Correlation between hypoxia inducible factor -1α and renin expression in rats kidney induced by cobalt chloride

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Abstrak

Latar belakang: *Kobalt klorida dapat digunakan sebagai senyawa yang dapat menimbulkan kondisi mimikri hipoksia tanpa kadar rendah oksigen di dalam tubuh, dan menstabilkan hypoxia inducible factor-1α. Kami memutuskan untuk mengobservasi apakah terdapat regulasi ekspresi renin oleh HIF-1α. Dengan demikian kami menyelenggarakan beberapa penelitian untuk memastikan kemungkinan dan memulai dengan penelitian induksi tikus secara intraperitoneal kobalt klorida (*CoCl$_2$*) untuk membangkitkan kondisi mimikri hipoksia dan mendapatkan konsentrasi dan pola ekspresi HIF-1α dan mRNA.*

Metode: *Dua puluh empat ekor tikus dibagi menjadi 4 grup: kontrol, 2, 8, dan 24 jam inkubasi pasca injeksi intraperitoneal 30 mg/kg berat badan* CoCl$_2$*. Setelah tikus dikorbankan, organ ginjal digunakan untuk pemeriksaan parameter berat ginjal, kadar RNA, kadar protein HIF-1α (ELISA) dan mRNA renin (RT-PCR).*

Hasil: *Hasil menunjukkan bahwa terdapat perbedaan rasio berat ginjal/berat badan tikus, namun secara statistik tidak bermakna (p > 0,05). Secara statistik tidak terdapat perbedaan bermakna kadar protein HIF-1α antar kelompok (p > 0,05). Ekspresi relatif mRNA renin meningkat tajam (30 x kontrol), mulai pada 8 jam inkubasi pasca induksi intraperitoneal* CoCl$_2$ *dan terus meningkat sampai inkubasi 24 jam (2465 x kontrol). Korelasi antara protein HIF-1α dan ekpresi relatif mRNA renin menggunakan analisis* Pearson *menunjukkan positif kuat (R = 0,91) (p = 0,09).*

Kesimpulan: *Terdapat kemungkinan yang besar bahwa gen renin diregulasi oleh HIF-1α.* ***(Med J Indones. 2012;21:128-32)***

Abstract

Background: Cobalt chloride can be used as an agent to stabilize hypoxia inducible factor-1α (HIF-1α) and to imitate hypoxia without low levels of oxygen inside the body. We intended to investigate if there was any regulation of renin expression by HIF-1α. Therefore, we conducted several studies to clarify this possibility starting with the induction of hypoxic mimicry in rats by intra-peritoneal (IP) injection of cobalt chloride (CoCl$_2$) to obtain the levels and pattern of HIF-1α and renin mRNA and protein expression.

Methods: Twenty-four rats were randomly divided into four groups, control group and incubation groups 2, 8, and 24 hours after intra-peritoneal injection of 30 mg CoCl$_2$ per kg BW. After the rats were sacrificed, kidneys were excised, weighed and kidney weight compared to BW. Tissue parameters were measured such as RNA concentration, HIF-1α protein by ELISA, and renin mRNA by RT-PCR.

Results: Differences between the groups in the ratios of kidney weight to BW and in the concentrations of HIF-1α protein were statistically not significant (p > 0.05). Relative expression of renin mRNA increased markedly starting 8 hours after CoCl$_2$ IP injection (30 times over controls) and further rising until 24 hours (2465 times over controls). Correlation between HIF-1α and renin mRNA by Pearson analysis was strongly positive, but not significant (R = 0.91; p = 0.09).

Conclusion: Renin gene regulation in renal hypoxic mimicry strongly correlates with HIF-1α. ***(Med J Indones. 2012;21:128-32)***

Keywords: Cobalt chloride (CoCl$_2$), hypoxia inducible factor-1α (HIF-1α), renin

Hypoxia inducible factor (HIF) is a master transcription factor that regulates transcription of several genes to maintain energy homeostasis.[1] In normoxic condition, HIF-1α is degraded through ubiqitin-proteasomes.[1,2] Degradation of HIF-1 begins with the hydroxylation of prolines 402 and 564 in an oxygen-dependent-degradation domain (ODDD) catalyzed by prolyl-hydroxylase (PHD).[2,3] Hydroxyprolines 402 and 564 in HIF-1α are recognized by the tumor suppressor vonHippel-Lindau protein (pVHL), which acts as an E$_3$ ubiquitin ligase. After ubiqitinylation, HIF-1α is degraded proteasomally.[4] In hypoxic condition, lack of oxygen as a PHD co-substrate decreases the enzyme activity and increase HIF-1α.[2] Prolyl-hydroxylase activity needs the presence of substrates and co-substrates such as oxygen, 2-oxoglutarate, and vitamin C to catalyze the reaction, which produces hydoxylated HIF-1α, succinate and CO$_2$.[5] Besides lack of oxygen, 2-oxoglutarate analogues and CoCl$_2$ can inhibit prolyl-hydroxylase.[6] Intraperitoneal-CoCl$_2$ injection to rats stabilizes HIF-1α and induces hypoxic mimicry.[6]

Kidney infections lead to the disappearance of blood vessels, which causes ischemia and hypertension.[1,7,8] Since ischemia can trigger renin secretion,[1,7-10] we considered that HIF-1α may regulate renin expression.

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Hence, we investigated renin regulation by HIF-1α through intra-peritoneal injection of cobalt chloride (CoCl$_2$) into the rats to induce hypoxic mimicry and obtain the levels and pattern of HIF-1α and renin mRNA and protein expression.

METHODS

Animals

This experimental animal study was conducted in the Department of Biochemistry and Molecular Biology, Faculty of Medicine, Universitas Indonesia, in 2011 and aimed to observe the levels and pattern of HIF-1α and renin during renal hypoxic mimicry. Twenty-four male Sprague-Dawley rats with 150 – 200 gram of body weight from BALITVET Bogor were divided randomly into four groups of 6 rats each: control group and three CoCl$_2$ treated groups. The treatment was performed by intra-peritoneal injection of CoCl$_2$ (30 mg/kg BW). Incubation times after induction were 2, 8 and 24 hours before the rats were sacrificed. After the rats were sacrificed, kidneys were removed and kept in a deep-freezer at -84 ºC until used. Each kidney was homogenized with a micro-pestle and RNA was isolated using "TriPure DNA RNA protein isolation" kit (Roche 11 667 165 001, Indonesia). From tissue homogenates we obtained the concentration of HIF-1α protein. The RNA isolates were used in Real Time RT-PCR to measure the relative expression of HIF-1α and renin. HIF-1α protein was measured by Western blot. HIF-1α and renin mRNA relative expression were measured by "iScript™ One Step RT-PCR with SYBR® Green" kit) (BioRad # 170-8892, USA).

Sample preparation

Preparation of kidney homogenate

100 mg of kidney tissue were put into 1.7 mL microtubes, then 0.5 mL TriPure Isolation Reagent was added. The tissue was homogenized at 15-25ºC using a micro-pestle. Another aliquote of 0.5 mL TriPure Isolation Reagent was added to the homogenate, then centrifuged at 12000 g for 10 minutes. The supernatant was used for measurements.

RNA isolation from kidney tissue

Total RNA was extracted with TriPure (DNA-RNA-protein) Isolation Reagent (Roche 11 667 165 001, Indonesia).

Separation: Kidney homogenates were incubated at 15-25ºC for 5 minutes to ensure that nucleoprotein

complexes completely dissociated. Then, 0.2 mL of chloroform (CHCl$_3$) were added, shaken vigorously for 15 seconds and incubated again at 15-25ºC for 2-15 minutes. Centrifugation at 12000 g and 2-8ºC for 15 minutes separated 3 phases. The clear aqueous phase was on top and contained RNA. Interphase and organic phase at the bottom were colored red and contained DNA and proteins.

Isolation of RNA: The clear phase at the top was moved to another microtube. The residual red phases were stored at -20°C. To the clear phase 0.5 mL isopropanol was added, the microtube closed and 15 times shaken up and down. Subsequently, the samples were incubated for 10 minutes at 25ºC to precipitate RNA, centrifuged at 12,000 g at 2ºC for 10 minutes and the supernatant was discarded. To clean the RNA, one mL of 75% EtOH was added to the precipitate, vortexed and precipitated again by centrifugation at 7500 g and 2ºC for 5 minutes. The supernatant was discarded. Subsequently, the precipitate was half-dried to become a semi-dry RNA pellet and re-suspended in 50 µL of RNAse-free water containing DEPC (DEPC-treated RNAse-free water). The precipitate was dissolved several times by pipette the solution, and then incubated at 55-60ºC for 15 minutes.

Measurement of parameters

Primer design of HIF-1α, renin, and β-actin genes

We used Primer-3 program to design the primers of HIF-1 α and renin genes. The respective sequences were obtained from the NCBI Gene Bank with the code (NC_005105.2) for rat HIF-1α and code (NC_005112) for renin. First, a Bioinformatics *in silico* search through the NCBI web site found the sequences of renin promoter, TATA box, and the structural renin gene. From this information, we made the design of the forward and reverse primer sequences of the promoter containing the HRE with the Primer-3 program. The primers were used for measurement of mRNA relative expression.

cDNA amplification by Real Time - PCR

We used iScript One-Step RT-PCR Kit with SYBR Green (BioRad), primers of HIF-1α and renin, microtubes, RT-PCR tubes, microcentrifuge, single and multichannel micropipettes, and RT-PCR with the CFX program (MiniOpticon BioRad®) as follow: cDNAs were synthesized in 10 minutes at 50ºC; iScript reverse transcriptase was incubated for 5 minutes at 95ºC. Forty PCR cycles were run for 10

seconds each at 95ºC, 30 seconds at 59ºC, and 30 seconds at 72ºC. Melting curve analysis: 1 minute at 95ºC, 1 minute at 55ºC, 10 seconds at 55ºC, totally 80 cycles, increasing 0.5ºC for each cycle. By using RT-PCR the amount of cDNA copies can be determined as quantitative gene expression of HIF-1α or renin. We used aquabidest as negative controls to exclude false positive results. As an external standard gene we used β-actin gene. Through RT-PCR we can obtain efficiency and cycle threshold (Ct) values. Expression of HIF-1α and renin genes was counted by relative quantification and relative concentration of mRNA was calculated using the Livax formula.

Measurement of HIF-1α by ELISA

This technique was run using Surveyor™ Intracellular Human/Mouse Total HIF-1α Immunoassay (R&D,SUV1935.). A series of standard solutions was made: 31.25 pg/mL; 62.5 pg/mL; 125 pg/mL; 250 pg/mL; 500 pg/mL; 1000 pg/mL. Reagent diluent 2 was used as blank. One hundred microlitre of blank, standards, and sample homogenates of the kidneys were put into each well of an ELISA micro-plate, incubated with shaking at room temperature for 2 hours. After incubation, the wells were washed 3 times with 250 µL washing buffer. Each well was added with 100 µL total HIF-1α detection antibody at a concentration of 50 ng/mL. Wells were incubated for 2 hours with shaking. After washing wells were added with 100 µL streptavidine-HRP 1/200, and incubated for 20 minutes with shaking. After washing, each well was added with 100 µL mixture of reagent color A and B at 1:1 ratio. The micro-plate was kept in the dark for 20 minutes, 50 µL of stop solution was added and during the next 45 minutes, the absorbance was read with the ELISA reader at wavelength of 450 nm.

RESULTS

The average ratios between kidney and body weights are shown in table 1.

Table 1. Average kidney-body weight ratios in rats with CoCl$_2$ induced hypoxic mimicry

Rat groups	Right kidney ± SD (%)	Left kidney ± SD (%)
Control	0.36 ± 0.04	0.35 ± 0.04
2 hours	0.39 ± 0.02	0.37 ± 0.03
8 hours	0.37 ± 0.05	0.37 ± 0.03
24 hours	0.34 ± 0.04	0.34 ± 0.04

The ratios between kidney and body weights were calculated. There is an increase at 2 hours of incubation, slightly stronger in right than in left kidneys. At 8 hours of incubation weight ratios decrease again to about controls normal with no difference between left and right kidneys. At 24 hours of incubation, the kidney-body weight ratios decrease below controls, equally left and right.

HIF-1α protein

HIF-1α protein concentration increases in the 2 hours incubation group and reaches its peak at 8 hours of incubation (Figure 1). After 24 hours protein concentration is back to control values. Statistically, the differences between groups were not significant (p > 0.05).
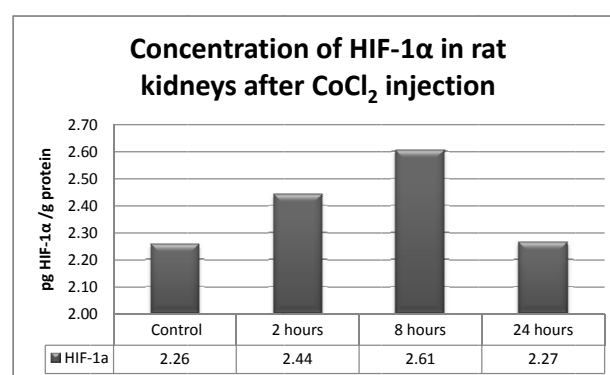

Figure 1. Concentration of HIF-1α protein in rat kidneys (pg/g tissue protein) after CoCl$_2$ *injection*

Renin and HIF-1α mRNA

HIF-1α primers were 5'-CTG CCT CTG AAA CTC CAA AGC CAC T-3' (forward) and 5'CTC ACT GGG ACT GTT AGG CTC AGG T-3' (reverse). Renin primers were 5'-CTT TGT ACCGACTTG GGT CA-3' (forward) and 5'-ATT TAG TCT CGT CCC GGA CA-3' (reverse) with a PCR product of 262 bp. Primers for the β-actin reference gene were 5'-ACC ACA GCT GAG AGG GAA ATC-G-3' (forward) and 5'-AGA GGT CTT TACGGATGT CAA CG-3' (reverse) with a PCR product of 277 bp.

Relative expression of HIF-1α mRNA

Averages of β-actin Ct and HIF-1α Ct of rat kidneys induced by CoCl$_2$ are shown in table 2.

Table 2. Average of HIF-1α and β-actin Ct in rat kidney tissue after CoCl$_2$ injection

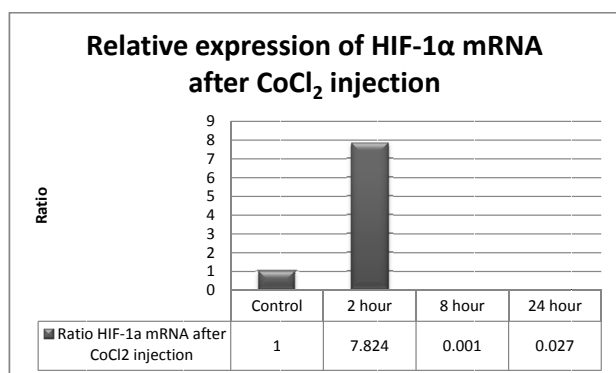| Rat groups	Average of β-actin Ct	Average of HIF-1α Ct
Control	27.855 ± 4.085	24.233 ± 0.517
2 hours	30.883 ± 1.928	24.294 ± 2.757
8 hours	23.290 ± 4.680	29.198 ± 0.794
24 hours	27.587 ± 2.685	29.173 ± 1.448

Figure 2. Relative expression of HIF-1α mRNA) in rat kidneys induced by CoCl₂ *injection*

Relative expression of HIF-1α increased at 2 hours of incubation after CoCl₂ intra-peritoneal injection (7.8 times over controls). In the group of 8 hours incubation after CoCl₂ injection HIF-1α relative expression was decreased markedly, 1/1000 of controls (Figure 2). In the 24 hours incubation group, HIF-1α mRNA increased slightly vs. 8 hours, but remained still far below the control group (27/1000).

Renin

Renin mRNA relative expression starts to increase markedly (30 times over controls) at 8 hours after IP CoCl₂ injection and further increase until 24 hours (2465 times over controls; figure 3).
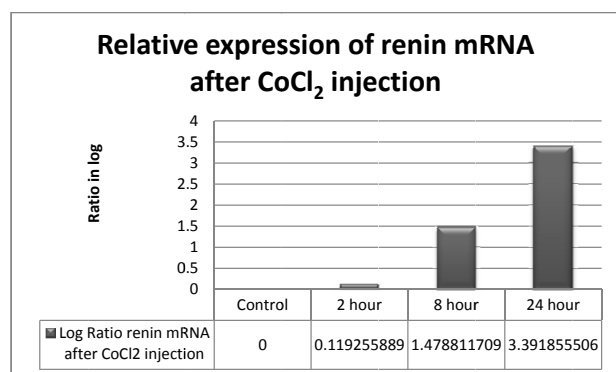

Figure 3. Relative expression of renin mRNA (in logarithmic scale) in rat kidneys induced by CoCl₂ injection

Correlation between HIF-1α protein and renin mRNA by Pearson analysis was strongly positive (R = 0.91) (p = 0.09) (Figure 4).

DISCUSSION

HIF-1α is synthesized but the stability of protein is regulated by its degradation process.[9] The key step of HIF-1α degradation process is PHD activity that can be modulated by oxygen, cobalt chloride, and desferroxamine.[9-11] Oxygen is one of the PHD substrates and if enough oxygen is present, PHD hydroxylates prolines of HIF-1α. Subsequently, hydroxylated HIF-1α will be degraded. Conversely, low oxygen levels in hypoxia inhibit PHD and stabilize HIF-1α. Another regulation of HIF-1α is on the level of its biosynthesis.[9] HIF-1α synthesis can be triggered by activation of p44/42MAPK pathway, a signal transduction pathway through tyrosin kinase and activation of phosphoinositol threephosphate kinase (PI3PK) and mitogen-activating protein kinase (MAPK). MAPK pathway increases the activity of HIF-1α transcription. Morerover, some cytokines such as IL-1β and TNF-α can stimulate the binding of HIF-1α to DNA. The reason why there were no significant different in expression of HIF-1α protein in oxidative stress and hypoxia is that the process of translation is depressed (suppressed) for energy efficiency.[9,11,12]
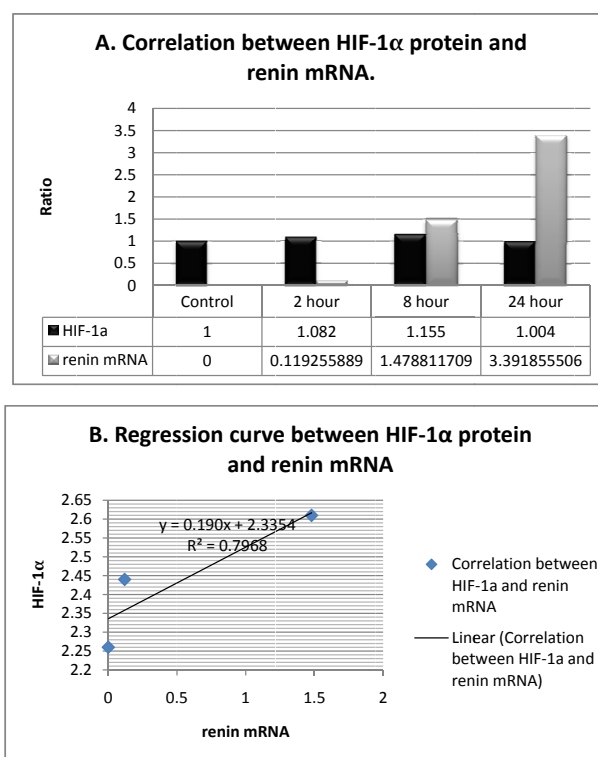

Figure 4. A: Correlation between HIF-1α protein and renin mRNA in kidney tissue induced by IP CoCl₂ *injection isstrongly positive (Pearsson correlation 0.91; p = 0.09); B: Regression curve of HIF-1α protein and renin mRNA*

In this research, we saw that renin gene was regulated by HIF-1α as we had seen in our previous research.[10] It was already known that HIF-1α is an acute response to hypoxic condition or to hypoxic mimicry induced by CoCl₂, besides renin is the following response to repair kidney homeostasis. Kidney respond to CoCl₂ quickly increased HIF-1α and followed by increase of renin. Correlation

between HIF-1α protein and HIF-1α mRNA relative expression by Pearson (SPSS 17) was 0.134, which is means the correlation was weakly positive. In other side, correlation between HIF-1α protein and renin mRNA in control, 2 hour and 8 hour groups by Pearson analysis was strongly positive (R = 0.91) (p = 0.09) (Figure 4).

Renin is a component of the renin-angiotensin-aldosteron-system (RAAS). The RAA system plays a role to maintain homeostasis of body fluids and blood pressure. Renin cleaves angiotensinogen to be angiotensin I. Angiotensin I (ANG I) then is cleaved to angiotensin II (ANG II) by angiotensin converting enzyme (ACE). Angiotensin II binds to its receptor at targeted cells and leads to biological actions or effects such as vasoconstriction, increase of vascular volume by increasing sodium and water retention in renal tubules. Until now, 4 types of ANG II receptors have been revealed: AT_1R, AT_2R, AT_3R, and AT_4R. AT_1R is the main receptor of ANG II. AT_2R is only found in fetuses and neonates. The characteristics of AT_3R and AT_4R are not yet known.[13] Recently, Matsuura et al[14] proved that $CoCl_2$ induction suppressed AT_1R expression and thus, biological effects of ANG II. It was considered that mimicry of hypoxia using $CoCl_2$ did not generate hypertension different from hypoxia that causes renin elevation and hypertension. Hypoxia acts as a key role in cardiovascular defects such as systemic or pulmonary hypertension.[14-17] Kramer et al[15] found that acute hypoxia leads to renin mRNA expression and renin activity in the plasma (plasma renin activity/ PRA). The increase of renin expression and PRA leads to hypertension. Kramer suggested that the increase of renin mRNA expression and PRA in acute hypoxia were caused by the increase of catecholamine concentration in circulation.[15] Our studies found that renin was one of several genes, which are regulated by HIF-1α (in progress of publishing).[10] The elevation of HIF-1α increases renin expression. Therefore, in hypoxia or hypoxic mimicry, besides elevation of catecholamines renin elevation is regulated by the increase of HIF-1α protein.

In this research, we found that in $CoCl_2$ intra-peritoneal induction of rats, there is a strong correlation between the level of HIF-1α protein and renin mRNA in the kidney tissues. This result is supporting our previous study that renin expression is regulated by HIF-1α. From this research, we conclude that $CoCl_2$ causes an increase of HIF-1α protein and renin mRNA relative expression.

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