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Human RELMβ is a mitogenic factor in lung cells and induced in hypoxia

Aparna Renigunta[a], Christiane Hild[a], Frank Rose[a], Walter Klepetko[b], Friedrich Grimminger[a], Werner Seeger[a], Jörg Hänze[a,*]

[a] *University of Giessen Lung Center (UGLC), Medical Clinic II, Biochemistry, Friedrichstr. 24, Justus-Liebig University, D-35392 Giessen, Germany*
[b] *Cardiothoracic Surgery, University Hospital, Währinger Grütel 18-20, 1220 Vienna, Austria*

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Abstract RELMβ (resistin-like molecule) represents the most related human homologue of mouse RELMα, also known as hypoxic-induced mitogenic factor (HIMF). In this study, we isolated RELMβ cDNA from human lung tissue and performed regulatory and functional expression studies. RELMβ mRNA was upregulated in hypoxia in human lung A549 cell line as well as primary cultured adventitial fibroblasts and smooth muscle cells (SMC) of pulmonary arteries. Upon transfection of a RELMβ encoding expression plasmid into these cells, we observed significant induction of proliferation particularly in SMC and A549 cells, which could be blocked by phosphatidyl-inositol 3-kinase (PI3K) inhibitors LY294002 and wortmannin. The results suggest that human RELMβ may contribute to hypoxic-induced pulmonary vascular remodeling processes or hypoxia related fibrotic lung disease.
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1. Introduction

RELMβ (resistin-like molecule) and resistin are the two human members of the resistin gene family also named FIZZ2 and FIZZ3 (found in inflammatory zone) encoding small cysteine-rich secreted proteins [1,2]. In mouse, a third member RELMα (FIZZ1) has been identified not present in human [3,4]. Mouse RELMα is also described as hypoxia-inducible mitogenic factor (HIMF) and strongly contributes to hypoxic-induced pulmonary vascular remodeling by triggering proliferation of smooth muscle cells (SMC) and exerting strong vasoconstrictive effects on pulmonary vasculature. Additionally, angiogenic properties have been described for HIMF [3]. Since RELMβ is the closest human homologue of mouse HIMF, we were interested in RELMβ gene expression especially in human lung. We isolated its cDNA from human lung homogenate, and studied its hypoxic dependent regulation in pulmonary vascular cells and human lung epithelial A549 cell line. We observed significant induction of RELMβ in hypoxia and found proliferative effects particularly in A549 cells and SMC from pulmonary artery. Induction of proliferation appeared to be mediated by phosphatidyl-inositol-kinase (PI3K) pathway, since LY294002 and wortmannin inhibited proliferation strongly in RELMβ transfected cells.

*Corresponding author. Fax: +49 641 99 42429.
E-mail address: joerg.haenze@uglc.de (J. Hänze).

Abbreviations: RELM, resistin like molecule; HIMF, hypoxic-induced mitogenic factor; FIZZ, protein found in inflammatory zone; SMC, smooth muscle cells; FB, fibroblast

2. Materials and methods

2.1. Cell culture and human lung tissue

Culturing of human epithelial lung cell line A549 was performed according to the protocol given by the American type culture collection. Human cell preparations were established from excess lung tissue originating from human donor lungs employed for transplantation. This protocol was approved by the Justus-Liebig University Ethics committee. Cells were isolated by careful dissection of parenchymal connective tissue as described. Primary adventitial fibroblasts (FB) and SMC from the pulmonary artery were isolated and cultured as described [5]. In brief, primary FB were isolated from human pulmonary artery by careful dissection of parenchymal connective tissue. The adventitia of <1 mm^3 tissue pieces was removed and placed into 12-well cell culture plates with 500 μl culture medium. Primary SMC were isolated from human pulmonary artery by carefully preparing <1 mm^3 pieces of media, devoid of adventitial tissue as assessed by microscopic control. Experiments were performed with cells in passage 3 or 4.

2.2. Transfection

For transfection, cells were grown to ~80% confluence. 0.2 μg Plasmid-DNA was mixed with synthetic peptide Tat-RGD (GML GIS YGR KKR RQR RRP PQT GGC RGD MFG C) which was employed as an enhancer of transfection. 2.5 μg of Tat-RGD was used per well for A549 cells and 0.8 μg/well for FB and SMC. The volume was made up to 25 μl with Hank's buffered saline. This mixture was incubated at room temperature for 15 min. Then 0.5 μl/well Lipofectamin 2000® (Invitrogen, Carlsbad, CA, USA) was added and incubated at room temperature for further 15 min. This mixture was added to the cells. Followed by incubation at 37 °C for 4 h, the medium was replaced and cells cultured under standard conditions for 24 h.

2.3. Isolation and construction of expression plasmid for RELMβ cDNA

For cloning of the full length RELMβ cDNA we performed RT-PCR using RNA extracts from human lung tissue with primers derived from human RELMβ cDNA sequence (Accession No.: AF323084): PHIMF-f: ccc cag gac act gac tct gta and M-HIMF-f: aaa ctg agt tct cag cct cct c. The purified PCR product was ligated into pGEM-T-easy plasmid (Promega) and positive clones were sequenced. Inserts from pGEM-T-easy plasmid were amplified by PCR for subcloning into pCMV expression plasmid (Clontech, Paolo Alto, USA) using the following primers: HIMF-kozak-kpn1: ctt ggt acc gcc gcc acc ATG GGG CCG TCC TCT TGC CTC C; HIMF-HA-TGA-xho1: gaa ctc gag tca gcc gcc acc agc gta atc tgg aac atc gta tgg gta gcc acc ggt cag gtg gca gca gcg ggc agt ggt cc. The forward primer contained a *Kozak* sequence as an optimized translation start and the reverse primer carried a hemeagglutinin A (HA) tag in frame at the C-terminus.

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2.4. RELMβ-mRNA analysis by RT-PCR

RNA was extracted from human lung tissue or cells incubated for 24 h in normoxic or hypoxic (1% oxygen concentration) conditions using guanidine thiocyanate-acid phenol (RNAzol B, WAK-Chemie, Germany). Total RNA for human tissues screening were purchased (Premium RNA; Clontech, Paolo Alto, USA). 1 μg of RNA per sample was copied to cDNA using reverse transcriptase (MMLV-RT, Invitrogen, Carlsbad, CA, USA) with 100 ng 15-mers of oligo-dT primer. As a negative control, MMLV-RT was omitted (data not shown). The following primers were used: hRELMβ+: 5′ CCC TTC TCC AGC TGA TCA AC 3′, hRELMβ−: 5′ CCA CGA ACC ACA GCC ATA G 3′, HPRT+: 5′ TCG AGA TGT GAT GAA GGA GAT GGG A 3′, HPRT−: 5′ TCA AAT CCA ACA AAG TCT GGC CTG T 3′. The cycling conditions were 95 °C for 15 m, followed by 40 cycles of 94 °C for 10 s, 52 °C for 30 s, 72 °C for 30 s and a final extension with 72 °C for 10 m. Real-time PCR was performed using the ABI Prism 7700 detection system (Applied Biosystems, Foster City, CA, USA) with SYBR-Green as fluorescent dye, enabling real-time detection of PCR products according to the manufacturer's protocol. HPRT mRNA was used as internal control for calculation of ΔCT (Threshold cycle) values and relative quantification.

2.5. Western-blot

Cells were lysed using Laemmli buffer and denatured for 5 m at 95 °C and run on a sodium dodecyl sulfate–polyacrylamide gel. After electroblot of the gel to a nylon membrane (PVDF, Pal), HA specific bands were visualized by a monoclonal mouse antibody against HA added at a dilution of 1:10 000 (Sigma–Aldrich) and chemiluminescence (ECL, Amersham, Freiburg, Germany) by using a second biotin-coupled anti-mouse antibody and a complex of biotin and streptavidin coupled with horseradish peroxidase. As a loading control, samples were also analyzed by Western-blot analysis for cytoplasmic β-actin with a mouse monoclonal anti β-actin antibody (Abcam, 1:10 000 dilution).

2.6. MTT assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed as a measurement of number of viable cells. Cells were seeded in 96-wellplates and transfected by expression plasmid. After 24 h, MTT (0.2 mg/ml) was added to each well, and incubation continued for 1–2 h at 37 °C. The extent ofMTT reduction to formazan within cells was quantified by spectrophotometric measurement at 490 nm.

2.7. BrdU incorporation

Cellular DNA synthesis (cell cycle S-phase) was assessed by incorporation of the thymidine analogue 5-bromo-20-deoxyuridine (BrdU) into the DNA of replicating cells using a commercially available colorimetric immunoassay according to the recommended protocol of the company (Roche, Mannheim, Germany). After addition of BrdU to cells they were fixed and incorporated BrdU was measured by ELISA using a specific BrdU antibody. The values given in the figures represent the raw data obtained by photometric measurement at 450 nm.

3. Results

For analysis of human RELMβ gene expression, we initially isolated a full-length cDNA of RELMβ from human lung tissue RNA by RT-PCR strategy. The nucleic acid sequence (Submitted to EMBL Accession No.: AM050721) differed at positions 59 and 97 after start of translation from a published sequence (Accession No.: AF323084), resulting in two amino-acid exchanges (proline to leucine and lysine to glutamine). The alignment of cDNAs of human RELMβ and mouse HIMF (NCBI Accession No.: BC029248) reveals sequence identity of 69%. Comparison of the amino acid sequences yields a sequence similarity of 58.6% and a sequence identity of 49.6%.

Tissue screening of RELMβ expression revealed expression in lung, heart, kidney and adrenal gland, highest expression in intestine, whereas in brain and liver no signal was detectable (Fig. 1A). Since HIMF plays an important role in hypoxic adaptive processes in lung physiology in mouse, we analyzed hypoxic gene regulation of RELMβ, a HIMF homologue, in human lung cells. To this end, we employed the lung epithelial A549 cell line, as well as primary cultured SMC and adventitial FB from human pulmonary arteries (Fig. 1B). RELMβ mRNA was analyzed in extracts of these cells by realtime-RT-PCR. When related to the house keeping gene hypoxanthin-phospho-ribosyl-transferase (HPRT) mRNA, we found highest basal RELMβ mRNA levels in SMC followed by A549 and FB cells. Strongest RELMβ mRNA hypoxic induction was observed in A549 cells followed by SMC and FB. For further functional analysis, we transfected these cells by an expression plasmid recombinant with RELMβ cDNA and tagged with hemeaglutinin A (HA) for protein analysis by anti-HA antibody. Control cells were transfected with the corresponding empty plasmid. Transfected cells were studied with regard to proliferation by measuring the overall cellular mitochondrial respiratory chain activity with MTT assay and cellular DNA synthesis by BrdU incorpration (Fig. 2A and B). Induction of proliferation by RELMβ was observed in all investigated cell types, being stronger in A549 and SMC than

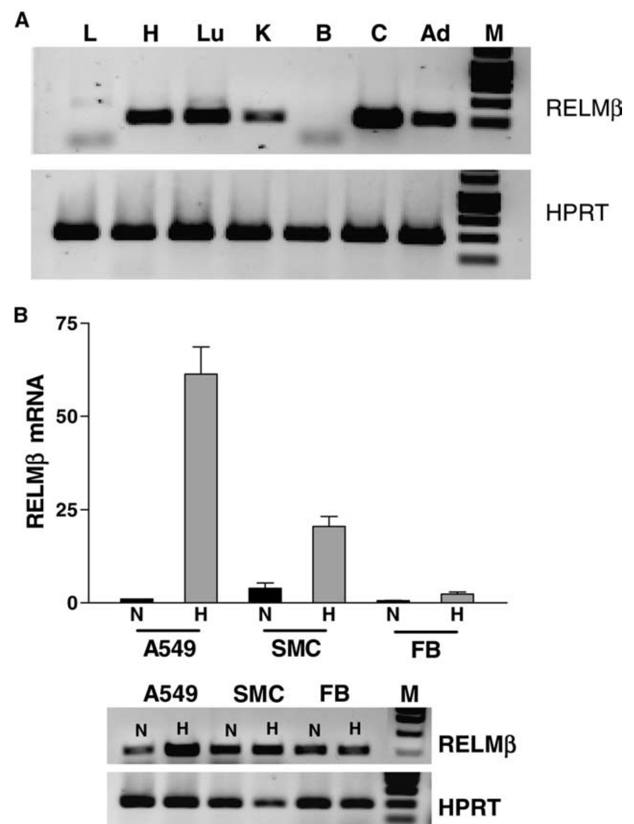

Fig. 1. (A) RELMβ mRNA expression as analyzed by RT-PCR in various human tissues (L: liver; H: heart; Lu: Lung; K: kidney; B: brain; C: colon intestine; Ad: adrenal gland). As a control, house keeping RT-PCR product of HPRT mRNA is shown. (B) Quantification of RELMβ mRNA in A549 cells, SMC and FB cultured in normoxia (N) or hypoxia (H) for 24 h by real-time RT-PCR in comparison to HPRT mRNA (mean ± S.E.M., $n = 3$). A representative gel electrophoretic analysis of the integrity and specificity of the PCR products from the realtime RT-PCRs is shown below the bar graph.

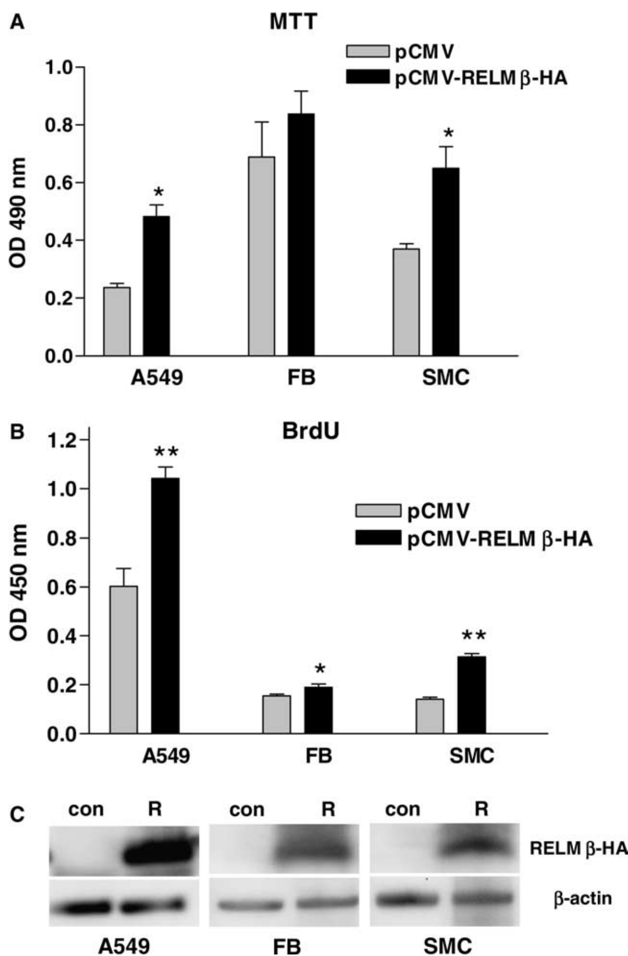

Fig. 2. Analysis of proliferation by MTT test or BrdU incorporation of A549 cells, SMC and FB cells transfected with RELMβ-HA tagged expression plasmid (pCMV-RELMβ-HA) or empty plasmid (pCMV). Significant induction of proliferation in cells overexpressing hRELMβ is observed. (A) MTT results (mean ± S.D., $n = 4$ $^{*} < 0.05$ Mann–Whitney-test). (B) BrdU results (mean ± S.D., $n = 8$ for A549, $n = 6$ for FB, $n = 8$ for SMC, $^{*} < 0.05$, $^{**} < 0.01$ Mann–Whitney-test). (C) As a control for RELMβ overexpression, cellular extracts were analyzed by HA-Western-blot showing signals only in case of pCMV-RELMβ-HA transfected cells (R) but not in empty plasmid transfected cells (pCMV). Shown is a representative blot of $n > 3$ experiments.

FB cells. Transfection and expression of HA-tagged RELMβ cDNA was confirmed by HA Western-blot analysis (Fig. 2C) showing immunoreactivity in RELMβ transfected cells but not in empty plasmid transfected cells. Since the mitogenic effect of HIMF in mouse cells could be blocked by inhibitors of PI3K/Akt signal transduction, we performed experiments employing wortmannin or LY294002 for inhibition of PI3K. In A549 cells which were transfected by RELMβ expression plasmid, we observed significant reduction of proliferation (>50%) when treated with wortmannin or LY294002. In cells transfected with empty vector, we observed no effect on proliferation using these inhibitors. As a further control, we included non-transfected cells in the experiments. These cells revealed higher S-phase activity when compared to the transfected cells and demonstrated a significant but slight decrease (∼15%) of proliferation upon PI3K inhibitor treatments (Fig. 3).

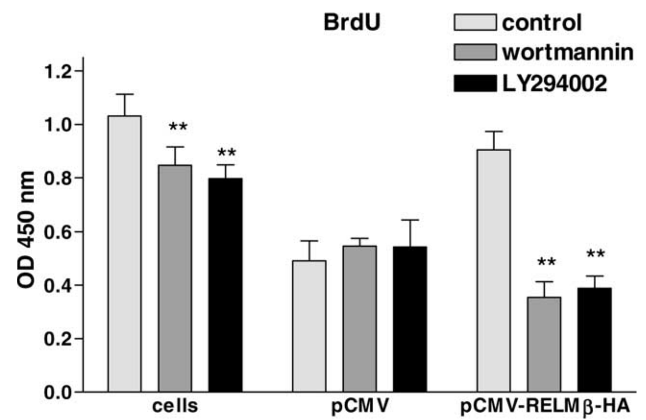

Fig. 3. Analysis of proliferation as measured by BrdU incorporation in A549 cells. Cells were either non-transfected (cells) or transfected by pCMV-HA, or by pCMV-RELMβ-HA and then incubated with LY29004 (40 μM) or wortmannin (100 nM) added to the medium (mean ± S.D., $n = 5$, $^{**} < 0.01$ Mann–Whitney-test).

4. Discussion

In this study, we have analyzed the gene expression, hypoxic regulation and proliferative effects of RELMβ in human lung cells. Our study was inspired by investigations on the related HIMF gene in mouse, also described in other context as RELMα or FIZZ1 [3]. Human RELMβ represents the gene with the highest homology with mouse HIMF, however, it is not considered as the human ortholog gene of mouse HIMF, which appears not to exist [4]. In this report, we have analyzed the expression, hypoxic regulation and pro-proliferative potency of RELMβ on human lung cells. We analyzed the expression pattern of human RELMβ on mRNA level by RT-PCR in several human tissues and found highest expression in lung, heart, colon, and adrenal gland, followed by kidney. Two studies examined HIMF expression in mouse. At protein level HIMF was only detectable by Western-blot in lung from hypoxic treated mice [3]. At mRNA level as analyzed by Northern-blot [6], HIMF was abundantly expressed in lung and less but significantly expressed in heart and skeletal muscle, whereas no signal was observed in brain and liver in accordance with our study. In conclusion, so far described differences and common grounds exist for expression of RELMβ in human, and of HIMF in mouse.

We found hypoxic induction of RELMβ in A549 cell line and primary vascular cells from human pulmonary arteries. The hypoxic induction was strongest in A549 cells followed by SMC and FB. Probably, this observation reflects that the expression of a given gene is regulated by specific factors which differ between cells. Hypoxic gene induction may be HIF-dependent via hypoxia-responsive-elements (HRE) [7]. Bioinformatic analysis of the human RELMβ gene revealed five putative HRE (RCGTG) binding sites. One site was located in the intervening intron sequence 2, and four at the 3′ flanking region (Table 1A). These putative regulatory DNA sequences need further experimental proof for their functional relevance. As comparison, putative HREs of the corresponding mouse HIMF gene are shown (Table 1B). Two HRE sites in the 5′ flanking region were detected which also require experimental investigation.

Table 1A
Putative HRE sequences of human RELMβ gene (EMBL Accession No.: AF352731)

HRE sequence	Location
gacgt **ACGTG** cagggaga	IVS2 4033/4016
cacta **ACGTG** gcatctag	3′FS +314/+331
tgtcc **ACGTG** atctcatt	3′FS +417/+434
aacat **ACGTG** tgcatgtg	3′FS +674/+691
catag **GCGTG** ggcaagga	3′FS +1303/+1286

Table 1B
Putative HRE sequences of mouse RELMα (HIMF) gene (EMBL Accession No.: mm_ensembl11:CHR16_11_1177_04)

HRE sequence	Location
tacag **ACGTG** gatgctct	5′FS −3805/−3788
tgtgt **GCGTG** tgtgtgtg	5′FS −624/−610

HRE core sequence: RCGTG (R is A or G), IVS: Intervening sequence (intron), FS: flanking sequence (numbering: 3′FS: + is downstream from end of transcription; 5′FS: − is upstream from start of transcription; IVS2: numbering with regard to complete gene sequence).

In transfected cells overexpressing RELMβ, significant proliferative effects were observed. Thus, this study suggests that RELMβ in the human system is similarly regulated as described for HIMF in mouse, and acts as a hypoxia-driven pro-proliferative factor. Moreover, the pro-proliferative effect of RELMβ could be suppressed by two PI3K inhibitors, a characteristic also observed for HIMF in mouse cells. PI3K has been demonstrated to mediate proliferation in human pulmonary SMC via serine/threonine kinase Akt which directly affects cell cycle regulation [8]. Thus, the signalling mechanisms by so far unknown receptors appear to be similar between RELMβ and HIMF. Consequently, RELMβ in human may play a role in hypoxic-induced pulmonary remodelling leading to pulmonary hypertension, and in fibrotic lung diseases associated with hypoxia.

Further features of HIMF were explored in mice experiments. Beside proliferative effects, HIMF was demonstrated to have angiogenic (subcutaneous mouse in vivo matrigel plug model) and vasoconstrictive properties in pulmonary artery [3]. In addition, HIMF was shown to play a role in lung development, by regulation of apoptosis and participation in alveolarization and lung maturation [9] and compensatory lung growth after pneumectomy [10]. Also, HIMF (FIZZ1) was shown to be strongly increased during allergic pulmonary inflammation [6]. The exploration of these characteristics regarding RELMβ in the human system remains open. So far, functional aspects of RELMβ and resistin have been mainly described in mice with respect to resistence to insulin [2,11,12]. Resistin, mainly expressed in adipocytes, and RELMβ, expressed particularly expressed in epithelial cells from intestine, were demonstrated to mediate resistance to insulin leading to increased glucose production.

In sum, our study revealed new findings for human RELMβ as a factor which is induced in hypoxia and which exerts pro-proliferative effects. These effects were observed in human primary vascular cells originating from pulmonary arteries, and in lung A549 cell line. Thus, features of human lung RELMβ resemble those of HIMF in mouse lungs.

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