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Original Research Article

Expression of HIF-1, VEGF-β and Caspase-3 in Myocardium of Rats Subjected to Hypoxic-Hyperglycemic Condition

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Abstract

Introduction: Studies of the protective effects of insulin on endothelial impairments are still very limited.
Aim: This study is intended to find out the effect of insulin injection on expression of HIF-1, VEGF-β, and caspase-3 in myocardial tissues of rats subjected to hypoxic-hyperglycemic condition.
Materials and methods: Wistar rats (n=30) were subjected to hyperglycemic by alloxan injection and be hypoxic by exposuring the animal to a normal and low oxygen tension alternatingly. The test animals then grouped into two. The first group was treated with insulin NPH, the second one was not.
Results: Results showed that expression of HIF-1 and caspase-3 did not significantly affected by insulin treatments. However, expression of VEGF-β was significantly increased by insulin injection.
Conclusion: In conclusion, insulin has potential to be used as a protective agent against endothelial impairments in rats experienced hypoxia and hyperglycemia.
Keywords: diabetes, hyperglycemia, hypoxia, HIF-1, VEGF-β, Caspase-3, myocardium.

1. Introduction

Hyperglycemia in diabetes mellitus has implications for many pathogenic mechanisms that affect endothelial function [1]. Hyperglycemia can directly induces apoptotic cell death in the myocardium cause both micro- and macroangiopathy that lead to coronary heart disease (CHD) [2,3]. On ischemic myocardium, the detrimental effect of hyperglycemic condition involved several mechanisms, including oxidative stress, inflammation, apoptosis, endothelial dysfunction, hyper coagulation, platelet aggregation and impairment of ischemic preconditioning [4]. This situation has made worth to assume that patients with diabetes are more likely to die from cardiovascular disease than that of non-diabetic [5].

Pathophysiological complications of diabetes are thought to be closely associated with hyperglycemia and hypoxia. It has been indicated that hyperglycemia and hypoxia are the risk factors to accelerate the onset and progression of various complication of diabetes [6]. In normal subjects the hypoxic condition will be responded by tissues by expressing hypoxia-inducible factor-1alpha (HIF-1α). However, in diabetes hyperglycemia impairs hypoxia-dependent protection of HIF-1α that defects response of the tissues to low oxygen tension [7, 8].

HIF-1α was known to mediate other transcriptional responses to low oxygen including expression of angiogenic, metabolic, and cell cycle genes [9]. Among regulators of angiogensis, vascular endothelial growth factor (VEGF) is one the targeted genes that is regulated by HIF-1α [10]. In individuals with cerebral ischemia, VEGF known to inhibit activation of caspase-3 so that protect neurons from hypoxic injury [11].

Hyperglycemia is caused by glucose metabolism defects and the hypoxic condition is considered to augment

the impairments in such metabolism. It is also suspected that hypoxia causes weight loss, increased insulin resistance, and low levels of leptin in diabetes [12]. In addition, hyperglycemia is one of the factors that can cause the destabilization of HIF-1α transcription which results in impairments of cell response to hypoxia [13].

Given that hyperglycemia is considered a major determinant of complications in diabetes, the use of insulin to control of glucose levels is considered [14]. Insulin treatment has been reported to protect cardiomyocytes from hypoxia-induced apoptosis in rats through sphingosine kinase 1/sphingosine 1-phosphate/S1P receptor axis pathway [15]. However, report on whether the insulin treatment in subjects with hyperglycemia and hypoxia is useful for improve the protective mechanism of endothelial impairment is still lacking.

Current research is aimed to investigate the effect of insulin on expression of HIF-1, VEGF, and caspase-3 in myocardial tissues of alloxan-induced hyperglicemic mice under low oxygen tension *in vivo*.

2. Material and Method

2.1 Experimental Animals

In this experiment, male Wistar rats aged 8 weeks, weighing between 150-200 g, obtained from Lampung Veterinary Center, Bandar Lampung, Indonesia were used. The animals were handled according to the Ethical Clearance from Faculty of Medicine, University of Lampung, Indonesia (Ethical Approval No. 1128/UN26/8/DT/2015). They were maintained under controlled temperature (23 – 25°C), 08:00 -20:00 light and 20:00-08:00 dark cycle, fed with a standard laboratory diet (granular pellet Type 521 from Charoen Pokphand Indonesia Ltd., Mojokerto, West Java, Indonesia) and water *ad libitum*.

2.2 Controlled Measures

In this experiment test animals were conditioned to experience hyperglycemia and hypoxia. The hyperglycemic condition of experimental animals was made by intraperitoneally injecting 10 ml of alloxan monohydrate (Sigma Aldrich, Cat. No. A7413-10G) at the dose of 120 mg/kg body weight, followed by high calories diet for 7 days. Before and after alloxan injection, bool glucose levels of each animal were measured using strip glucometer (from Roche, Germany).

Hypoxic treatment of the test rats was performed during sleeping hours of the animals, between 8:00 - 12:00 a.m, for three days successively. The procedure of exposure to induce hypoxia is carried out by alternately replacing the composition of the air inhaled periodically from normoxia (21% O_2) for 90 sec with hypoxia (5% O_2) for 30 sec.

2.3 Experimental Design and Treatment

Test animals (n=30) that were conditioned to be hyperglycemic and hypoxic were grouped into two, 15 rats each. Group 1 was set to receive Insulin Neutral Protamine Hagedorn (Insulin NPH), whereas group 2 was set to receive no insulin. Insulin injections were carried out during the treatment of hypoxia, three times a day at 10th, 13th and 19th hours, with consecutive doses of 1, 1, and 2 units.

2.4 Parameters Measured

After 72 hours of treatment, the mice were killed and the heart was taken for examination of HIF-1, VEGF-β, Caspase-3 expression in myocardium of the animals using immunohistochemical techniques. All experiment steps of this study lasted from April to May 2018.

2.5 Immunostaining

To detect expression of HIF-1, VEGF-β and Caspase-3 in myocardial tissue of the rats, a conventional immunohistochemistry technique was performed. In brief, four micron-thick sections were deparaffinized using xylol for 5 minuntes. Antigen retrieval by microwave for 5 min and aquadest was used to rinse the slides. Endogenous peroxidase activity was blocked using 0.3% H_20_2 in methanol for 20 minutes. After being changed three times by PBS for 5 minutes, the slides were incubated with polyclonal antibody, at 4°C, overnight.

To visualize expression of HIF-1, a HIF-1 Alpha Polyclonal Antibody (from Bioss Inc. USA, Cat. No. bs-0737R) was used. VEGF Polyclonal Antibody (from Bioss Inc. USA, Cat. No. bs-1665R) was used to visualize expression of VEGF-β. Whereas, Caspase 3 Polyclonal Antibody (from Bioss Inc. USA, Cat. No. bs-2593 R) was used to visualize Caspase-3.

After being incubated with Histofine Simple Stain Rat MAX PO (MULTI), from Nacalai, USA, Cat. No. 414191F for 60 min and washed three times with water, the slides were stained with diaminobenzidin (DAB) solution, 10 minutes at room temperature. After being washed three times with running tap water for 3 minutes, the slides were counter stain with Mayer's hematoxylin for 0.5 minutes.

2.6 Data Analysis

Given only two treatment groups were compared in this study, the data analysis was carried out using Student t test, at a significance level of 0.05.

3. Results

Baseline characteristics of experimental animals which include body weight, heart rate, blood glucose level before and after alloxan injection are presented in Table 1.

Table 1: Baseline characteristics of experimental (insulinized) and control (non-insulinized) rats

Variables	Values	
	Insulinized group (mean ± SD)	Non-insulinized group (mean ± SD)
Body weight (mg)	156.87 ± 8.11	161.07 ± 4.2
Heart rate (bpm)	160.53 ± 15.01	162.80 ± 8.48
Pre-alloxan blood glucose (mg/dl)	111.80 ± 7.06	114.53 ± 7.38
Post-alloxan blood gluocse (mg/dl)	174 ± 8.80	173.67±10.10

Insulin injection effects on the expression of HIF-1α, VEGF-β, and Casoase-3 in myocytes of hypoxia/hypergylicemic treated rats are presented in Table 2, 3 and 3 respectively. The Student's t statistical test applied to the data in Table 1 showed that the number of HIF-1α expressing myocytes in both test rat groups has no significant difference (p = 0.181). Photomicrographs visualizing the expression of HIF-1α in the myocytes of both rat groups are presented in Figure 1.

Statistical test (Student's t) applied to the data in Table 3 showed that the number of VEGF-β expressing myocytes in insulinized rats is significantly higher that of non-insulinized (p = 0.001). The expression of VEGF-β in the myocytes of both rat groups are visualized in micrographs presented in Figure 2.

Table 2: Effect of insulin injection on the expression of HIF-1α in myocytes of rats experiencing hyperglycemia and hypoxia

Treatments	N	Number of HIF-1 expressing cells (mean ± SD)	t-test (p-value)
Insulinized	15	63.33 ± 24.97	0.181
Non-insulinized	15	77.33 ± 12.79	

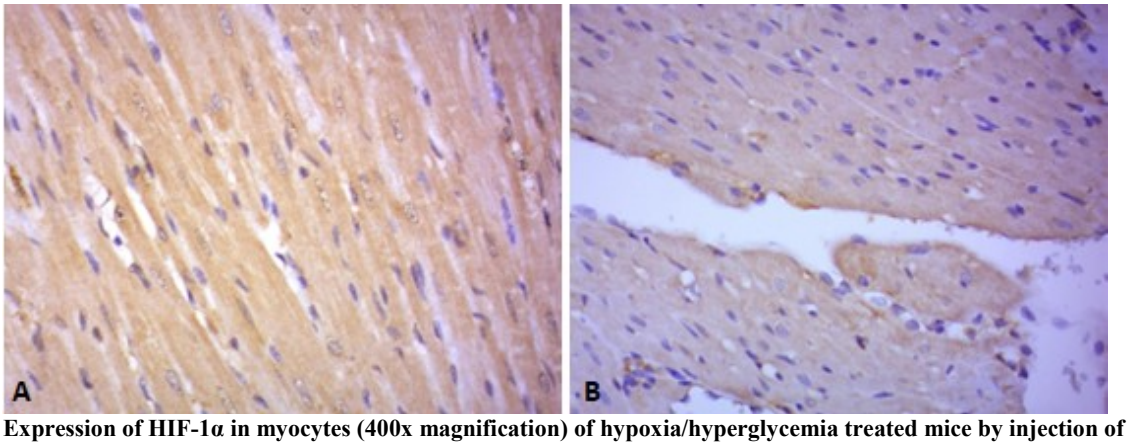

Figure 1: Expression of HIF-1α in myocytes (400x magnification) of hypoxia/hyperglycemia treated mice by injection of insulin (A) and without insulin (B)

Table 3: Effect of insulin injection on the expression of VEGF-β in myocytes of rats experiencing hyperglycemia and hypoxia

Treatments	N	Number of VEGF-β expressing cells (mean ± SD)	t-test (p-value)
Insulinized	15	37.33 15.80	0.001
Non-insulinized	15	13.67 ± 8.76	

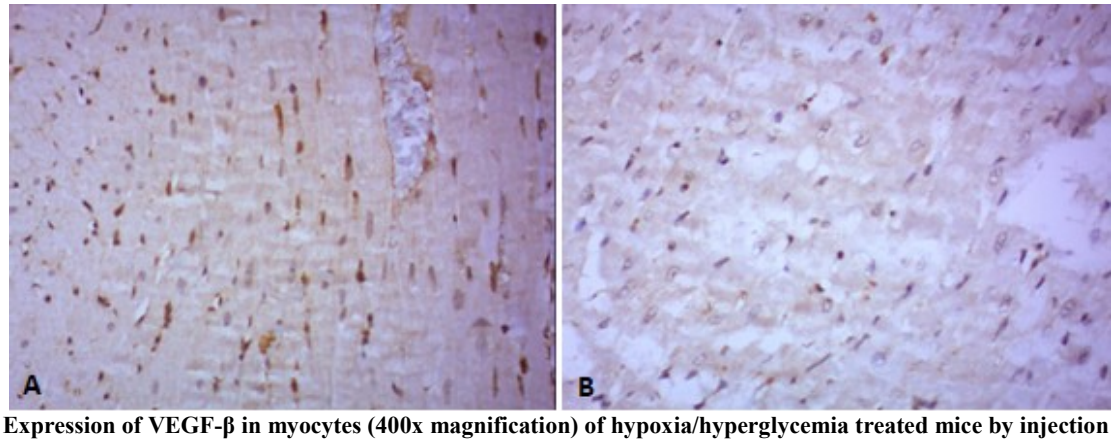

Figure 2: Expression of VEGF-β in myocytes (400x magnification) of hypoxia/hyperglycemia treated mice by injection of insulin (A) and without insulin (B)

Student's t-test performed in analyzing data in Table 4 showed that the number of Caspase-3 expressing cells in both insulinized and non-insulinized rats were not significantly different (p = 0.370). The expression of Caspase-3 in the myocytes of both rat groups are visualized in micrographs presented in Figure 3.

Table 4: Effect of insulin injection on the expression of Caspase-3 in myocytes of rats experiencing hyperglycemia and hypoxia

Treatments	N	Number of Caspase-3 expressing cells (mean ± SD)	t-test (p-value)
Insulinized	15	41.33 ± 24.75	0.370
Non-insulinized	15	58.00 ± 23.05	

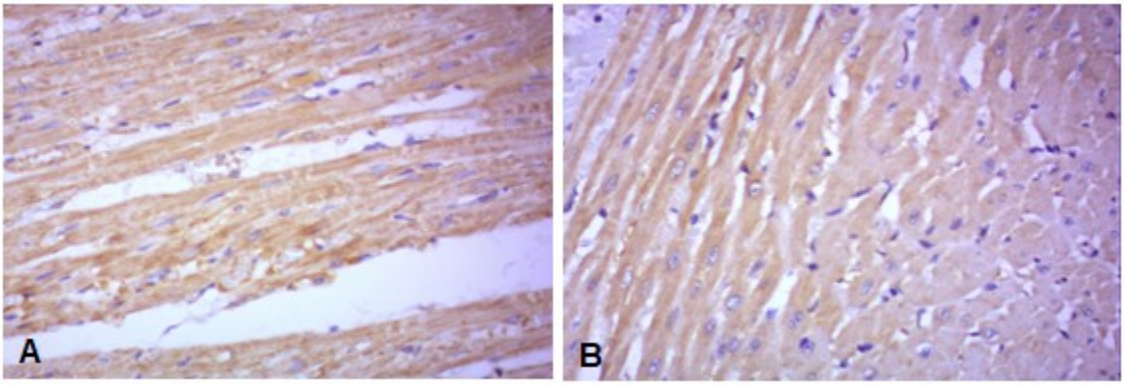

Figure 3: Expression of Caspase-3 in myocytes (400x magnification) of hypoxia/hyperglycemia treated mice by injection of insulin (A) and without insulin (B)

4. Discussion

Overall, current study showed that intermittent hypoxia exposure for 4 hours a day and alloxan injection to the test rats effectively increased expression of HIF-1 and Caspase-3, but reduced expression of VEGF-β in the myocardial cells of the animals. Furthermore, insulin injection to the hypoxic/hyperglycemic-treated rats showed no significant effects on the expression of HIF-1 and Caspase-3, but effectively in increasing expression of VEGF-B in myocytes of the animals.

Why do HIF-1 and Caspase-3 not respond to the insulin treatment? It may not be because the insulin is not effective, but the conditions of hypoxia and hyperglycemia that are not achieved as expected. In this experiment, blood glucose levels achieved by alloxan injection were 174 ± 8.80 mg/dl in insulinized group and 173.67±10.10 mg/dl in non-insulinized group. These levels of blood glucose may not be exactly categorized as hyperglycemia because in normal mice, blood glucose levels range 85 – 132 mg/dl [16]. In fasted rodent, blood sugar levels are normally <199 mg /dl [17].

In addition, in this study the duration of alternating exposure between normoxia (90 seconds) and low oxygen tension (30 seconds) only lasted 4 hours per day for 3 days. This too short exposure may not be enough to create hypoxia conditions in experimental myocytes. In fact, to create hypoxic conditions, Darna and colleagues (2017) do alternating exposure between normoxia and low oxygen tension up to 72 events per day (three minutes each event), for 7 days [18].

Although it is well known that low oxygen tension can induce the expression of HIF-1α, it is suspected that hyperglycemia and HIF-1α can influence each other. HIF-1α is known to induce the expression of glycolytic enzymes and glucose metabolism, causing glucose accumulation in cells, but high glucose is also known to inhibit HIF-1α expression [19].

Our data suggest that hypoxic/hyperglycemic condition has managed to increase expression of Caspase-3 in cardiomyocytes of the rats. However, given the elevated expression of Caspase-3 as depicted in Table 4 and Figure 3 was not significant enough, then the insulin injection to the animals has insignificant effects accordingly. As indicated previously, the exposure of experimental animals to low oxygen tension will increase the expression of caspase-3 [20], as an indicator of cell apoptosis, especially in cardiac myocytes [21].

Our results, as indicated in Table 3 and Figure 2 suggest that insulin injection to hypoxic/ hyperglycemic treated rats is managed to increase expression of VEGF-β. When cells experienced low oxygen tension, the expression of HIF-1 will increase that leads the releasing of VEGF-β. Furthermore, VEGF-β will be bound by VEGF-β receptors and trigger the tyrosine kinase pathway so that angiogenesis occurs. Such fenomenon was reported by Fan *et al* (2009) that an increase in VEGF-β was directly proportional to the increase in HIF-1 [22]. However, Oltmanns *et al* (2006) in their study found that VEGF-β levels will decrease after a 25% decrease in oxygen for 30 minutes [23].

Another explanation for the success of insulin injection increases the expression of VEGF-B because insulin has a direct influence on enhancing the transcription of VEGF-β genes [24]. Conversely, in the high-glucose condition, it was revealed that VEGF could inhibit the

secretion of insulin. Therefore it can be assumed that VEGF plays a role in the metabolism of glucose [25].

5. Conclusion

Although administration of insulin in this experiment did not show a significant effect on the expression of HIF-1 and Caspase-3, the insulinization is effective in enhancing VEGF-β expression in myocardium of rats experienced hypoxia and hyperglycemia. Thus, it can be concluded that insulin has pontential to be used as a protective agent against hyperglycemia-induced endothelial impairments.

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