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RESEARCH ARTICLE

CYTOGLOBIN EXPRESSION IN RAT KIDNEY DURING EXPOSURE TO SYSTEMIC CHRONIC HYPOXIA

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ABSTRACT

Background: The kidneys in physiological conditions are always in a state of relative hypoxia. Cytoglobin (Cygb) is the newest globin protein found of the globin family. One of the functions of Cygb is in oxygen supply. Cygb expression is found to increase in hypoxic conditions, which are thought to be an adaptation response to hypoxia.

Objective: This study aimed to analyze the expression of Cygb in rat kidneys which were exposed to chronic systemic hypoxia.

Methods: Twenty five male Sprague-Dawley rats, weighing 150-200 g were used in this experiment. Rats were divided into 5 groups: The control group was exposed to normoxia; the hypoxia groups (10% oxygen / 90% nitrogen) for 1 day; 3 days; 7 days and 14 days. After treatment, rats were sacrificed and their kidneys were taken. Cygb mRNA expression was measured by qRT-PCR, while Cygb protein expression was measured by the ELISA method.

Results: The expressions of Cygb mRNA and protein were found to be highest on day 3 of hypoxia and was correlated very strongly and significantly ($r2 = 0.96$; $p < 0.05$).

Conclusion: The highest expression of Cygb on day 3 of chronic systemic hypoxia exposure is suggested as an attempt to restore oxygen supply to the kidneys.

Keywords : cytoglobin mRNA, cytoglobin protein, kidney, systemic chronic hypoxia

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INTRODUCTION

Actually, the kidneys, per mass unit receive higher blood flow than the other tissues. The blood supply to the kidneys is more than 80 mL/min/100 g tissue, while the oxygen fraction extracted by all organs is lower than the kidneys.[1] But, as a consequence of the arrangement of blood vessels in the kidney which form a parallel arrangement between the renal arteries and veins, causes the oxygen to diffuse out directly from the arterial system to the venous system before it reaches the capillaries. It caused the partial pressure of oxygen (pO_2) in the cortex of kidney only reaches 25-50 mmHg and not exceed than 10-25 mmHg in the medulla of kidney. [2,3] This means renal medulla has a lower pO2 than the renal cortex. Due to low pO2 in the kidney, the kidney obtain energy mainly from anaerobic system. Thus, the kidney cortex is more susceptible toward hypoxia compared to the medullas which are adapted to hypoxia condition.

Body adaptation toward hypoxia has been explored in many research studies in recent years. Previous research found that there is protein called hypoxia inducible factor-1 (HIF-1) that plays a role in adaptation to changes in O2 level. [3-5] One of the globin protein that might have a role as a carrier protein is cytoglobin (Cygb).[6-9] The role of Cygb so far has not known clearly yet. Cygb is supposed to function in O2 supply, as an antioxidant, antifibrotic, regulation of apoptosis and NO and nitric signaling.[10]

Jusman proved that the liver tissue of rats that has exposed to chronic systemic hypoxia showed that expression of Cygb can be regulated by HIF-1α.[11,12] It is one of the mechanisms of adaptation to chronic systemic hypoxia to maintain a state of homeostasis.

Another study conducted by Prijanti has shown that kidneys that experienced hypoxia will express HIF-1α.[13] Therefore, the question arises whether the kidney tissue of rats that experience chronic systemic hypoxia would cause an increase of Cygb expression in the kidney tissue as an adaptation to improve the oxygen supply to the kidney tissue. This study aims to analyze the expressions of Cygb mRNA and protein in the kidney tissue of the rat which are exposed to the systemic chronic hypoxia.

MATERIAL AND METHODS

This was an in vivo experimental study using twenty-five male Sprague-Dawley rats, weighing 150-200 g. Rats were divided into 5 groups: the control group (normoxia); hypoxia groups (10% oxygen/90% nitrogen) for 1 day; 3 days; 7 days; and 14 days. Kidney organs were collected after the rats were sacrificed. The protocol was approved by Ethical Committee of Center Research and Health Development, Ministry of Health Republic Indonesia (Balitbangkes RI) No. LB.032.02/KE/4783/08.

The materials used for this research are the kidneys of twenty-five male rats (Rattus sp. Spraque-Dawley strain) which are exposed to chronic systemic hypoxia treatment. While the consumable items used are special gas mixtures in gas tank that containing 10% oxygen and 90% nitrogen (PT Samator, Jakarta); Tripure isolation kit (Roche); PBS 0.1 M pH 7.4; DEPC (DEPC-treated RNase-free water); iScript One-Step RT-PCR with SYBR Green (Cat. No. 170-8893, BioRad Laboratories, Hercules, CA, USA); Cygb primer and PUM primer as housekeeping gene; RNA template (used 100 ng) and

nuclease-free water. A series of BSA standard solution (0; 0.2; 0.4; 0.6; 0.8; and 1mg/mL); Enzyme-Linked Immunosorbent Assay Kit for Cygb; 10 nM Tris buffer with pH 8; distilled water; rats' food and water; sawdust; animal cage; dry ice and ice.

Preparation of kidney tissue homogenate

Kidney tissue was homogenized with 0.1 M PBS pH 7.4 (1:10 dilution), centrifuged for 10 min at 4oC, then collect the supernatant.

Total RNA isolation

The total RNA was isolated from homogenate of rat kidney tissue using Tripure isolation kit (Roche).

Measurement of the relative expression of Cygb mRNA

Measurement of Cygb mRNA was done using iScript One-Step SYBR Green (BioRad, Cat. no 170-8893). The primer for Cygb was designed using primer3 based on GeneBank NM_130744.2. The sequences of the Cygb primers are as follows: F: 5 'ACC TGC AGA ATG ACC CAG AAC 3'; R: 5 'GGA AGT TGG CAT CCC ACT CAA 3'. Meanwhile, for the PUM primers (housekeeping gene) are as follows: F: 5 'AGG ACA GCA GCA GGT TCT CCG T 3'; R: 5 'AGC AGC AGG CGG CAA CAC AT 3'.

Measurement of Cygb protein

Cygb protein was measured using Cygb ELISA Kit (Cat no. SEC426Ra, USCN).

RESULTS

The relative expression of Cygb mRNA in kidney tissue

The Cygb mRNA expression showed that cycle threshold (Ct) of Cygb and Pum have normally distributed in each group. Data has efficiency value = 2, that each group has the same values that have been determined by the CFX program used in RT PCR instrument.

Table 1. Efficiency and Ct of Cygb and housekeeping gene

Rat group	Pum		Cygb	
	Efficiency	Ct	Efficiency	Ct
K	2	34.032 ± 0.627	2	25.782 ± 0.325
H1	2	34.250 ± 0.579	2	24.702 ± 0.618
H3	2	33.750 ± 0.632	2	23.202 ± 0.632
H7	2	33.992 ± 0.580	2	25.150 ± 0.580
H14	2	33.253 ± 0.790	2	25.926 ± 0.790

The relative expression of Cygb mRNA using the Livak formula is shown in Figure 1. It showed that the expression of Cygb mRNA on day 3 was the highest (an increase of almost 5 fold compared to control) and also compared to hypoxia treatment on the day 1, day 7 and day 14. Cygb mRNA reaches its peak on day 3 and then decreased until the end of hypoxia treatment.

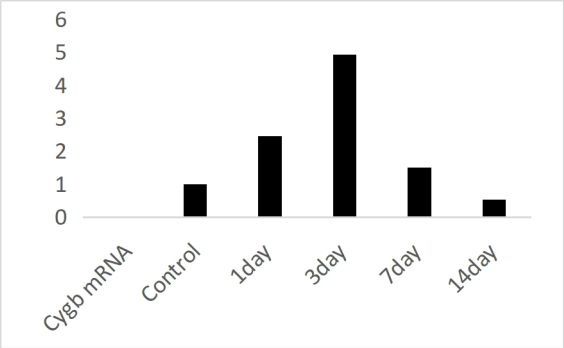

Figure 1. The relative expression of Cygb mRNA in kidney tissue of rats exposed to chronic systemic hypoxia for 1, 3, 7 and 14 days. (Data were normalized to control)

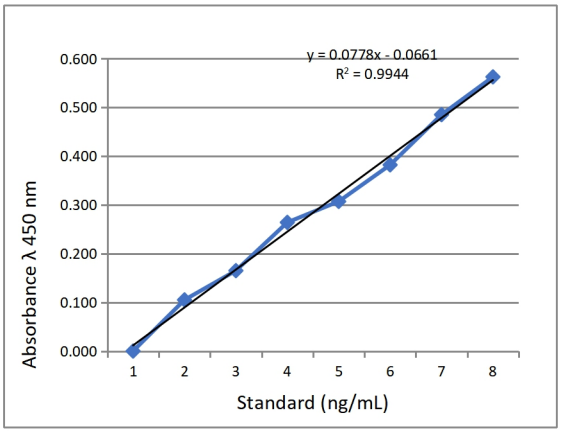

Figure 2. The standard curve of Cygb protein. The standard curve showed value R^2 =0.9944 with y = 0.0778x – 0.661.

Concentration of Cygb protein in kidney tissue

Concentration of Cygb was measured using a series of Cygb standard solutions (Figure 2). The Cygb concentration in kidney tissue showed a maximum level on the day-3 of hypoxia. (Figure 3.). Statistical analysis (ANOVA) showed significant different between the controlled group (normoxia) with hypoxic group day-3 and day-14 (p=0.04); between day-1 with day-3 and day-14 (p=0.040 and 0.001 respectively); between day-3 with day-7 and day-14 (p= 0.040 and 0.01 respectively); and between day-7 with day-14 (p=0.024)

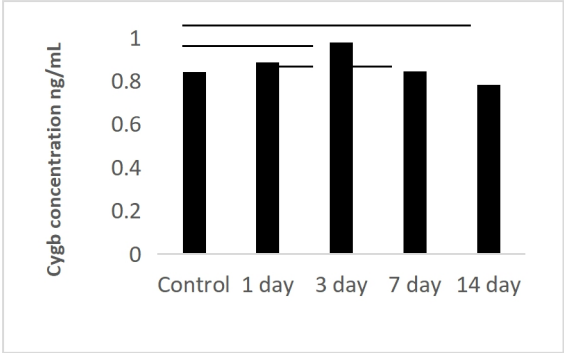

Figure 3. The concentration of Cygb protein in kidney tissue of rats exposed to chronic systemic hypoxia

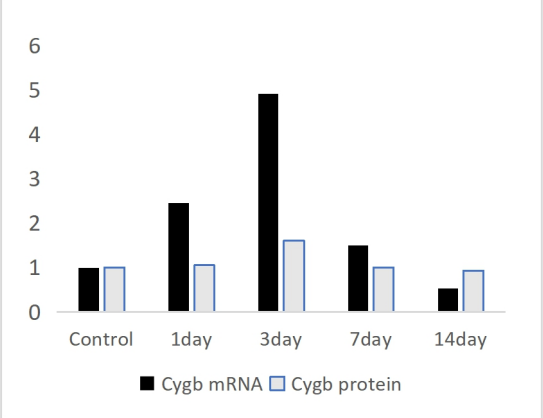

Figure 4. The comparison of Cygb mRNA with Cygb protein expression (Data were normalized to control group)

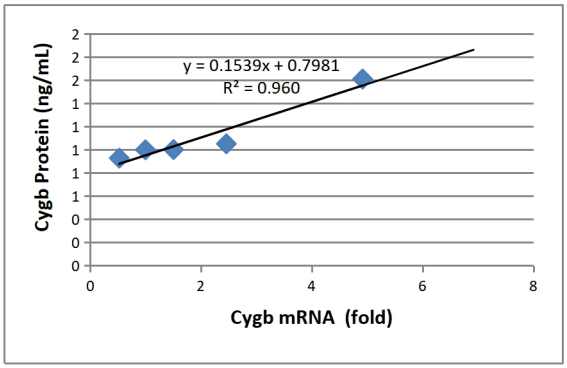

Figure 5. The strong and significant correlation of Cygb mRNA and protein in the rat kidney tissue on exposure to chronic systemic hypoxia ($p<0.05$; Pearson)

This study showed that the Cygb mRNA and Cygb protein in kidney tissue performed the quite same pattern, reached its peak on the day 3 after slightly increase on the day1 and after the day 3 it tends to

decrease during hypoxia treatment. Figure 4 showed comparison between the Cygb mRNA and protein. It was also showed a very strong correlation between Cygb mRNA and the Cygb protein (R2 = 0.96, p= 0.009) during the treatment of chronic systemic hypoxia (Figure 5).

DISCUSSION

The kidneys are organs that even in normoxic conditions tend to experience hypoxia. In normoxia, the renal cortex requires adequate supply of O_2 to optimize the glomerular filtration and tubular function in the secretion and reabsorption of compounds that are important to the body. The outer kidney medulla is part of the kidney which contains many tubules which are susceptible to damage due to regional hypoxia. While the inner medulla has better resistance to hypoxia. That means, the cortex, outer medulla, and the inner medulla have different responses to hypoxia. The inner medulla has better adaptation than the cortex and outer medulla.

Therefore the question arises, how is the kidney response to hypoxic conditions? One protein that plays a role in hypoxic conditions is hypoxia-inducible factor-1 (HIF-1). HIF-1 is a transcription factor that plays a role in regulating the expression of various genes for proteins which is responsible for the adaptation to hypoxia. One protein that is under the regulation of the HIF-1 protein is Cygb. [10-12]

This study found that Cygb mRNA expression in rat kidney tissue of rats which were exposed to systemic hypoxia (10% O_2 and 90% N_2) increased 4.9 folds on the day-3 of hypoxia compared to the control and decreased with continued hypoxia until day-14. This increase in mRNA Cygb expression is likely to be induced by HIF-1 protein which increased in hypoxic conditions. Jusman's research proves that Cygb can be induced by HIF-1α proteins in rat liver tissue that has been exposed to chronic systemic hypoxia.[11,12]

Prijanti also proved that there was an increase in HIF-1α mRNA and protein expression in cytoplasm of renal cortical tubule cells in sustained hypoxia by immunohistochemical examination. There is a strong correlation between expressions of HIF-1α mRNA and protein (Pearson; R2 = 0.907; p <0.05).[13]

Our study proved that Cygb mRNA and protein in kidney tissue increased significantly in the same pattern and had a very strong correlation (Pearson; R2 = 0.96; p=0.009), during treatment of chronic systemic hypoxia. Both Cygb mRNA and protein expression reached maximum values on the third day of hypoxia. It is assumed that cells undergo adaptation to control hypoxic conditions until day-3, while on the day-7 and day-14 hypoxia, mRNA and protein expression decreases. Even on the day-14, Cygb mRNA expression is lower than the control group. This is probably caused by majority ofcells have underwent apoptosis, therefore only a limited living cells expressed Cygb. This was supported by Winani's research which has proven that chronic systemic hypoxia increased kidney MDA level on the day-3 of hypoxia, which meant there was an accumulation of free radical damage.[14] Thus, the increase in MDA level in kidney tissue was in line with the relative expression of Cygb mRNA. It was suggested that the kidneys are adapted to cope the oxidative stress resulting from hypoxia.

Hendrawan et al. also proved that in rat myocardial cells exposed to chronic

systemic hypoxia, many cells experienced apoptosis on the day-14 of hypoxia.[15]

As it is known that the cortex and the outer part of the medulla are the parts of the kidney that are most susceptible to damage due to regional hypoxia, while the inside of the medulla can adapt due to chronic systemic hypoxia. Nishi et al proved that there was an increase of Cygb mRNA and protein expression and the number of cells that produce Cygb in the renal cortex during chronic systemic hypoxia. Increased Cygb mRNA expression can lead to increased synthesis of Cygb protein. [15]

Our study did not separate the cortex, the outer medulla and the inner medulla of kidney tissue. We suggested that the expression of Cygb mRNA and protein increased maximally on the day-3 of hypoxia, as a mechanism for overall kidney tissue adaptation to hypoxia. Most likely this adaptation begins with the stabilization of HIF-1 which has been proven by Prijanti that there was an increase in HIF-1 on day-3 of systemic hypoxia.[13] This HIF-1 will regulate the expression of Cygb mRNA which in turn will continued by increasing the expression of Cygb protein in an effort to increase O_2 flow to the tissue and overcome oxidative damage caused by hypoxia.[12,13] Jusman study showed that inhibition of HIF-1 alfa protein with ibuprofen will decrease the Cygb mRNA expression in fibroblast of keloid.[16]

In hypoxia after day-3, on the day-7 and continued on the day-14, there was a decrease in Cygb expression because many cells had undergone apoptosis, so they did not express Cygb.

CONCLUSION

We concluded that expression of mRNA and Cygb protein in kidney tissue which peaked on day 3 of hypoxia and decreased with continued hypoxia is an adaptation response to chronic systemic hypoxia.

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