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Hypoxia increased Malondialdehyde from Membrane Damages is Highly Correlated to HIF-1 α but not to Renin Expression in Rat Kidney

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Abstract. Systemic hypoxia lead to deplete amount of ATP in tissues that is caused by inefficiency of ATP production in anaerobic glycolysis. Furthermore, degradation of ATP will activate xanthine oxidase activity, that has side effect free radical production and oxidative stress. In addition, hypoxia is known lead to HIF-1 α stabilities by reducing prolyl hydroxylase activity and renin expression. Higher renin expression can cause hypertension. This research aim is to reveal how strong oxidative stress damages of systemic hypoxia (MDA) correlated to HIF-1 α and renin expression in kidney tissue. This experimental study conducted using rats induced by hypoxic states for 1 day, 3 days, 7 days, and 14 days with gas mix 10% O₂, 90% N₂ to proof systemic hypoxia, produce free radical damaged (malondialdehyde/MDA), increase expression of HIF-1 α and renin mRNA relative expression. MDA was measured by spectrophotometric method, HIF-1 α by Immunohisto chemistry, and renin mRNA using RT-PCR. Results showed that in systemic hypoxia MDA were increased significantly in 7 and 14 days of hypoxia, but in 14days MDA level was lower than 7 days hypoxia. HIF-1 α increased during hypoxia. Relative expression of renin mRNA was increased during systemic hypoxia and the peak was at 3day hypoxia. MDA has strong correlation to HIF-1 α than to renin mRNA relative expression. We conclude that MDA as product of membrane damage caused by free radicals has strong correlation to HIF-1 α in systemic hypoxia.

Key word: Malondialdehyde, renin mRNA, HIF-1alpha, hypoxia, oxidative stress

1. Introduction

Previous decades, studies revealed that in renal ischemia there was an protein increased called renin [1]. Renin also increase when there was atherosclerosis intra renal [2]. Further studies reveal that during ischemia or hypoxia there was a transcription factor protein called HIF-1 alpha that regulate synthesis of some targeted proteins to adapt against hypoxia. It was understood that HIF-1 alpha is a master transcription factor for controlling the synthesis of several key proteins for cell survival [3]. Semenza and colleges who were firstly isolated and purified HIF-1 alpha in 1995 [3]. During transcription of certain proteins and exert its regulation HIF-1 alpha bind to a consensus sequence found in promoter region of the targeted gene named as HIF-1 Response Element (HRE) [4].

Our previous study in 2012 about renin expression, result show that inhibition of prolyl hydroxylase lead renin mRNA relative expression increased markedly 30 times than control, starting 8 hours after CoCl₂ intra peritoneal injection, and further, increased until 24 hours produced 2465 times than controls. There was a strong correlation (R=0.91) between HIF-1 alpha and renin mRNA relative expression but not significant (p=0.09) [5]. Prolyl hydroxylase could be inhibited also by hypoxia. Reduce of O₂ level means reduce substrate of PHD, therefore inhibit enzyme activity, it makes HIF-1 α is stabilized. Low oxygen level contributes to increase ROS production from mitochondrial respiratory chain, and from higher activity of xanthine oxidase [6]. ROS production impact on membrane to suffer from lipid



peroxidation and produce MDA as membrane damages intermediate. Following to previous studies we conduct research to proof that oxidative stress lead to membrane damage as MDA, and reveal its correlation to HIF 1 α and renin expression.

2. Experimental Methods

This experimental study used rats treated with hypoxic state that divided into several groups: 1, 3, 7, and 14day hypoxia, compared with a control group (atmosphere oxygen concentration). Each group consist of 5 rats, therefore total rats were 25. After treatment rats were sacrificed to obtain kidney tissues.

2.1. DNA and RNA isolation

Isolation of RNA and DNA run using TriPure Isolation Reagent (Roche) Kit.

2.2. Making Homogenate

One hundred mg rat kidney tissue was put into 1.7 mL micro-tube, than added with 0.5 mL TriPure Isolation reagent®. Using micropestel it blended to be a homogenate at 15-25° C. When it became homogenized it was added more of 0.5 mL TriPure Isolation Reagent®, and then centrifuged at 12000g for 10 minutes. We used the supernatant as homogenate. Homogenate underwent DNA-RNA isolation (TriPure DNA, RNA, protein Isolation Kit®) (Roche). Using the TriPure protocol we obtained DNA, RNA and protein isolates separately. RNA was used to obtain HIF-1 α and renin mRNA relative expression using SBRGreen RT-PCR (BioRad) [7,8].

2.3. Separation

Homogenate previously made was incubate for 5 minutes at 15° – 25° C, to ensure complete dissociation of nucleoprotein. Homogenate was added 0.2 mL chlorophorm (CHCl₃) and shake for 15 seconds, and then incubated for 2-15 minutes at 15° – 25° C. After incubation, micro-tube was centrifuged at 12,000 g for 15 minutes, at 2° – 8° C. The result show 3 phase: liquid phase has no color in the upper site contain RNA, interphase and organic phase in the bottom contain DNA and protein.

2.4. RNA isolation

Upper phase with no color was remove into new microtube. Other phase and bottom phase were kept in – 20° C. Clear phase was added with 0,5 isopropanol, closed, and follwed by gentle mixing 15 times. After that microtubes were incubated for 10 minutes at 25° C to precipitated the RNA. This process followed by centrifugation for 10 minutes at 12000 g, 2° C, and then the supernatant were removed. Precipitate was added 1 mL 75% Ethanol. RNA precipitate in Ethanol isolated by vortex, and precipitated by centrifugation for 5 minutes at 7500g, 2° C, and the supernatant was removed. Further more the pellet let to be half dried. After reach half dried, the RNA precipitate was resuspended into 50 μ L RNA free water that contained DEPC (*DEPC-treated RNase-free water*). The pellet resuspended by pippeting several times, and then incubated for 15 minutes, at 55°-60° C [7].

2.5. DNA isolation

For DNA isolation, the inter and bottom phase was added with 0,3 mL 100% Ethanol into micro tube, and then mixed gently for 15 times. After mixing micro tube were incubated for 3 minutes at 25° C. For DNA precipitation, micro tubes were centrifuged for 5 minutes at 2000g, 2° C. Supernatant were removed into new micro tube and kept at -20 ° C for protein isolation. Precipitate washed by adding 1 mL of Na-citrate in 10% Ethanol into each micro tubes, and then samples were incubate for 30 minutes at 25°C, while mixing several times. After that micro tubes were centrifuged at 2000 g, for 5 minutes 2°C, and the supernatant were taking away. This washing was repeated 3 times. DNA precipitate as result was washed with 75% Ethanol, and then incubated for 10-20 minutes at 25°C while mixing. The following procedure was centrifugation at 2000 g for 5 minutes at 2°C, and the DNA precipitate was dissolve with 0.3 mL 0.8 NaOH [8].

2.6. Malondialdehyde measurement

Malondialdehyde as end product of lipid peroxidation will reacted with Tyobarbituric acid at 100°C produced substance with pink color and gave absorbance at 530 nm. Materials consist of kidney

homogenates, 20% trichloroacetic acid (TCA) solution, 0.67% tyobarbituric acid (TBA) solution, diluted tetraethoxypropan standards (1: 80.000). Equipments: centrifuge, balance, micro pipettes, tips, spectrophotometer, glassware. Protocols: standards dilution were made firstly before measurement, to produce standard curve. Standards and samples reacted with 1 mL 20% TCA, and mixing by vortex. It followed by centrifugation at 3000 rpm for 10 minutes. Supernatant (liquid phase) was taken and reacted with 2 mL 0.67% TBA, incubated for 10 minutes at 100°C, in water bath. After cooling down in ice box, absorbance were measured at 530 nm [9].

2.7. Renin mRNA relative expression by Real Time PCR.

Primer design: Primer of HIF-1 α and renin were created using Primer 3. The sequences were obtained from Gene-Bank NCBI with code NC_005105.2. for HIF-1 α , and NC 005112 for renin. Amplification of cDNA: The kit was used: *iScript One-Step RT-PCR Kit with SYBR Green* (BioRad®), primer of HIF-1 α and renin, micro-tubes of RT-PCR, blue, yellow and white tips. Protocols: cDNA amplification was done using RT-PCR with CFX program (MiniOpticon BioRad®). *iScript One-Step RT-PCR Kit with SYBR Green* (BioRad®) protocols: 1) cDNA synthesized for 10 minutes at 50°C. 2) iScript Reverse Transcriptase was activated at 95°C. 3) PCR cycles was 40 cycles was run for 10 seconds at 95°C; 30 seconds at 59°C (optimized first); 30 seconds at 72°C. 4) Melting curve analyzing for 1 minute at 95°C; 1 minute at 55°C; 10 seconds at 55°C; 80 cycles, increased 0.5°C each cycle. Aquabidest was used as negative control. Beta –Actin used as reference. Results calculated to obtain mRNA relative expression was used the following formula Pfaffl and Livak:

$$\text{Ratio (Pfaffl)} = \frac{(E_{\text{target}})^{\Delta C_t^{\text{target}}}}{(E_{\text{ref}})^{\Delta C_t^{\text{ref}}}}$$

target (calibrator – test) ref (calibrator – test)

Target was HIF-1 α or renin gene; Test was hypoxia kidney; calibrator was control kidney; reference was beta-actin gene; E-target was gene of HIF-1 α or renin efficiency; E-ref was beta-actin gene efficiency; and $\Delta C_t^{\text{target}} = [C(t)\text{calibrator gene of HIF-1}\alpha \text{ or renin}] - [C(t)\text{test gene of HIF-1}\alpha \text{ or renin}]$; $\Delta C_t^{\text{ref}} = [C(t)\text{calibrator gen beta-aktin}] - [C(t)\text{test gen beta-aktin}]$

Formula of Livak:

$$\text{Ratio (Livak)} = 2^{-\Delta\Delta C_t}$$

$\Delta\Delta C_t$ was ΔC_t test - ΔC_t calibrator; ΔC_t test was C_t target gene - C_t reference gene; ΔC_t calibrator was C_t target calibrator gene - C_t referens calibrator gene; Test was hypoxia kidney, Calibrator was control; Target was HIF-1 α or Renin; reference gene was beta-actin

2.8. ELISA.

This method was optimized by titration of antigen: 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, 1/1024, 1/ 2048, 1/4096. Protocol: Kidney homogenates were measured for protein concentration first, dilution with carbonate –bicarbonate buffer with pH=9.8 (coating buffer). Two hundred μ L sample with twice dilution was added into first well of micro-plate, and then diluted by moving to the next well 100 μ L that already filled with 100 μ L coating buffer, it was continued until the last well. At the last well, after mixing, 100 μ L of liquid at the last well was remove away. Micro-plate was incubated over night at 2°C, and shake. Microplate wells were wash 3 times with 200 μ L PBS=tween 20 pH 7.2. After that wells were blocked with 10% bovine serum albumin, followed by reaction with specific antibody anti renin/ HIF-1 α (Santa Cruz). Further, second antibody labelled with peroxidase (1:2000) was put into wells. The last was putting substrate ABTS, and it let to stay for 10 minutes followed by absorbance measurement at 405 nm [10].

3. Result

3.1. Rats induced to hypoxic state.

Rats were induced to hypoxic state at 1 day, 3 day, 7 day and 14 day using combination of 10% oxygen and 90% nitrogen. The results included increasing of HIF-1 α and renin protein, and renin mRNA relative expression.

3.2. HIF-1 α mRNA relative expression during hypoxia

During hypoxia HIF-1 α mRNA increased until 7 day but decrease in 14 day, but was still higher than control (Figure 1).

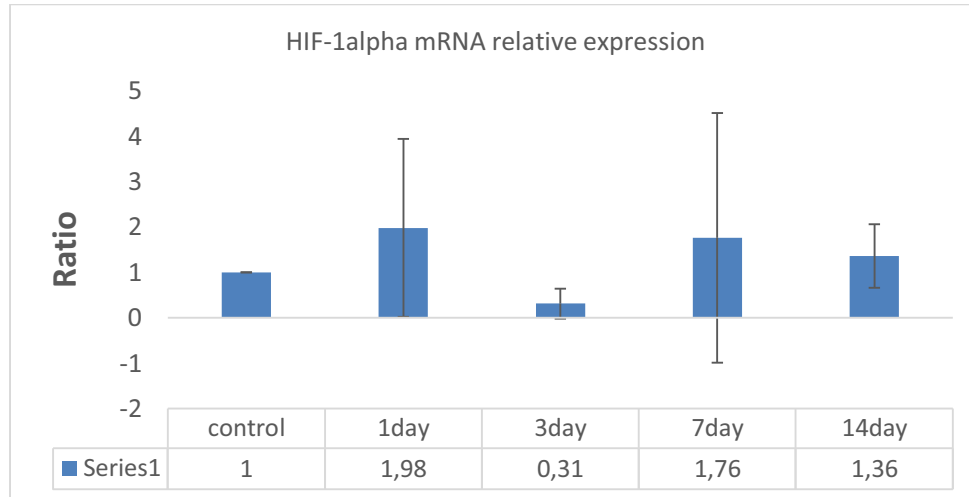


Figure1. HIF-1 α mRNA relative expression in the kidney tissues during hypoxia. HIF-1 α mRNA relative expression, measured using RT-PCR give result in ratio. In all group HIF-1 α mRNAs were increased except 3day group show the lowest level.

3.3. HIF-1 α protein level during systemic hypoxia

In hypoxia HIF-1 α should be increased, we found in the hypoxia-treated rat kidney compare to normal control show an increase of HIF-1 α level, but the differences were not significant ($p > 0.05$) (Figure 2).

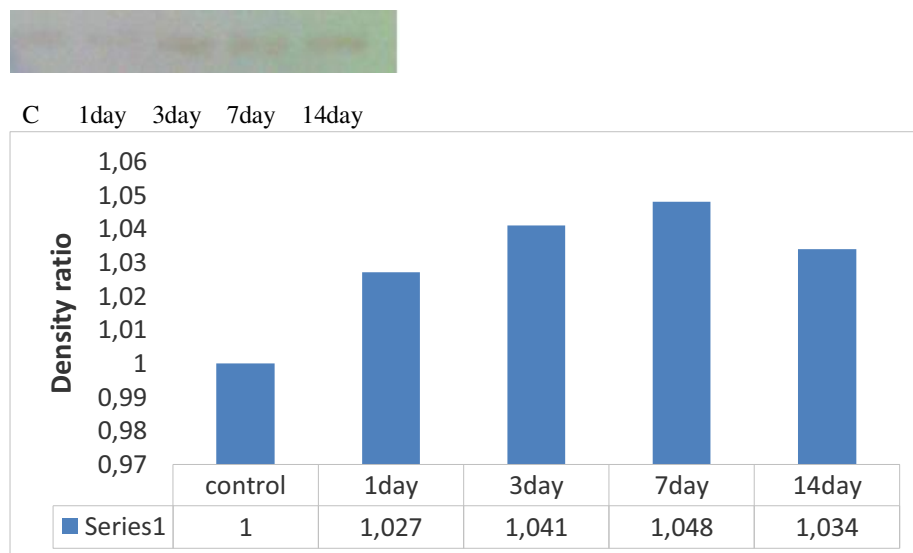


Figure 2. Western blot of HIF-1 α during hypoxia and control. C= control group or normal oxygen level; 1day= 1 day hypoxia rat kidney tissue; 3day= 3 day hypoxia rat kidney tissue; 7day= hypoxia rat kidney tissue; 14day= 14 day hypoxia rat kidney tissue. Western blot examination showed, all Groups expressed HIF-1 α in different intensity (above). The intensities (photo shop program) measured showed not significant different ($p > 0.05$) (below).

3.4. Renin mRNA relative expression and protein density in rat hypoxia kidney

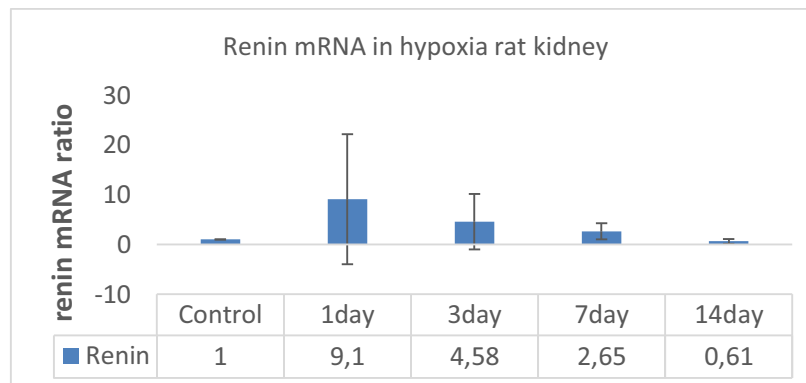
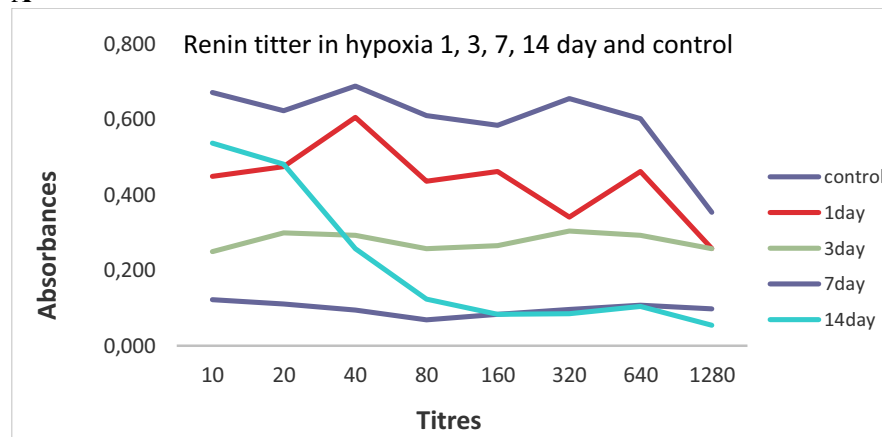


Figure 3 Renin mRNA relative expression in rat hypoxia kidney. Renin mRNA relative expressions were increased markedly in 1day and decreased gradually until 14day. 1, 3, and 7day groups were still higher than control, except 14day group.

A



B

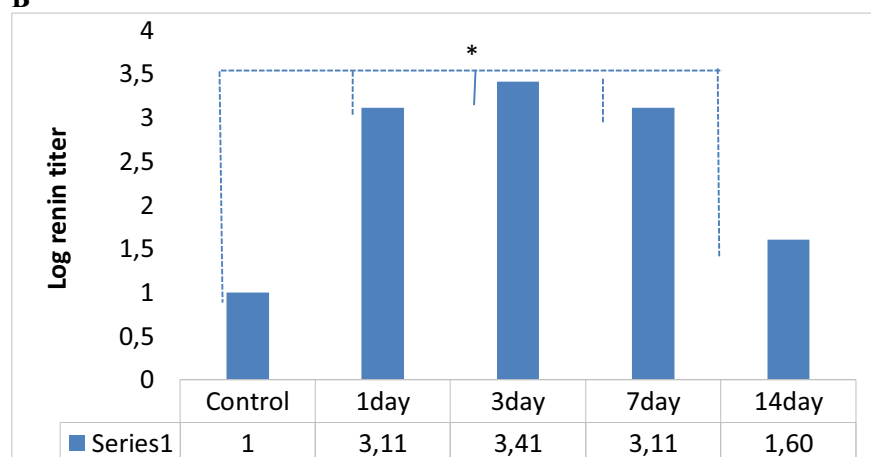


Figure 4. (A) Tittre of renin (B) Log titer of renin protein during hypoxia. Control: control group (normal oxygen level). The renin mRNA relative expression and the titer of renin were increased during hypoxia, and reach peak at 3day. Both of mRNA or protein were in similar pattern. Pierson correlation between mRNA relative expression and renin titer is $R^2 = 0.802$.

Relative expression of renin mRNA showed some increase in hypoxic state of 1day, 3day, and 7day, but in 14 day decrease below normal control. In addition, renin titers also increase in treated groups compared to normal control ($p < 0.05$) (figure 3). The correlation between HIF-1 α and renin mRNA, $R = 0.581$ $p = 0.305$ (Figure 4). There was a not significant correlation between HIF-1 alpha protein and renin mRNA relative expression. Otherwise, the correlation between HIF-1 alpha protein and renin protein showed strong correlation, $R = 0.77$ (figure 5).

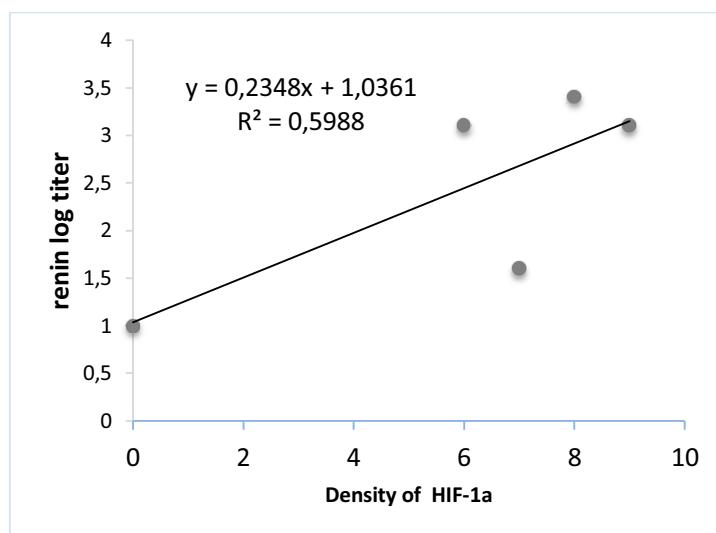


Figure 5. Correlation between HIF-1 α intensity and Renin titer. HIF-1 α protein synthesis and stability were followed by the increase of renin mRNA relative expression with correlation $R^2 = 0.599$.

3.5. Oxidative stress marker: Malondialdehyde (MDA)

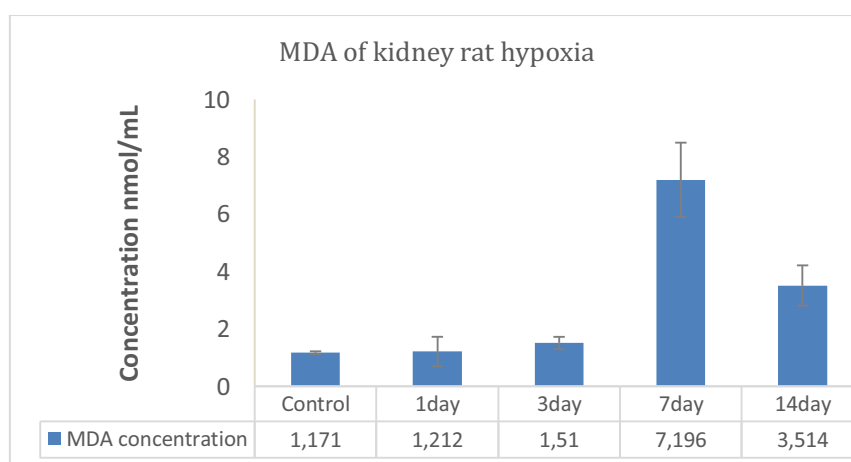


Figure 6. Malondialdehyde concentration (nmol/mL) in kidney tissue of systemic hypoxic rats. 1day=1day hypoxia, 3day= 3 day hypoxia, 7day= 7 day hypoxia, 14day= 14 day hypoxia group. ANOVA showed; 7 and 14day hypoxic kidney tissue MDA concentrations were higher than control, 1day hypoxia, and 3day hypoxia $p < 0.05$.

Correlation between MDA and HIF-1alpha mRNA

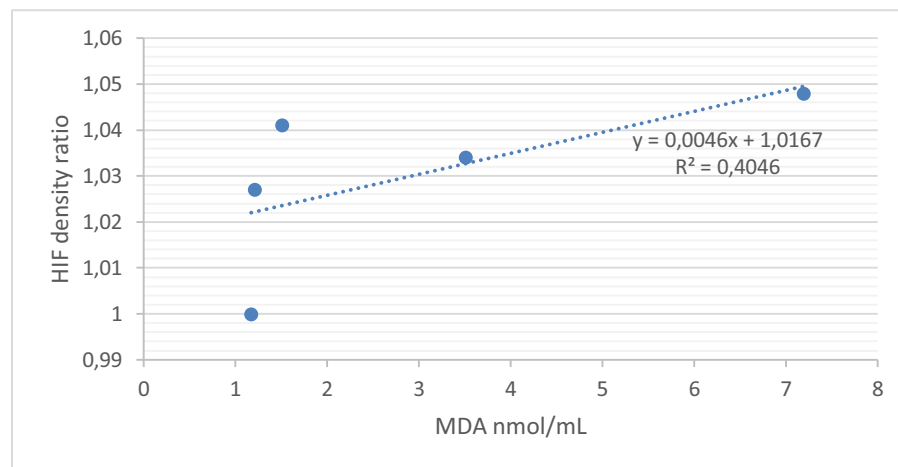


Figure 7. MDA and HIF-1alpha correlation

Correlation between MDA and HIF-1alpha show $R=0.64$, strong correlation

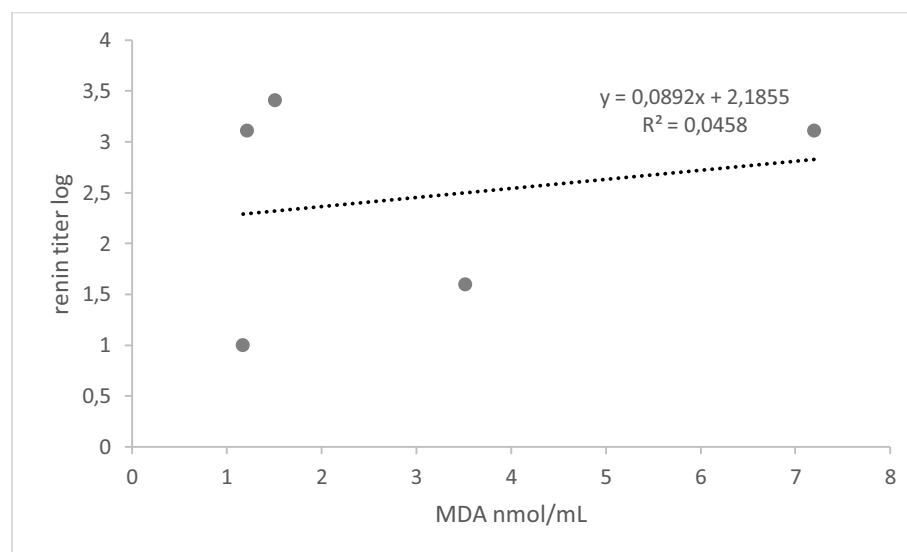


Figure 8. MDA and renin titer correlation. There is no correlation between MDA concentration and renin protein (log of titer).

4. Discussion

Rats induced to hypoxic state.

Hypoxic state treatment showed us an increase of HIF-1 α and the increase of renin mRNA and protein. During hypoxia, synthesis and stabilization of HIF-1 α proceed together, through PI3K/ERK, Akt, mTOR can trigger HIF-1alpha transcription.[11].

The accumulation of HIF-1 followed by the increase of renin mRNA and protein, but it did not mean that only HIF-1 activate the transcription of renin gene. It is already understood that renin secretion can be triggered by Chloride ion in kidney tubular detected by jugstglomerular cells (JGCs), on the other hand renin secretion can be stimulated directly by sympathetic nervous to kidney, through beta-adrenergic receptor on JGCs.[12]

In this study, we found that oxidative stress marker, malondialdehyde showed strong correlation to HIF-1 α protein (figure 7). We consider that systemic hypoxia triggers oxidative stress and also increased of transcription factor of hypoxia, HIF-1 α . In addition, there was a strong correlation between HIF-1 α and renin, $R=0.77$ in systemic hypoxia. This result show similarity with study of inhibition of prolyl hydroxylase using CoCl_2 that produced HIF-1 α stability and caused increased of renin expression.[5] It is considered that hypoxia strongly correlated with renin expression in kidney. Synthesis of HIF-1 α can be stimulated through tyrosine kinase receptor signaling. Previous study shows that OH^\bullet (hydroxyl) radical or ROS stimulate signaling of tyrosine kinase receptor.[13] Because OH^\bullet radical is difficult to measure caused by it has short live, therefore we measure the surrogate marker of stress oxidative (malondialdehyde), and it present that the increase level of MDA correlated strongly with HIF-1 α protein expression.

Malondialdehyde was known as the end product of lipid peroxidation process, damaged of lipid membrane caused by free radical attack. In systemic hypoxia, there were several sources that produced free radical during hypoxia. Mitochondria will be influenced during hypoxia, the flow of electron in respiratory chain disturbed caused by decreased of Oxygen as acceptor of the electron, the break pushed electron flow out of the respiratory chain and attack oxygen molecule reside.[14] This condition produce superoxide free radicals. Superoxide free radical will be reduced by superoxide dismutase to release hydrogenperoxide. Hydrogenperoxide will be reduced by catalase and glutathione peroxidase to produce water and some oxygen. If the amount of endogenous antioxidant capacity was not enough to overcome the excessive free radical, it could release excessive amount of hydroxyl radical from hydrogen peroxides.[15] Therefore, higher amount of hydroxyl radical lead to increase of HIF-1 α synthesis. Other source was Xanthine oxidase activity that increased during systemic hypoxia due to increase the rate of ATP degradation instead of the metabolic change to be anaerobic glycolysis.[16]

5. Conclusion

First, this research showed that in prolong systemic hypoxia the kidney could accumulate HIF-1 α , increase of MDA and renin expression. In addition HIF-1 α lead to increase of renin mRNA and protein expression. Secondly, we found that MDA and HIF-1 α had strong correlation, otherwise there was no correlation between MDA and renin instead of HIF-1 has strong correlation with renin expression.

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