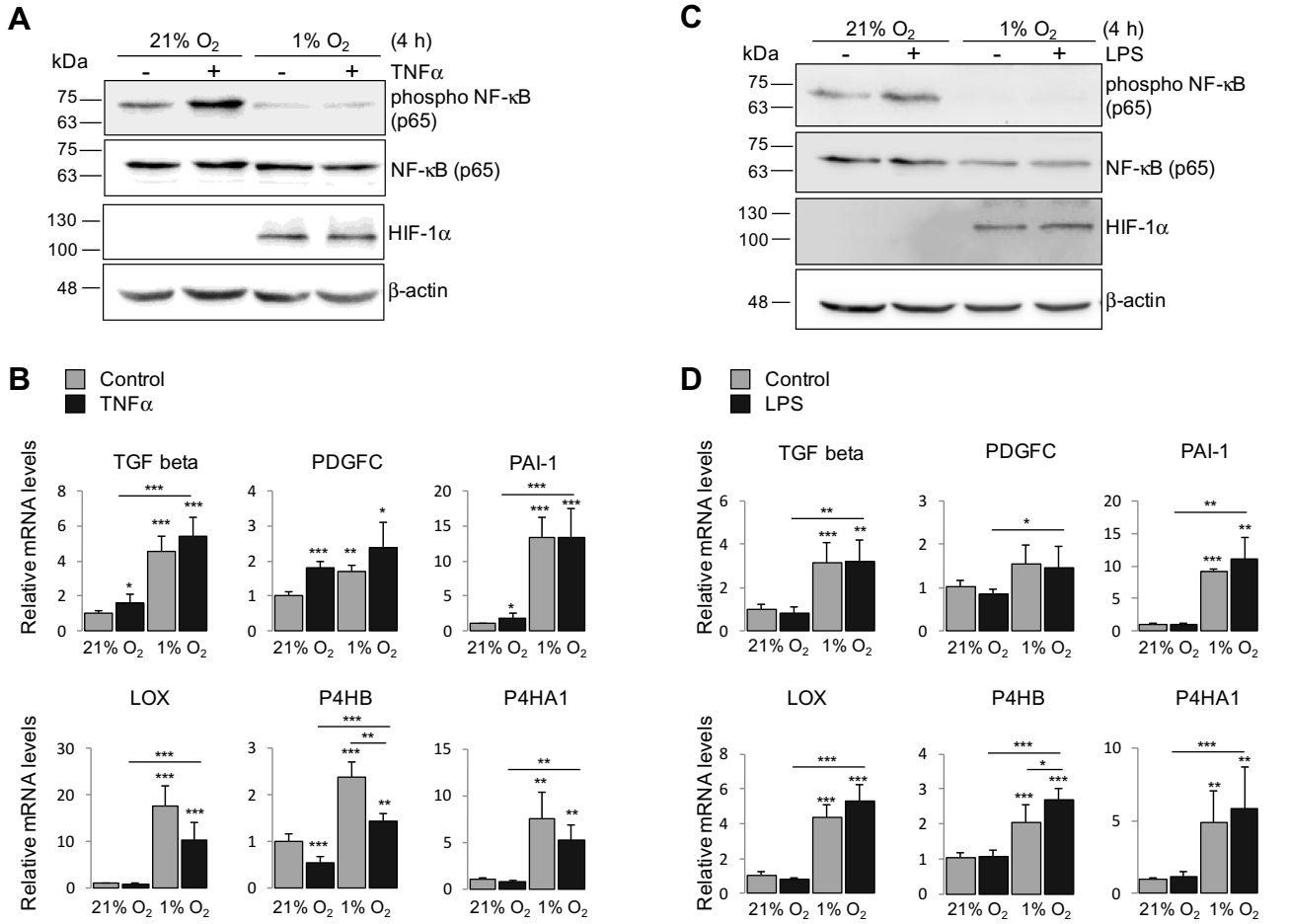

Figure 2 The effect of inflammatory mediators and hypoxia on Huh7 cells. Huh7 cells were treated with TNFα (10ng/mL) or LPS (10μg/mL) under 21% O_2 or 1% O_2, for 4 hrs (**A** and **C**) or 24 hrs (**B** and **D**). (**A** and **C**) TNFα or LPS induce the phosphorylation of the p65 NF-κB (phospho NF-κB p65) subunit under normoxia, but not under hypoxia. HIF-1α protein levels increase under hypoxia, but are not affected by TNFα or LPS. Western blot analysis with anti-phospho-NF-κB p65 (Ser536), NF-κB, HIF-1α and β-actin antibodies. (**B** and **D**) Quantitative real-time PCR determination of TGF-β, PDGFC, PAI-1, LOX, P4HB, and P4HA1 mRNA levels. Values represent the mean ± SD of 2–4 experiments performed in duplicate. Statistical analysis was done by *t*-test (*P<0.05, **P<0.01, ***P<0.001).

Interestingly, quantitative real-time PCR analysis showed that although incubation under hypoxia for 24 hrs caused a significant increase of the mRNA levels of TGF-β, PDGFC, PAI-1, LOX, P4HB, and P4HA1, the effect of the inflammatory mediator TNFα was less pronounced, as TNFα increased the mRNA levels of the fibrosis mediators TGF-β, PDGFC and PAI-1 only under normoxia, while it decreased the mRNA levels of the fibrosis marker P4HB under normoxia and hypoxia (Figure 2B).

Similarly, treatment of Huh7 cells with LPS (10μg/mL) for 4 hrs increased phosphorylation of the NF-κB p65 subunit only under normoxia. Moreover, hypoxia but not LPS treatment increased the protein levels of HIF-1α (Figure 2C). Furthermore, analysis by quantitative real-time PCR showed that incubation for 24 hrs under hypoxia, but not LPS increased the mRNA levels of fibrosis mediator (TGF-β and PAI-1) or fibrosis marker (LOX, P4HB, P4HA1) genes (Figure 2D).

Discussion and Conclusion

In Huh7 cells hypoxia and/or HIF-1 induce the expression of the fibrosis mediator and fibrosis marker genes and this induction is independent of the activation of the NF-κB pathway.

Interestingly, although the HIF-1 and the NF-κB pathways have been shown to be positively interconnected, mainly in cells of the immune system,[5] we found that incubation with the proinflammatory factors TNFα or LPS, activated NF-κB but did not affect HIF-1α protein levels. In addition, hypoxia inhibited the activation of NF-κB by TNFα or LPS in Huh7 cells.

Similar lack of cooperation between HIF-1 and NF-κB was also reported in mouse colon cancer cells,[7] as well as in primary human airway smooth muscle cells.[6]

In conclusion, our data indicate that hypoxia and HIF-1 play an important role in the development of fibrosis in hepatocellular carcinoma, independent of the

activation of the NF-κB pathway. As fibrosis is a hallmark of liver pathologies, anti-HIF targeting therapies could prove promising for the treatment of hepatic disease.

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Disclosure

The authors report no conflicts of interest in this work.

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