의학박사 학위논문

Effect of Bilirubin on the Expression of Hypoxia-induced factor 1α (HIF-1α) in Renal Proximal Tubular Cell Line

빌리루빈이 신장 근위세뇨관 세포주에서 **Hypoxia-induced factor 1α (HIF-1α)**의 발현에 미치는 영향

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이 논문을 의학박사 학위논문으로 제출함

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A thesis submitted to the Department of Immunology

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Abstract

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Introduction: Renal dysfunction has a common process of tubular cell death and tubulointerstitial inflammation followed by fibrosis, whereas initiation of such dysfunction is likely to have multi-hit characteristics. Renal tubulointerstitial hypoxia causes fibrogenesis and also stimulates infiltration and maturation of immune cells to generate inflammation. Hypoxia-inducible factor (HIF) is influenced by the level of reactive oxygen species (ROS) and plays an integral role in the body's response to hypoxia. Bilirubin has been reported to be an effective antioxidant,

and has the beneficial effects on renal diseases. However, the exact mechanisms controlling HIF expression under hypoxia are not resolved. The aim of this study is to elucidate the effect of bilirubin on HIF-1 expression under hypoxia and the possible mechanism of HIF-1 regulation by bilirubin in human proximal tubular cells *in vitro*.

Methods: The human proximal tubular kidney (HK2) cells treated with actinomycin D were cultured for 15 h in 5% oxygen with or without bilirubin. HIF-1α messenger RNA (mRNA) and protein expression were measured in the cells cultured with 0.1 mg/dL bilirubin and compared to the control cells. Next, in order to elucidate the molecular mechanism of bilirubin on HIF system, the changes of ROS and HIF system were measured after treating the cell with the inhibitors of PI3K/mTOR, PI3K/AKT, and ERK 1/2 pathways which affect the transcription and translation of HIF-1α expression. Then, HIF-1α expression and phosphorylation of P70S6 kinase were measured after treating the cell with 10μM exogenous hydrogen peroxide (H_2O_2) to investigate the effect of NADPH oxydase system which is another major source of ROS generation in mitochondria possibly contributing to HIF system. Finally, NOX4 gene, which is also known to affect the expression of HIF system, was blocked by small interfering RNA (siRNA) and then HIF-1α mRNA expression was evaluated.

Results: The messenger RNA (mRNA) expression of HIF-1α that was suppressed by actinomycin D was increased by 1.69 ± 0.05 folds in the cells cultured with 0.1 mg/dL bilirubin, compared to the control cells. The inhibitor (miltefosin) of PI3K/AKT pathway attenuated HIF-1α

expression increased by bilirubin whereas the inhibitors of PI3K/mTOR and ERK 1/2 pathways did not. HIF-1α expression was decreased by 10μM exogenous H_2O_2; scavenger of ROS with or without bilirubin in the HK2 cells increased HIF-1α concentration more than that in the cells without bilirubin. Exogenous H_2O_2 decreased the phosphorylation of P70S6 kinase, which was completely reversed by bilirubin treatment. Knockdown of NOX4 gene by small interfering RNA (siRNA) increased HIF-1α mRNA expression.

Conclusions: Bilirubin enhanced HIF-1α mRNA transcription as well as the upregulation of HIF-1α protein translation through 1) inhibition of oxidaive stress, at least partly by downregulating subunits of NADPH oxidase and 2) activation of PI3K/AKT pathway in HK2 cells cultured under hypoxia. These findings suggest that bilirubin could be a cytoprotective molecule with antioxidative properties in renal tubular injury.

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Keywords: Proximal tubular cell, Bilirubin, HIF-1α, ROS

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Introduction

Increased consumption of renal oxygen and/or decreased renal oxygen supply (also called renal hypoxia) with increased oxidative stress is believed to be an important contributor in renal disease progression (1). In experimental animal models with kidney disease, the oxygen content is reduced throughout the kidney, which is confirmed by oxygen microelectrode, blood oxygen level-dependent magnetic resonance imaging (BOLD-MRI), and pimonidazole staining (1). Microelectrode measurement revealed that the oxygen tension (pO_2) in whole renal parenchyme, especially in the renal medulla, was lower in the type I diabetic animal model induced by streptozotocin than in the non-diabetic control (2). In a remnant kidney model, tubular hypoxia, a representative model of chronic progressive renal disease, was developed in the early phase of the disease without any histological interstitial change (3). Experiments with acute kidney injury models induced by cyclosporin (4), gentamicin (5), ischemia-reperfusion injury (6), or other stimuli, as well as with chronic renal injury models, including chronic tubulointerstitial disease (7), showed that hypoxia has an important pathophysiological role in the progression of renal diseases. In addition, hypertensive rats had lower pO_2 in the lumens of proximal and distal tubules than normotensive Wistar Kyoto rats (8). Renal tubulointerstitial hypoxia stimulates fibrogenesis with the production of collagen I and α-smooth muscle actin, induces epithelial-mesenchymal transdifferentiation (1), and stimulates

infiltration and maturation of immune cells to generate inflammation (9).

The kidney has the lowest physiological pO_2 compared with other organs despite receiving the largest fraction of the cardiac output (10). This can be attributed to diffusional shunting of oxygen between descending arterial vasa rectia and ascending venules in the renal medulla (11), and the oxygen content between 10 and 20 mmHg in the renal outer medulla and less than 10 mmHg in the renal inner medulla (10, 12). The averaged pO_2 value was 39−40 mmHg in the proximal and distal tubules of normotensive Wistar Kyoto rats (11). The tubular cells within S3 segment of proximal tubule had higher oxygen demand with abundant Na/K ATP−ase. Moreover, compromised blood flow to the outer medulla easily leads to hypoxic injury, particularly to the S3 segment of the proximal tubule (10).

Hypoxia−inducible factor (HIF) is a master gene involved in the regulation of hypoxia; it induces the expression of genes controlling oxygen delivery, vascularization, and glucose metabolism (13). The primary mechanism of HIF activity regulation is through oxygen−dependent proteasomal degradation of the HIF−α subunit (10, 14). Under normoxia, the proline residues of HIF−α are hydroxylated by prolyl hydroxylase (PHD) and recognized by von Hippel Lindau protein, culminating in polyubiquitination and proteasomal degradation (13). In addition, lysine acetylation in normoxia targets HIF−α for degradation by proteasome. Under hypoxia, the PHD activity and acetylation are downregulated and HIF−α escapes degradation (13).

Even if hypoxia is the main mechanism of HIF−1 activation, there is an increasing body of evidence demonstrating that a number of non−hypoxic stimuli are also highly capable of turning on this transcription factor (15). Phosphoinositide 3−kinase (PI3K) pathway and its downstream effectors, mTOR and P70S6 kinase, mediate the increased HIF−1α translation (15). P70S6K regulates the translation of a group of messenger RNAs (mRNAs) possessing a 5'−terminal oligopyrimidine tract, such as HIF−1α mRNA (15), which is the main effector mechanism responsible for HIF−1α induction by angiotensin II (16) and lipopolysaccaride in macrophages (17). Moreover, activation of p42/p44 MAPK is sufficient to promote the transcriptional activity of HIF−1 (18).

Another important mechanism of HIF−1 activation is oxidative stress, the presence of reactive oxygen species (ROS) in excess of the antioxidant capacity in the kidney induces renal cell damage, accumulation of which leads to acute kidney injury and kidney failure (19). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) 2 and 4 are the major sources of O_2^- and H_2O_2 in the kidney and play a crucial role in the regulation of growth and death in tubular cells (20). NOX2 is predominantly localized to the plasma membrane and its activity is regulated by cytosolic factors, including p47phox, p67phox, p40phox, and Rac. Nox4 is predominantly localized on the intracellular membranes of organelles, including thenucleus, mitochondria, and the endoplasmic reticulum (ER), thereby leading to increased production of ROS, mitochondrial dysfunction, and apoptosis in renal cells (21). The

cytochrome b558 α-subunit, p22phox (one of the key electron transfer elements of the NADPH oxidases) is a critical component of the superoxide-generating NADH/NADPH oxidase system (22). NOX4 has been implicated in the basal production of ROS in the kidney and in pathologic conditions such as diabetic nephropathy and CKD; upregulation of NOX4 may be important in renal oxidative stress and kidney injury (20). Although there is growing evidence indicating the involvement of NADPH oxidase in renal pathology, there is a paucity of information on the role of NADPH oxidase in the regulation of HIF-1. ROS have been reported to be involved in the oxygen-sensing mechanism (23). Superoxide inhibited hypoxia-dependent increase in the HIF-1α levels in the renal interstitial cells (23) and hydrogen peroxide (H_2O_2) blunted the increase in HIF-1α under hypoxia (24). Several experiments have reported that the subunits of NADPH oxidase, which are one of the major sources to generate ROS, were related to HIF-1α expression in response to hypoxia in the renal interstitial cells (23) or the A549 cells (25).

Bilirubin, an end product of heme catabolism, is a potent circulating antioxidant and its level is directly proportional to the total serum antioxidant capacity in humans (26). Recently, there have been increasing evidences about the beneficial effects of the physiological level of bilirubin on diabetes, diabetic complication, and renal diseases. Higher serum bilirubin is associated with reduced risk of diabetes mellitus (27-29) and diabetic nephropathy (27, 30, 31); it is correlated with lower incidence of hypertension in normotensive men

(32) and lower incidence of end-stage renal disease in IgA nephropathy (33). Bilirubin attenuates renal injury in cyclosporine-induced nephropathy (34) and ischemia- reperfusion injury (35, 36), reduces the streptozotocin-induced pancreatic damage in the Gunn rat (12), and protects rodents against diabetic nephropathy by inhibiting oxidative stress and the downregulation of NADPH oxidase (37). Furthermore, bilirubin is suggested as an effective agent to reduce mortality and counteract hypotension elicited by endotoxin through mechanisms involving a decreased NOS2 induction secondary to inhibition of NAD(P)H oxidase (38).

In this study, we investigated the effect of bilirubin on HIF-1 expression in the proximal tubular cells under 5% oxygen concentration and the possible mechanism of HIF-1 regulation by bilirubin in human renal proximal tubular cells.

Methods

Cell culture

The proximal tubular epithelial cells derived from the human kidney (HK2) cells (ATCC CRL−2190) were cultured in Renal Epithelial Basal Medium (Lonza Walkersville, MD, USA) with recommended supplements. We used HK2 cells passaged 10−25 times for the experiments. The cells were cultured in room air with 5% CO_2 condition or in 5% O_2 condition with 95% CO_2 condition. We used 5% O_2 condition (O_2 pressure approximately 38 mmHg) as similar O_2 tension as in the proximal tubules of a normal kidney. With 70−80% cell confluence under 21% O_2 condition, the cells were cultured for 15 h under 5% O_2 condition with or without H_2O_2, or bilirubin (Sigma #B4125, MO, USA) by dissolving the cells in dimethyl sulfoxide. The cells were treated with the chemicals wortmannin (100 μM; Merck 681675, NJ, USA), Ly294002 (20 μM; Merck 440202, NJ, USA), rapamycin (10 μM; Merck 440202, NJ, USA), miltefosine (5 μM; Merck 440202, NJ, USA), catalase (1000 U/ml; Sigma #C9447, MO, USA), diphenylene iodonium (DPI, 10 μM; Calbiochem #3003260, CA, USA), and actinomycin D (0.15 μg/ml; Sigma #856258, MO, USA) for 15 min before culturing in 5% O_2 condition. Transfection of small interfering RNA (siRNA) for *NOX4* gene (Santa Cruz sc−41586, CA, USA) or *p22phox* gene (Santa Cruz sc−36149, CA, USA) into 2×10^6 HK2 cells in 10−cm−diameter culture dishes was performed as per the manufacturer's recommendation 24 h prior to culturing under 5% O_2

condition.

Detection of ROS generation

Oxidation-sensitive 2',7'-dicholorofluorescein diacetate (H_2DCF-DA; Sigma, MO, USA) was used to measure the intracellular production of ROS. Cells were incubated with 10 μM H_2DCF-DA at 37℃ for 30 min, washed, collected by scraping, and resuspended in phosphate-buffered saline (PBS). The fluorescence intensity was measured using a fluorescence spectrophotometer at excitation and emission wavelengths of 490 nm and 526 nm, respectively.

Western blotting

Western blotting was conducted as described in the previous study (34). The cells were harvested and lysated. The protein concentration was measured using a bicinchoninic acid assay kit (Thermo Fisher Scientific, IL, USA). The protein samples were run on sodium dodecyl sulphate (SDS)-polyacrylamide mini-gels (Bio-rad Mini Protean III) and then transferred onto nitrocellulose membranes by electroelution. Antibodies used in this study included anti-HIF 1α(#610958), anti-caspase 9 (#551246)(BD Bioscience, NJ, USA), anti-ERK 1/2 (#4696), anti- phospho-ERK 1/2 (#9101), anti-AKT (#9272), anti-phospho-AKT (Ser473) (#9271), anti-phospho-mTOR (#5536) (Cell Signaling Technology, MA, USA), anti-mTOR (Thermo Scientific Pierce, PA1- 518, IL, USA), anti-RAPTOR (Abcam ab5454,

CA, USA), anti-actin (sc-1616), anti-NOX 4 (sc-20141), anti-p22phox (sc-20781), anti-p47 phox (sc-14015), and anti-p67phox antibody (sc-15342) (Santa Cruz, CA, USA). Incubation with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, CA, USA) was followed by band visualization using an enhanced chemiluminescence substrate (Thermo Fisher Scientific, IL, USA). The density of the bands was quantified by the GS-700 Imaging Densitometry (Bio-rad, CA, USA), and their values were normalized to that of the actin protein in the control.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from the cells was isolated using the Trizol-Reagent (GIBCO, CA, USA). The RNA was dried, re-dissolved, and quantified by spectrophotometry. cDNA was generated from 200 ng of total RNA by the SuperScript® III First-Strand Synthesis System (Invitrogen 18080-051, CA, USA), according to the manufacturer's instructions. The mRNA expressions of HIF-1α, actin, GLUT-1, p22phox, p67phox, and NOX4 were determined by RT-PCR using the following primers:

HIF-1α (forward) 5'-CAGTTTCTGTGTCGTTGCTGC-3'
(reverse) 5'-ACTTTCCTCAGTCGACACAGC-3',
GLUT-1 (forward) 5'-ACAAACAGCGACACGACAGTG-3'
(reverse) 5'-TCATCATCGGTGTGTACTGCG-3',
p22phox (forward) 5'-CTGCTTGATGGTGCCTCCGAT-3'
(reverse) 5'-ACTTTGGTGCCTACTCCATTG-3',

p67phox (forward) 5'-CCACTGTGTTCTCACACCACA-3'
(reverse) 5'-GCTTGTTCCCTGCAACTACCT-3',
NOX4 (forward) 5'-TACAGGCACAAAGGTCCAGAA-3'
(reverse) 5'-CAAGATACCGAGATGAGGATC-3',
and actin (forward) 5'-CGGGGTCACCCACACTGTGCC-3'
(reverse) 5'-GTACTTGCGCTCAGGAGGAGC-3'.

PCR was performed using the TaKaRa Ex Taq (Magnesium-free) buffer (Takara Bio Inc. RR01AM, Shiga, Japan). The density of the bands was quantified by densitometry, and the values obtained were normalized to that of the gene of the control sample and compared between the samples.

Statistical analysis

The results were calculated as mean ± standard deviation. The statistical analyses were performed using SPSS (version 21.0, IBM, NY, USA). The difference of continuous variables between the groups was analyzed by using a method of analysis of variance or Student t-test, according to the number of groups. The level of statistical significance was defined as a p value of <0.05.

Results

The effect of bilirubin on HIF−1α protein expression

In HK2 cell cultured under 5% O_2 condition, the HIF−1α protein expression was increased by bilirubin treatment at 0.01−0.2 mg/dL concentration (Figure 1A). We selected the concentration of 0.1 mg/dL for this study, which of bilirubin decreased the expression of p22phox protein (Figure 1B) (12). Bilirubin steadily increased HIF−1α expression in HK2 cells after 1−h treatment (Figure 1C), until 5 h (Figure 1D).

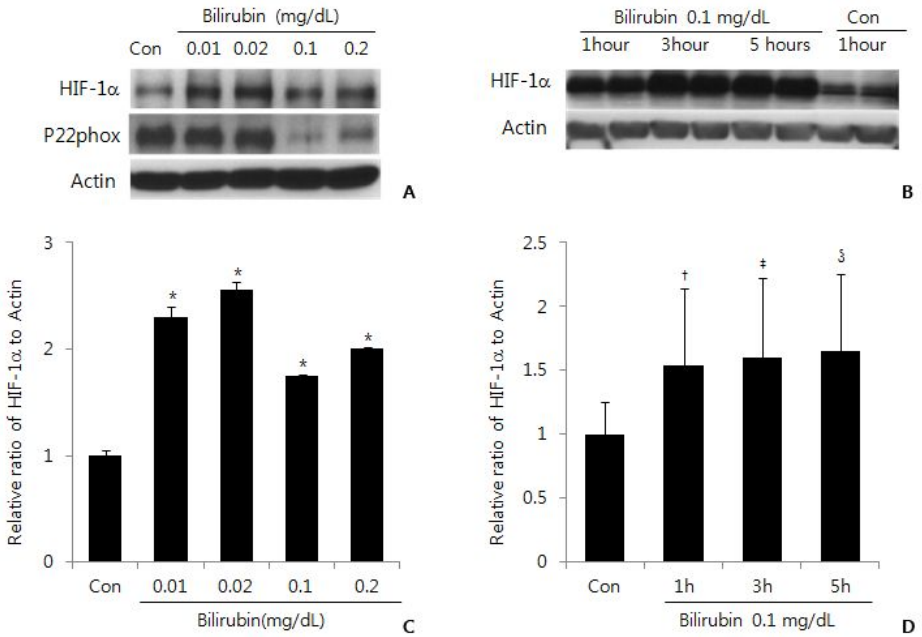

Figure 1. The effect of bilirubin on HIF−1α protein expression in HK2 cell cultured under 5% O_2 condition.

A, B. Western blotting, **C, D**. The relative ratio of HIF−1α protein to actin normalized to control.

A. Bilirubin was added to culture media with each indicated concentration for 1 h.

B. Bilirubin was added to culture media with each indicated concentration for 1, 3, or 5 h.

Con: Control samples of human HK2 cells cultured under 5% oxygen condition for 1 h.

Data are representative of three separate experiments run in triplicate.

* $p < 0.001$, † $p = 0.025$, ‡ $p = 0.020$, and § $p = 0.015$ as compared to control (Con). The vertical bar indicates 95% CI of the mean value.

The effect of bilirubin on HIF−1α mRNA transcription

Actinomycin D restrains gene transcription by inhibiting RNA chain elongation by RNA polymerase. It inhibited the increase in HIF−1α protein concentration by bilirubin treatment in HK2 cells cultured under 5% O_2 condition. The expression of HIF−1α protein in cells with 0.1 mg/dL bilirubin was increased by 1.57 ± 0.21 folds (Figure 2A), which was otherwise decreased by 0.66 ± 0.09 folds by actinomycin D (Figure 2B), as compared to that in the control cells.

The mRNA expression of HIF−1α was increased by 1.69 ± 0.05 folds in the cells with 0.1 mg/dL bilirubin under 5% O_2 condition, as compared to that in the control cells (Figure 3A and 3B). The mRNA of GLUT−1, a downstream gene of HIF−1α, was also increased by bilirubin treatment (Figure 3A and 3C). Under 21% O_2 condition, bilirubin slightly increased the mRNA expression of HIF−1α and GLUT−1 (Figure 3).

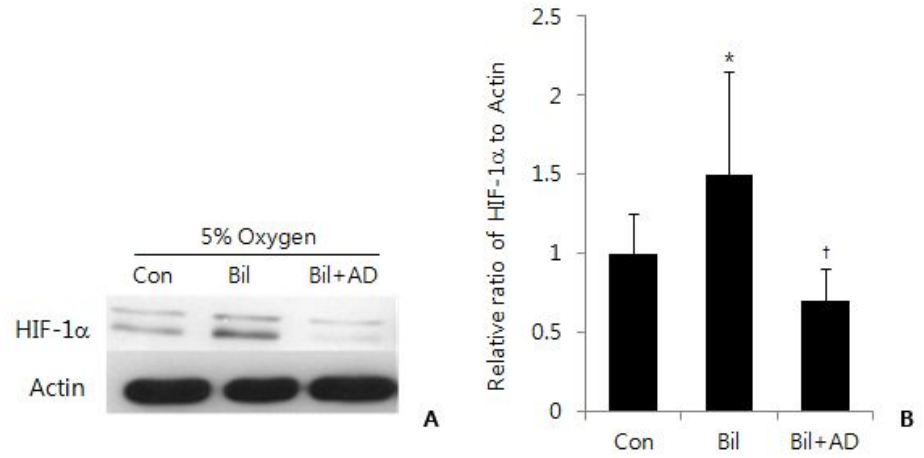

Figure 2. The effect of bilirubin on the expression of HIF-1α protein.

A. Western blotting with the proteins from cells cultured with (0.1 mg/dL) or without bilirubin or actinomycin-D (0.15 μg/ml before bilirubin treatment),

B. The relative ratio of HIF-1α protein to actin normalized to control in western blotting.

BiI: Bilirubin 0.1 mg/dL was added to culture media with each indicated concentration for 1 h.

Bil+AD: Actinomycin-D (0.15 μg/ml) was added 15 min before bilirubin treatment,

Con: Control samples of human HK2 cells cultured under 5% oxygen condition for 1 h.

Data are representative of three separate experiments run in triplicate.

* $p = 0.008$ compared to Con. † $p = 0.001$ compared to Bil.

The vertical bar indicates 95% CI of the mean value.

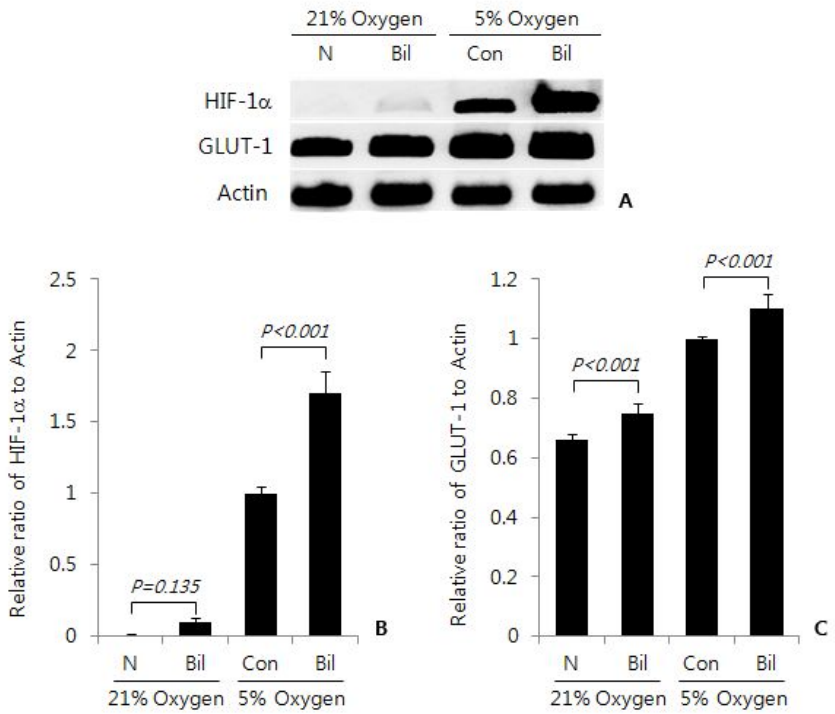

Figure 3. The effect of bilirubin on the mRNA expression of HIF-1α and GLUT-1.

A. RT-PCR for mRNA expression of HIF-1α, GLUT-1, and actin.

B. The relative ratio of HIF-1α mRNA to actin normalized to control in RT-PCR.

C. The relative ratio of GLUT-1 mRNA to actin normalized to control in RT-PCR.

BiI: Bilirubin 0.1 mg/dL was added to culture media with each indicated concentration for 1 h.

Con: Control samples of HK2 cells cultured under 5% oxygen condition for 1 h.

N: Control samples of HK2 cells cultured under 21% oxygen condition for 1 h

Data are representative of three separate experiments run in triplicate.

The vertical bar indicates 95% CI of the mean value.

The mechanism of increased HIF-1α translation by bilirubin: Inhibitors of PI3/AKT/mTOR pathway

We added inhibitors of PI3K (wortmannin and Ly294002), mTOR complex-1 (rapamycin), and PI3K/AKT pathway (miltefosine) 15 min prior to bilirubin treatment. Only PI3/AKT pathway inhibitor (miltefosine) effectively suppressed HIF-1α protein expression increased by bilirubin treatment in the HK2 cells cultured under 5% O_2 condition, whereas the inhibitors of PI3K/mTOR and ERK 1/2 pathways did not (Figure 4).

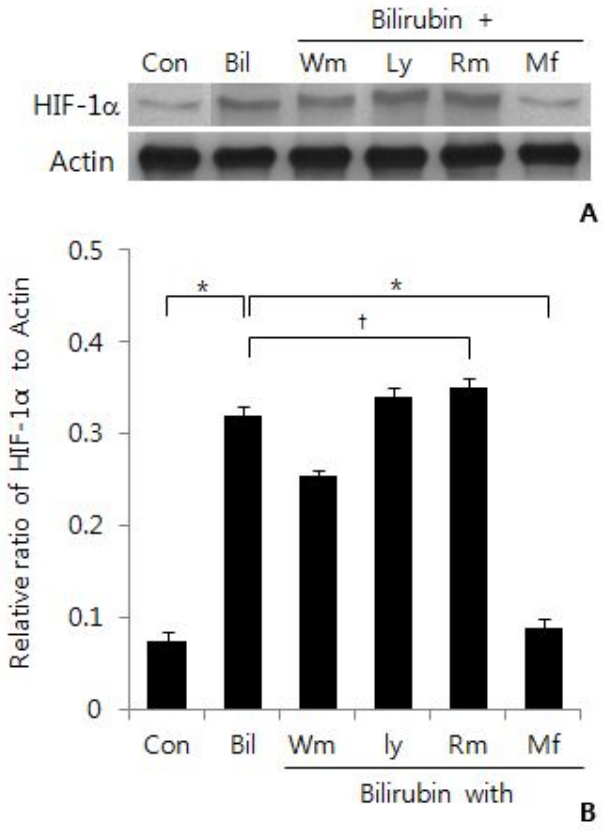

Figure 4. The effect of bilirubin on the expression of HIF-1α protein in presence of inhibitors for various pathways.

A. Western blotting with the proteins from cells cultured with (0.1 mg/dL) or without bilirubin and with wortmannin (100 nM), Ly294002 (20 μM), rapamycin (10 nM), or miltefosine (5 μM).

B. The relative ratio of HIF-1α protein to actin normalized to control in western blotting.

BiI: Bilirubin (0.1 mg/dL) was added to culture media with each indicated concentration for 1 h.

Con: Control samples of human HK2 cells cultured under 5% oxygen condition for 1 h.

Wortmannin, Ly294002, rapamycin, or miltefosine was added 15 min before bilirubin treatment.

Wm: wortmannin, **Ly**: Ly294002, **Rm**: rapamycin, **Mf**: miltefosine

Data are representative of three separate experiments run in triplicate.

* $p < 0.001$, † $p > 0.05$ compared to bilirubin treatment.

The vertical bar indicates 95% CI of the mean value.

The mechanism of increased HIF−1α translation by bilirubin: Endogenous and exogenous H_2O_2

The endogenous H_2O_2 concentration was more in 5% O_2 condition than in 21% O_2 condition; the concentration decreased with bilirubin treatment of the HK2 cells (Figure 5A). The expression of HIF−1α protein was decreased by exogenous H_2O_2 (10 μM) in HK2 cells cultured under 5% O_2 condition (Figure 5B). Cells to which both bilirubin and scavenger of superoxide or H_2O_2 were added increased the HIF−1α concentration more as compared to the cells treated with bilirubin only (Figure 5C and 5D). Exogenous H_2O_2 decreased the phosphorylation of P70S6 kinase in HK2 cells under 5% O_2 condition; this effect was completely reversed by bilirubin treatment (Figure 6A and 6B). However, the addition of bilirubin did not alter the phosphorylation of mTOR protein (Figure 6D). ERK1/2 phosphorylation was not influenced by H_2O_2 or bilirubin (Figure 6A and 6C).

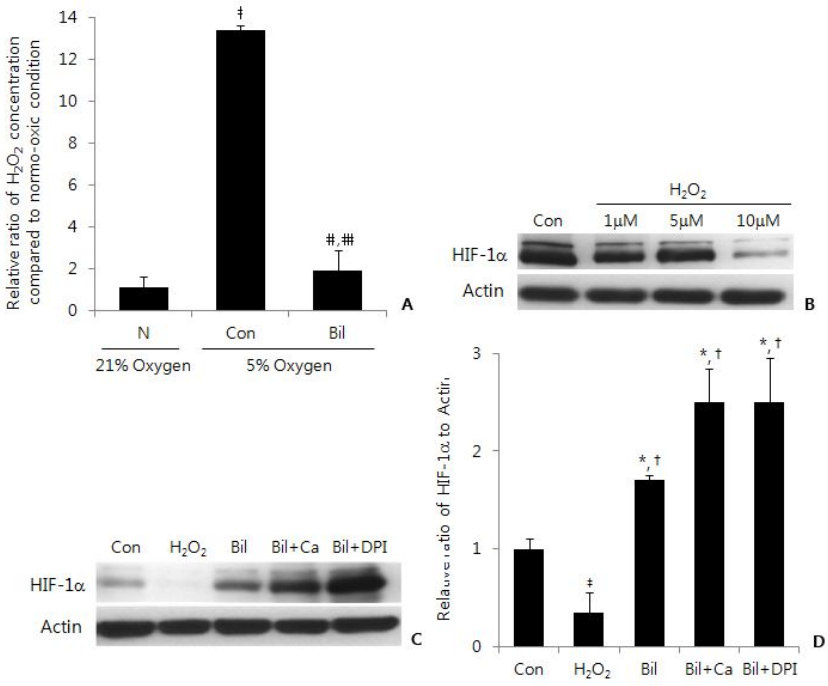

Figure 5. The effect of H_2O_2 on HIF−1α proteins expressions.

A. H_2O_2 production determined using H_2DCF−DA.

B, C. Western blotting with the proteins from cells cultured with (10 μM) or without H_2O_2, bilirubin (0.1 mg/dL), catalase (1000 U/ml), or DPI (10 μM).

D. The relative ratio of HIF−1α protein to actin normalized to control in western blotting.

N: Normoxic control cells cultured under 21% oxygen concentration.

Con: Hypoxic control cells cultured under 5% oxygen concentration

Bil: bilirubin, **Ca:** catalase, **DPI:** diphenyleneiodonium

Human HK2 cells cultured under 5% oxygen condition for 1 h.

Data are representative of three separate experiments run in triplicate.

∓,∓∓ $p < 0.001$ compared to N, ∓∓∓ $p = 0.017$ compared to Con, ∗, ‡ $p < 0.001$ compared to Con, † $p < 0.001$ compared to H_2O_2.

The vertical bar indicates 95% CI of the mean value.

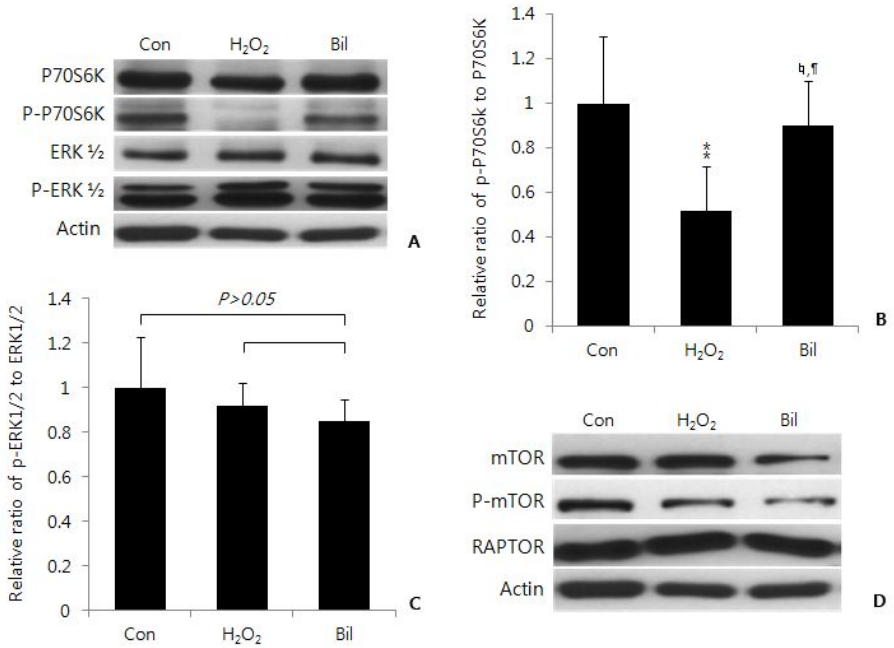

Figure 6. The effect of H_2O_2 on molecules involving HIF−1α protein abundance pathway.

A. Western blotting with the proteins from cells cultured with (10 μM) or without H_2O_2, bilirubin (0.1 mg/dL).

B, C. The relative ratios of phospho−P70S6K to P70S6K and phospho−ERK1/2 to ERK1/2 protein normalized to control in western blotting.

D. The effect of bilirubin on the expression of mTOR and RAPTOR. Western blotting of cells cultured with (10 μM) or without H2O2 or with (0.1 mg/dL) or without bilirubin. Human HK2 cells cultured under 5% oxygen condition for 1 h.

Con: Hypoxic control cells cultured under 5% oxygen concentration

Human HK2 cells cultured under 5% oxygen condition for 1 h.

Data are representative of three separate experiments run in triplicate.

‡ $p = 0.003$ and ϟ $p > 0.05$ compared to Con, ¶ $p = 0.014$ compared to H_2O_2.

The vertical bar indicates 95% CI of the mean value.

The mechanism of increased HIF-1α translation by bilirubin: NADPH oxidase

The protein abundance and mRNA expression of NADPH oxidase, an important source of ROS, effectively decreased by bilirubin treatment (Figure 7 and 8).

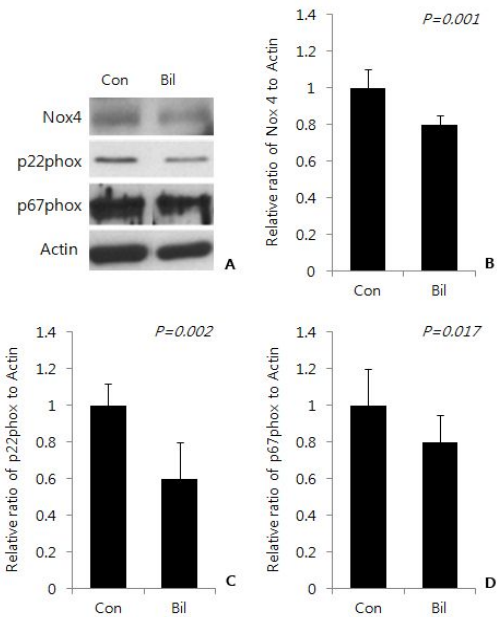

Figure 7. The effect of bilirubin on the protein expression of NADPH oxidase subunits.

A. Western blotting with the proteins from cells cultured with (0.1 mg/dL) or without bilirubin,

B-D. The relative ratio of proteins of NADPH oxidase subunits to actin normalized to control in western blotting.

Human HK2 cells cultured under 5% oxygen condition for 1 h.

Data are representative of three separate experiments run in triplicate.

The vertical bar indicates 95% CI of the mean value.

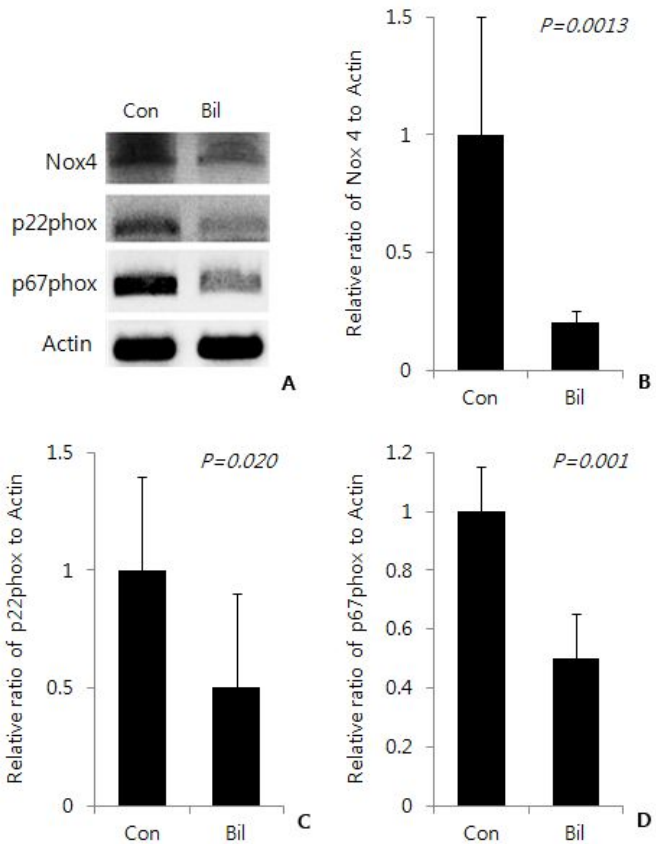

Figure 8. The effect of bilirubin on the mRNA expression of NADPH oxidase subunits.

A. RT-PCR from cells cultured with (0.1 mg/dL) or without bilirubin,

B-D. The relative ratios of mRNAs of NADPH oxidase subunits to actin normalized to control in RT-PCR. Human HK2 cells cultured under 5% oxygen condition for 1 h.

Data are representative of three separate experiments run in triplicate.

The vertical bar indicates 95% CI of the mean value.

The mechanism of increased HIF-1α translation by bilirubin: NADPH oxidase subunits

We also investigated what subunit involves primarily in HIF-1α mRNA expression through the inhibition of NOX4 and p22phox by siRNA transfection. Transfection of siRNA for NOX4 gene significantly increased HIF-1α mRNA expression as compared to that in the control cells. However, the extent of increase in HIF-1α mRNA expression by siRNA was lower than that of bilirubin treatment (Figure 9). The siRNA of p22phox did not increase HIF-1α mRNA expression of HK2 cells (Figure 10).

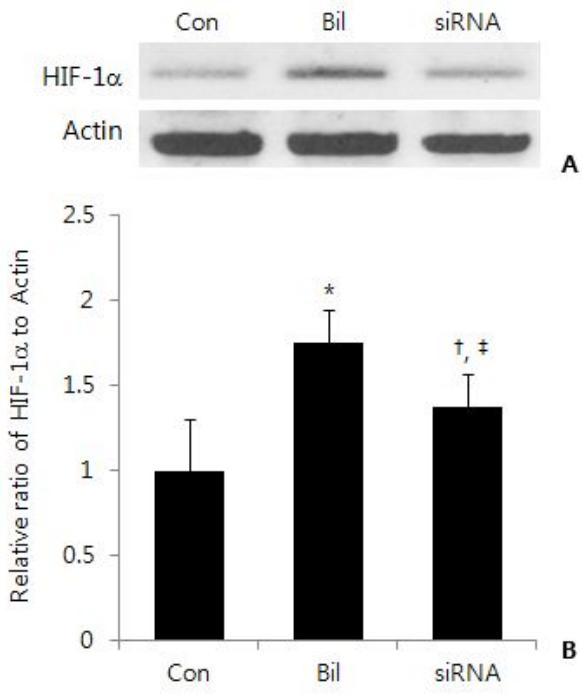

Figure 9. The effect of siRNA NOX4 on the expression of HIF-1α mRNA.

A. RT-PCR from cells cultured with (0.1 mg/dL) or without bilirubin and siRNA NOX4.

B. The relative ratios of mRNAs of HIF-1α to actin normalized to control in RT-PCR.

Human HK2 cells cultured under 5% oxygen condition for 1 h.

Data are representative of three separate experiments run in triplicate.

*p < 0.001 compared to Con, †p = 0.024 compared to Con, ‡p = 0.008 compared to Bil.

The vertical bar indicates 95% CI of the mean value.

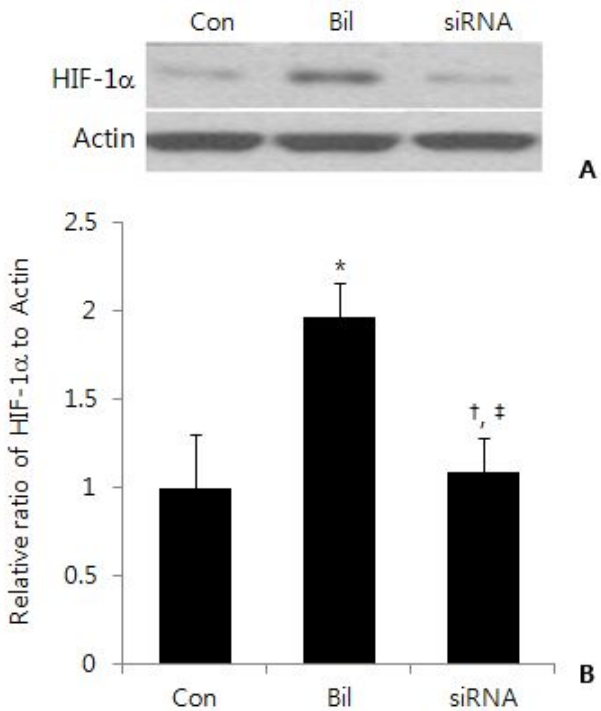

Figure 10. The effect of siRNA p22phox on the expression of HIF-1α mRNA.

A. RT-PCR from cells cultured with (0.1 mg/dL) or without bilirubin and siRNA p22phox.

B. The relative ratios of mRNAs of HIF-1α to actin normalized to control in RT-PCR.

Human HK2 cells cultured under 5% oxygen condition for 1 h.

Data are representative of three separate experiments run in triplicate.

* $p < 0.001$ compared to Con, † $p = 0.126$ compared to Con, ‡ $p < 0.001$ compared to Bil.

The vertical bar indicates 95% CI of the mean value.

The effect of bilirubin on apoptotic signals

Bilirubin treatment increased the phophorylation of AKT and decreased the cleavage of caspase 9 protein (Figure 11).

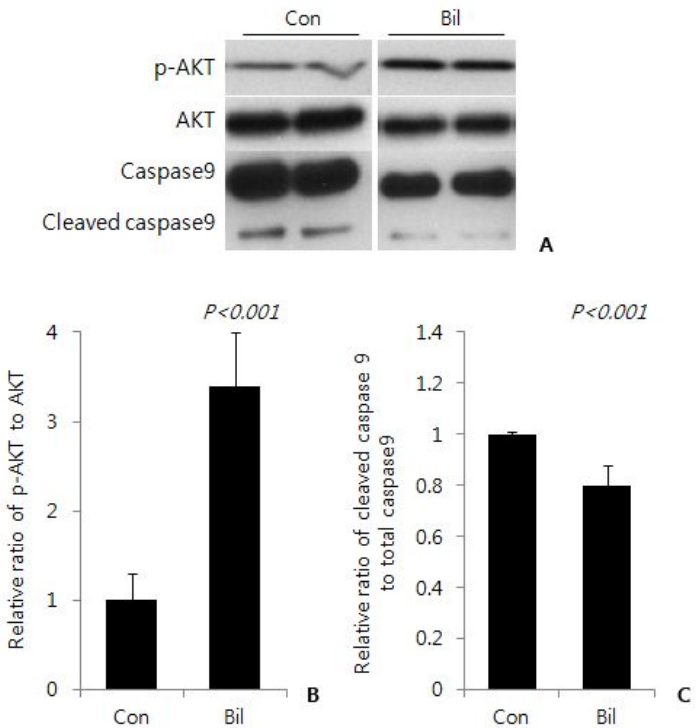

Figure 11. The apoptotic signals according to bilirubin treatment.

A. Western blotting from cells cultured with (0.1 mg/dL) or without bilirubin,

B. The relative ratios of proteins normalized to control in western blotting.

Human HK2 cells cultured under 5% oxygen condition for 1 h.

Data are representative of three separate experiments run in triplicate.

The vertical bar indicates 95% CI of the mean value.

Discussion

Bilirubin treatment increased the HIF−1α expression in HK2 cells cultured under 5% oxygen content, similar to the oxygen condition of proximal tubular cells in kidney, that is, approximately 38 mmHg. Bilirubin increased HIF−1α transcription, which was otherwise attenuated by actinomycin D treatment. The enhancement of HIF−1α protein expression by bilirubin was partly because of attenuation of decreased phosphorylation at P70S6 kinase by ROS and partly because of inhibition of *NOX4* expression , and resulted in decreased cleavage of caspase 9 protein. Induction of HIF−1α protein by bilirubin may be related to the activation of PI3K/AKT pathways.

In animal experiments, re−oxygenation was necessarily performed for a few minutes during sacrifice and tissue manipulation; this step may have resulted in HIF degradation in the kidney (6). As a result, HIF could not be detected in renal tissue of animals raised under hypoxia (6). The HIF protein concentration in animal experiments could only be verified with exaggerated stimuli such as anemia, cobaltous chloride, or carbon monoxide (6). Accordingly, we performed an *in vitro* experiment using human renal proximal tubular cells cultured in 5% oxygen concentration (around 38 mmHg pO2), to verify the effect of bilirubin and ROS on the expression of HIF in physiologic oxygen content in the kidney. Previous studies have shown that the pO2 values averaged 40−42 mmHg in the proximal and distal tubules, 30 mmHg in the outer medulla, and 48 mmHg in the efferent arteriole *in vivo* (11). HIF, a

mediator of transcriptional response to hypoxia, is expressed in different renal cell populations (6). HIF−1α was mainly induced in tubular cells including proximal tubules, distal tubules, and collecting ducts with stimuli of anemia, cobaltous chloride, and/or carbon monoxide (6). HIF−2α was expressed in the endothelial and interstitial cells as well as in a small set of glomeruli (6).

In this study, bilirubin induced HIF−1α protein transcription and H_2O_2 suppressed HIF−1α protein transcription. Addition of even a small amount (0.01 mg/dL) of bilirubin to the HK2 cell culture media was sufficient for successful induction. We used the dose of 0.1 mg/dL of bilirubin, which showed the inhibitory effect of NADPH subunits. ROS may be involved in hypoxia because the formation of ROS requires molecular oxygen (39). Treatment of purified HIF−1 with oxidative agent and H_2O_2 diminished HIF−1 DNA binding capacity, which suggests that reducing conditions may promote HIF−1α activity under hypoxia (39−41). The effects of ROS treatment on HIF−1α level or activity are a scientific debate. Destabilization (42) and stabilization of HIF−1 α by ROS (43) have been reported. Accordingly, under ROS treatment, controversial findings showing either induction (43) or reduction of HIF−1 target gene expression (24, 42) were found. A recent study postulated a time dependent biphasic regulation of HIF−1α abundance in the presence of H_2O_2 (44). At short reaction periods (< 30 minutes), a stabilization of HIF 1 α was observed, whereas a destabilization of HIF−1 α was evident at prolonged (≥1

hour) observation intervals (44). In this study, one hour exposure of exogenous H_2O_2 suppressed the hypoxic induction of HIF-1 protein and mRNA expression.

Addition of H_2O_2 to cells resulted in destabilization of the HIF-1α protein in Hep3B cells (42) and decreased target gene expression of HIF-1α in immortalized rat proximal tubular cells (24). Increased superoxide anion by xanthine/xanthine oxidase decreased HIF-1α protein in renal medullary intercalated cells (RMIC) (23). The possible mechanism of ROS on HIF-1α expression suggest that it may primarily occur at post-transcriptional level because increased superoxide levels did not affect hypoxia-induced upregulation of HIF-1α mRNA in RMIC (23) and abolished the increase of HIF-1α protein induced by CBZ-LLL (*N*-carbobenzoxyl-L -leucinyl-Lleucinyl-L-norvalinal), which is a selective ubiquitin- proteasome inhibitor (23). In this study, P70S6K phosphorylation mediating increased HIF-1α protein translation, a downstream effector of PI3K (16, 17), was attenuated by exogenous H_2O_2. Exogenous H_2O_2 decreased phosphorylation of mTOR complex-1 in HK2 cells cultured under 5% oxygen concentration. Therefore, ROS may have affected post-transcriptional regulation of HIF-1α protein through modulation of PI3K/mTOR/P70S6K pathway. The NADPH oxidase activity significantly contributed to this post-transcriptional regulation of HIF-1α in intercalated cells under physiological conditions (38). Deletion of p22phox protein by antisense oligodeoxynucleotide increased HIF-1α protein concentration in RMIC (23), whereas, in adenocarcinomic human alveolar basal epithelial

(A549) cells, NOX1 overexpression enhanced HIF-1α protein to hypoxic stimuli (25). The results of this study revealed that antisense oligodeoxynucleotide for NOX4 increased HIF-1α mRNA expression in HK2 cells. The different subunits of NADPH oxidase have different effect on HIF-1α expressions in different cell lines; this aspects need further studies for understanding the possible mechanisms involved. Bilirubin concentration of 0.1 mg/dL effectively decreased H_2O_2 level and suppressed mRNA and protein level of NADPH oxidase subunits, NOX4, p22phox, and p67phox in HK2 cells cultured under 5% oxygen condition. The possible mechanism of increased HIF-1α expression by biliribuin may include increase in the transcription of HIF-1α by suppression of NAPDH oxidase subunits, especially of NOX4, and modification of post-transcriptional regulation of HIF-1α protein by reverse P70S6 kinase activity decreased by ROS under 5% oxygen concentration. However, in this study, bilirubin was not found to enhance phosphorylation of P70S6 kinase through the activation of PI3K/mTOR pathway. Furthermore, the inhibitors of PI3K/mTOR and ERK 1/2 pathways did not attenuate increased HIF-1α protein by bilirubin treatment in HK2 cells. Therefore, the effect of bilirubin on HIF-1α may be associated with PI3K/AKT pathways. The effect of bilirubin on HIF-1α protein activation was assessed by measuring the expression of anti-apoptotic protein, phopho-AKT, and pro-apoptotic protein, cleavaged caspase 9; we found that HIF-1α activation was related to decrease in pro-apoptotic signal. There were debatable results of HIF-1α activation on fibrosis and progression of kidney

injury. In a remnant kidney injury model, HIF activation attenuated the progression of proteinuria and structural damage in podocyte, accompanied with reduction in inflammation and fibrosis (45). However, the negative aspect of HIF activation included genetic ablation of epithelial HIF-1α, which inhibited the development of tubulointerstitial fibrosis in unilateral ureteral obstruction animal model (46).

In conclusion, bilirubin enhanced HIF-1α mRNA transcription as well as the upregulation of HIF-1α protein translation through 1) inhibition of oxidaive stress, at least partly by downregulating subunits of NADPH oxidase and 2) activation of P13K/AKT pathway in HK2 cells cultured under hypoxia. These findings suggest that bilirubin could be a cytoprotective molecule with antioxidative properties in renal tubular injury.

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요약 (국문초록)

서론: 신장기능 저하의 초기 유발 인자는 다양하나 궁극적으로는 세뇨관 세포의 손상, 자멸사, 세포사와 세뇨관간질의 섬유화가 진행된다는 공통적 과정을 거치는 것으로 알려져 있다. 이 공통 병인기전의 중심에는 세뇨관내 저산소증에 의한 면역세포의 활성화 및 침윤이 나타나며, 이로 인한 염증반응이 중요한 기전으로 알려져 있다. Hypoxia-inducible factor(HIF)계는 저산소증과 활성산소족(Reactive Oxygen species, ROS)에 반응하고 저산소증에 의한 손상을 조절하는 데 가장 중요한 역할을 수행한다. 빌리루빈은 항산화 효과와 신보호 효과가 있음이 알려졌으나 아직까지 그 기전을 명확히 알려져 있지 않다. 따라서, 본 연구는 인간 근위세뇨관 세포배양 모델에서 빌리루빈이 HIF계에 미치는 영향을 알아보고, 그 분자생물학적 기전을 알아보고자 하였다.

방법: 인간 근위세뇨관 (HK2) 세포주에 HIF 발현을 억제하는 Actinomycin D를 투여하면서 빌리루빈을 처리한 군(0.01-0.2 mg/dL)과 처리하지 않은 군(대조군)으로 나누어, 5% 산소를 주며 1-5시간 배양한 후 ROS와 HIF계의 발현의 변화를 비교하였다. 첫 번째, 빌리루빈 효과의 분자생물학적 기전을 알아보기 위하여 HIF의 발현의 가장 중요한 기전으로 알려져 있는 PI3K/mTOR, PI3K/AKT, ERK 1/2 pathway의 억제제를 투여한 후 ROS의 변화와 HIF계 변화를 측정하였다. 두 번째, HIF계에 영향을 미치는 것으로 알려져 있는 미토콘드리아에서의 ROS 형성에 관여하는 NADPH oxidase와의 연관성을 밝히기 위해 10μM의 과산화 수소를 주면 HIF 발현의 변화를 알아보고 이와 연관된 P70S6 kinase의 인산화 과정에 빌리루빈의 역할을 알아보았다. 세 번째, HIF 발현에 영향을 미치는 *NOX4*에 대한 영향은 *NOX4* 유전자에 대한 siRNA 처리 후 HIF-1α mRNA의 발현정도를 빌리루빈 처리 여부에 따라 확인하였다.

결과: Actinomycin D에 의해 억제되는 HIF-1α의 전사 RNA(mRNA) 발현은 대조군의 세포에서보다 0.1mg/dL 빌리루빈을 투여한 군에서 1.69 ± 0.05배 증가하였다. 이러한 증가는 PI3K/AKT의 저해제인 Mitefosin 투여로 감소하였으나, PI3K/mTOR, ERK 1/2 의 특이적인 억제제들을 투여로는 변화되지 않았다. HIF-1α의 발현은 10μM 과산화수소(H_2O_2)의 투여로 감소하였는데, 여기에 빌리루빈을 단독 혹은 과산화기 제거제와 동시에 투여하면 HIF-1α의 발현이 다시 증가함을 확인하였다. 과산화수소(H_2O_2)의 투여로 P70S6 kinase의 인산화가 감소되는 현상도 빌리루빈 투여로 완전히 회복되는 것을 확인하였다. 세포내 활성산소족(ROS)의 중요한 공급원인 NADPH oxidase의 발현도 빌리루빈 투여로 감소함을 확인하는 것이 관찰되었다. 그 기전을 알아보기 위한 small interfering RNA(siRNA)을 이용한 NOX4 유전자를 제어한 실험에서 HIF-1α mRNA 발현이 증가함을 관찰할 수 있었다.

결론: 본 연구를 통하여 신장내 근위세뇨관의 생리적 상황과 유사한 5% 산소농도 하에서 배양된 근위세뇨관세포 (HK2 세포)에 빌리루빈을 투여하면 HIF-1α의 mRNA 및 단백 발현이 증가하여 항산화 효과가 나타남을 알 수 있었고, 이는 1)활성산소족의 주요 공급원인 NADPH oxidase의 구성 단백인 NOX4의 저해를 통해서, 그리고 2) PI3K/AKT 경로를 활성화시킴으로 이루어짐을 확인하였다. 본 연구의 결과는 빌리루빈이 항산화 효과를 통하여 신장 세뇨관 세포 보호효과를 가지는 물질이 될 수 있음을 보여준다.

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주요어: 근위세뇨관세포, 빌리루빈, Hypoxia induced factor,활성산소족

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