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IBMX protects human proximal tubular epithelial cells from hypoxic stress through suppressing hypoxia-inducible factor-1α expression

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ABSTRACT

Hypoxia predisposes renal fibrosis. This study was conducted to identify novel approaches to ameliorate the pathogenic effect of hypoxia. Using human proximal tubular epithelial cells we showed that a pan-phosphodiesterase (PDE) inhibitor, 3-isobutyl-1-methylxanthine (IBMX) dose and time dependently down-regulated hypoxia-inducible factor 1α (HIF-1α) mRNA expression, which was further augmented by addition of a transcriptional inhibitor, actinomycin D. IBMX also increased the cellular cyclic adenosine monophosphate (cAMP) level. Luciferase assay showed that blocking of protein kinase A (PKA) using H89 reduced, while 8-Br-cAMP agonized the repression of HIF-1α promoter activity in hypoxic condition. Deletion of cAMP response element binding sites from the HIF-1α promoter abrogated the effect of IBMX. Western blot and immuno-fluorescent study confirmed that the $CoCl_2$ induced increased HIF-1α protein in whole cell lysate and in nucleus was reduced by the IBMX. Through this process, IBMX attenuated both $CoCl_2$ and hypoxia induced mRNA expressions of two pro-fibrogenic factors, platelet-derived growth factor B and lysyl oxidase. Moreover, IBMX reduced production of a mesenchymal transformation factor, β-catenin; as well as protected against hypoxia induced cell-death. Taken together, our study showed novel evidence that the PDE inhibitor IBMX can downregulate the transcription of HIF-1α, and thus may attenuate hypoxia induced renal fibrosis.

1. Introduction

Acquiring about 20% of total cardiac output, kidneys generally are well perfused with blood [1]. However, very low interstitial oxygen tension makes them vulnerable to ischemic injuries. Indeed, any impairment of generalized or regional renal blood flow can trigger the ischemia/ hypoxia signaling in this organ [2]. After an ischemic injury, endothelium is ruptured leading to activation of immune-reaction and vasoconstriction pathways in renal interstitium [3]. As a result, both microcirculation and regional blood flow is compromised. Due to their high metabolic activity and large oxygen demand, particularly the tubular epithelial cells become readily hypoxic in this ischemic condition [4]. Therefore, tubular epithelial cells of the kidney represent a prime target for hypoxia mediated acute kidney injury (AKI); and subsequently in long standing cases, chronic kidney diseases (CKD) [2,5].

Adaptive changes in response to hypoxia are critical for cells survival [6]. To ensure optimal functionality in hypoxic condition, hypoxia-inducible factor 1 (HIF-1) orchestrates transcription of enormous genes [6,7]. However, similar to other cells, maladaptive response of HIF-1 signaling in tubular epithelial cells enhance production of pro-fibrogenic genes and matrix modifying factors those lead to increased production of interstitial collagen and decreased degradation of extra cellular matrix (ECM) [4]. Moreover, HIF-1 signaling also facilitates to lose the epithelial signature of the tubular epithelial cells (i.e. E-cadherin) and to acquire mesenchymal signature (i.e. β-catenin),

Abbreviations: ActD, actinomycin D; AKI, acute kidney injury; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; CKD, chronic kidney diseases; CRE, cAMP response element; CREB, cAMP response element binding protein; ECM, extra cellular matrix; EMT, epithelial-to-mesenchymal transition; HIF-1, hypoxia-inducible factor 1; IBMX, 3-isobutyl-1-methylxanthine; PDE, phosphodiesterase; PDGFB, platelet-derived growth factor B; PKA, protein kinase A; LOX, lysyl oxidases; NF-κB, nuclear factor kappa B; PI3K-Akt, phosphatidylinositide 3-kinases- protein kinase B

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and thus enhances tubular epithelial-to-mesenchymal transition (EMT) [4,8]. As a result of either or in combination of both pathogenesis renal fibrosis occurs.

In glomerular endothelial cells, hypoxia enhances production of platelet-derived growth factor B (PDGFB) chain synthesis [9]. Infusion or systemic overexpression of PDGFB induces prominent mesangioproliferative changes and renal fibrosis. Accordingly, targeted intervention against the various PDGF isoforms offers a promising novel therapeutic approach to renal disease [8]. On the other hand, HIF-1 induced upregulation of lysyl oxidases (LOX) in renal epithelial cells was associated with chronic renal diseases and progressive fibrogenesis, which facilitates EMT [10,11]. Therefore, it is conceivable that HIF-1 can be targeted therapeutically to ameliorate hypoxia induced renal pathogenesis.

Between its two subunits, HIF-1α is widely expressed in all nucleated cells and its protein level is regulated by oxygen tension [6,12]. However, the factors those regulate transcription of HIF-1α remain elusive [13]. Although, in general it is considered that the transcription and synthesis of HIF-1α are constitutive and remain unaffected by oxygen tension [12]; studies also have shown that hypoxia and reactive oxygen species can regulate the expression of HIF-1α mRNA via the phosphatidylinositide 3-kinases-protein kinase B (PI3K-Akt) and nuclear factor kappa B (NF-κB) mediated pathways [14,15].

Recent studies have shown that phosphodiesterase (PDE) inhibitors prevent hyrdrolyzation of cyclic guanosine monophosphate (cGMP) and thus prolong the signaling action of nitric oxide (NO). The NO-cGMP accumulation stimulates dilatation of glomerular afferent blood vessels and thus attenuates the pathogenesis of diabetic nephropathy and CKD [16–18]. Cyclic adenosine monophosphate (cAMP) is also a second messenger, which carry over the cellular response through activating protein kinase A (PKA). PKA then activates cAMP response element (CRE)-binding protein (CREB), which acts as a transcriptional factor for many genes [19,20].

PDE inhibitors block degradation of cAMP and enhance PKA mediated cellular responses [21]. Among the isozymes expressed in the kidney, at least PDE4- and PDE7-inhibitors hydrolyze cAMP. The PDE4- or PDE7-inhibitors confer protection against pro-inflammatory phenotypes, chronic obstructed pulmonary diseases and type 2 diabetes mellitus, while the combination produces superior effects. Indeed, some broad spectrum PDE inhibitors such as resveratrol show additive or synergistic effects and lead to more effective therapies against Alzheimer's disease and impaired glucose tolerance [22].

3-isobutyl-1-methylxanthine (IBMX) is also a broad spectrum PDE inhibitor, widely used to induce cAMP mediated effects. In kidney, administration of IBMX significantly attenuated the cAMP-induced increase in renal secretion of AMP [23], suggests that through activating cAMP, IBMX may play some protective roles in kidney diseases. Therefore, we conducted this study: i) to explore the effect of IBMX on HIF-1α; and ii) to evaluate the roles of IBMX on two markers of renal fibrosis, PDGFB and LOX, as downstream targets of HIF-1α in human proximal tubular epithelial HK-2 cell line.

2. Materials and methods

2.1. Cell culture

Human proximal tubular epithelial HK-2 cell line was purchased from JCRB Cell Bank, Osaka, Japan. Cells were grown and maintained in DMEM/F-12, HEPES, no phenol red (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS) (Nichirei Biosciences, Japan) and 1% penicillin–streptomycin (P/S; Invitrogen, Grand Island, NY, USA). For giving intervention, about 50% confluent cells were serum starved overnight in DMEM/F12 medium containing P/S. IBMX (Sigma-Aldrich, St Louis, MO, USA) was dissolved in DMSO. Where indicated appropriate amounts of DMSO (Wako Pure Chemicals, Osaka, Japan) or IBMX was directly added to the cells to achieve the desired condition. In some experiments, cells were pretreated with the indicated regents for indicated time, followed by addition of DMSO or IBMX to the cells. Otherwise mentioned all reagents were purchased from Sigma-Aldrich. Cells were grown in 5% CO_2 and 21% O_2 at 37 °C in a humidified environment. For hypoxia, O_2 tension of the incubator was reduced to 1%.

2.2. Quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA), and first-strand cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, CA, USA) [24]. Quantitative PCR was carried out by real-time PCR in a ViiA7 Real time PCR system (Life Technologies, Tokyo, Japan). Primers used in this study are: HIF-1α (Hu-HIF1α-FW: 5′-TGTACCCTAACTAGCCGAGGA-3′, and Hu-HIF 1α-RV: 5′-CTGTGCAGTGCAATACCTTCC-3′), PDGFB (Hu-PDGFB-FW: 5′-CTGCAACAACCGCAACGTG-3′, and Hu-PDGFB-RV: 5′-ATC TCGATCTTTCTCACCTGGAC-3′), LOX (Hu-LOX-FW: 5′-CTGAAG GCCACAAAGCAAGTT-3′, and Hu-LOX-RV: 5′-GGACTCAATCCCTG TGTGTGT-3′); and for internal control TATA-binding protein (TBP; Hu-TBP-FW: 5′-TTGTACCGCAGCTGCAAAAT-3′, and Hu-TBP-RV: 5′-TATATTCGGCGTTTCGGGCA-3′). Due to significant downregulation of TBP by the YC-1, mRNA expression for the experiments using YC-1 was normalized with β2 microglobulin (B2M) [25]. RPL13A was also used as a reference gene for mRNA stability test, and the primer sequences have been reported previously [25]. All qPCRs were carried out using Fast SYBR Green Master Mix (Life Technologies, Tokyo, Japan). The mRNA expressions are presented as fold change over the control groups.

2.3. Western blotting

Proteins of whole cell lysate and nuclear fraction were extracted using RIPA buffer and NE-PER kit (both from Thermo Fisher Scientific), respectively following manufacturer's instruction [26]. The protein concentration was measured by the Bradford method using Bio-Rad Protein Assay Dye Reagent Concentrate (BioRad Laboratories, Hercules, CA, USA). The equal amount of proteins were resolved in 8% SDS-PAGE gel and transferred at 30 V for overnight to Immobilion-P membrane (Merck Millipore, Carrigtwohill, County Cork, Ireland). Membranes were blocked with 5% skimmed milk for 1 h, probed with primary antibodies for HIF-1α (1: 800; Novus Biologicals, Littleton, Colorado, USA), β-catenin (1:1000; Merck Millipore), E-cadherin (1:1000, Abcam, Milton, Cambridge, UK), β-actin (1:1000; Sigma-Aldrich) and lamin A/C (1:1000; Cell Signaling Technology, Danvers, MA, USA) for overnight at 4 °C. The blots were exposed to HRP-conjugated secondary antibodies, and the immune complexes were detected using ECL Prime Western Blotting Detection Reagent (GE Healthcare, Chalfont St Giles, Buckinghamshire, UK). Images were acquired using a LAS-1000 Plus imaging system (Fujifilm, Kanagawa, Japan), and the intensities were analyzed using ImageJ ver. 1.47 g software following developer's instruction [27]. Where indicated, the protein levels are presented as fold change over the β-actin or combined lamin A/C intensities.

2.4. Immunofluorescent staining

HK-2 cells were grown in 2-well chamber slides. After the intervention for 24 h, cells were washed twice with PBS, and fixed with 4% paraformaldehyde solution for 10 min at room temperature and washed twice again with PBS. Cells were permeabilized using 0.1% Triton X-100 (MP biomedicals LLC, Illkirch, Cedex, France) for 15 min at room temperature, washed once with PBS and blocked with PBS containing 0.1% Tween-20 (MP biomedicals LLC, Illkirch, Cedex,