

Relative hypoxia and oxidative stress in spleen lymphocytes of immunized Balb/c mice as indicated by HIF-1 α , HIF-2 α , Nrf2 expression, and glutathione peroxidase activity

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ABSTRACT

Background: Lymphocytes activated by immunization must increase their metabolism to meet the energy requirements for mitosis, differentiation, and protein synthesis, which may subject the cell to conditions of relative hypoxia and oxidative stress. This study was conducted to investigate the increase in the levels of transcription factors involved in both conditions.

Methods: Male Balb/c mice were divided into the following four groups, each consisting of six animals: the control and three experimental groups. The experimental groups were immunized by injection of 0.2 ml of 2% sheep red blood cells (SRBC) suspended in phosphate-buffered saline (PBS). Lymphocytes were harvested from the spleens of each group at time intervals of 24-, 48-, and 72-h post-immunization. The buffy coat from splenocytes was separated using Ficoll Histopaque as the medium. The lymphocytes were separated from adherent cells by incubating the purified splenocytes in microtubes for 2-h. Cells were lysed by three freeze-thaw cycles (-80°C and 37°C) and used to analyze the levels of HIF-1 α and HIF-2 α (mRNA and protein), Nrf2 (protein), and glutathione peroxidase (GPx) activity.

Results: The treatment caused an increase in GPx activity and HIF-1 α protein concentration 24-h post-immunization, whereas the HIF-1 α mRNA levels remained static. Elevated Nrf2 protein levels were detected within 48-h after treatment. Meanwhile, the HIF-2 α mRNA and protein levels increased within 72-h after immunization.

Conclusion: Immunization with SRBC suspension induced relative hypoxia, elevated reactive oxygen species (ROS), and oxidative stress in the lymphocytes as indicated by the increase in both HIF-1 α and HIF-2 α protein and mRNA levels, GPx activity, and Nrf2 protein levels.

Keywords: HIF-1 α , HIF-2 α , Nrf2, oxidative stress, relative hypoxia

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Immunization is a method to elicit a specific defense response by exposing the organism to a foreign substance. The role of leukocytes is essential in this phenomenon. The immune response is categorized into nonspecific and specific immunity. Specific immunity, which is the desired outcome of every immunization, is mediated by the mononuclear type of leukocytes, primarily macrophages or monocytes and lymphocytes, which are widely known as peripheral blood mononuclear cells (PBMCs). To elicit a specific immune response, both types of cells must perform various activities, i.e. migration, phagocytosis, synthesis of various cytokines, mitosis, and differentiation, and ultimately produce antibodies. Each activity requires vast amounts of energy, which is generally acquired through aerobic metabolism. If the oxygen supply does not match these metabolic demands, then the PBMCs involved in the immunization response are said to be in a state of relative hypoxia.¹⁻³

Almost all states of hypoxia disrupt several metabolic pathways in aerobic organisms. In response to hypoxic conditions, two transcription factors, hypoxia-inducible factor-1 (HIF-1) and hypoxia-inducible factor-2 (HIF-2), regulate a diverse number of genes that help aerobic organisms adapt to these harmful conditions.^{4,5} In addition, it has been reported that hypoxia leads to increased levels of reactive oxygen species (ROS).⁶ To counteract the ROS, cells possess a group of enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GRed) that quench the free radicals. These enzymes are regulated by another transcription factor, the nuclear factor erythroid 2-related factor 2 (Nrf2).⁷⁻⁹

This study was conducted to explore whether the specific immune response mediated by lymphocytes in response to sheep red blood cell (SRBC) immunization leads to increased expression levels of HIF-1 α , HIF-2 α , and Nrf2 as well as GPx activity due to increased metabolic activity.

METHODS

This was an experimental study conducted at the Laboratory of Molecular Biology for Oxidative Stress, Department of Biochemistry and Molecular Biology, Faculty of Medicine,

Universitas Indonesia, from October 2016 to April 2017. The animals were acclimatized for 2 weeks before treatment, followed by immunization and lymphocyte isolation. Splenocytes obtained from this procedure were used for analyzing the protein levels of HIF-1 α , HIF-2 α , and Nrf2 by enzyme-linked immunosorbent assay (ELISA); mRNA expression analysis of HIF-1 α and HIF-2 α by quantitative real-time reverse transcriptase PCR (qRT-PCR) method; and analysis of GPx activity by spectrophotometry.

Immunization and lymphocyte isolation

The animal model was obtained from Pusat Studi Satwa Primata, Bogor. This study was approved by the Ethics Committee of the Faculty of Medicine, Universitas Indonesia (No. 775/UN2.F1/ETIK/2016). Male Balb/c mice were randomly divided into the following four groups, each consisting of six mice: control, 24-, 48-, and 72-h. Mice from each group were injected intraperitoneally with 0.2 ml of 2% SRBC suspension. Furthermore, mice from each group were sacrificed after each period as indicated. Spleen samples were removed and homogenized with 500 μ l phosphate-buffered saline (PBS). Splenocytes suspended in PBS were layered on top of Ficoll Histopaque 1.084 and centrifuged at 1,500 rpm for 30 min. The buffy coat was removed and washed with PBS. The isolated mononuclear cells were resuspended in PBS and incubated for 1–2 h to separate nonadherent cells from adherent cells. The nonadherent cell fraction that primarily contains lymphocytes was extracted and stored at -80°C until they were needed for analysis. Before analysis, the lymphocytes were lysed by three freeze–thaw cycles as follows: the frozen samples obtained from the -80°C chamber were directly placed in a 37°C incubator for 5 min for thawing, after which they were immediately placed in -80°C for 5 min, and this cycle was continued three times.¹⁰

Total protein quantification

To quantify total protein, 2 μ l of the lysed samples were placed on a nanodrop plate, and the absorbance was measured at a wavelength of 280 nm. The absorbance was compared with the bovine serum albumin (BSA) standard curve to determine the total protein concentration.

Analysis of HIF-1 α , HIF-2 α , and Nrf2 proteins

The target proteins were quantified by ELISA¹¹ antibodies obtained from Elabscience

Table 1. HIF-1 α , HIF-2 α , and β -actin primer sequences

Gene	Primer sequence
HIF-1 α forward	5'-GCA CTA GAC AAA GTT CAC CTG AGA-3'
HIF-1 α reverse	5'-GCA CTA GAC AAA GTT CAC CTG AGA-3'
HIF-2 α forward	5'-CTC CAG GAG CTC AAA AGG TG-3'
HIF-2 α reverse	5'-CAG GTA AGG CTC GAA CGA TG-3'
β -actin forward	5'-CT AGG CCA ACC GTG AAA AG-3'
β -actin reverse	5'-ACC AGA GGC ATA CAG GGA CA-3'

(mouse HIF-1 α E-EL-M0687; mouse HIF-2 α E-EL-M0786; mouse Nrf2 E-EL-M2607).

Analysis of HIF-1 α and HIF-2 α mRNA relative expression

RNA from the cells was isolated by Tripure Isolation reagent (Ameritech, USA) using the β -actin housekeeping gene as control. The primers used in this process are listed in Table 1. The mRNA relative expression was analyzed using the procedure provided by One Step Kappa (KapaBiosystem). The PCR was run for 40 cycles. The annealing temperature was 52°C for the HIF-1 α and HIF-2 α primers and 54°C for the β -actin primer. Then, the Livak method¹² was used to determine the amount of mRNA in the sample. All the primer sequences were obtained from NCBI (HIF-1 α NM_001313919.1; HIF-2 α NM_010137.3; β -actin NM_007393.5).

Analysis of GPx enzyme specific activity

The Ransel (Catalog no. RS505)¹³ kit assay was used to quantify the GPx activity. The principle of this quantification method involves the spectrophotometric determination of the nicotinamide adenine dinucleotide phosphate (NADPH) concentration remaining after the first and third minute of reaction, measured as absorbance at 340 nm. The amount of NADPH measured reflects the activity of GPx in converting reduced glutathione (GSH) into oxidized glutathione (GSSG).

Statistical analysis

The result obtained from ELISA was the absorbance of each sample, which was then

converted into protein concentration through a standard curve. The amount of the desired protein was standardized to the total protein content. Data obtained from the GPx activity analysis were also in the form of absorbance, which were then converted into the amount of GPx enzyme using the equation provided in the protocol¹³ and also standardized to the total protein content of each sample. Meanwhile, the fold change of target mRNA relative expression to the housekeeping gene was measured by the number of cycle threshold (Ct) and converted into relative expression by the Livak method.¹² All data yielded from each assay were then evaluated for a significant difference by one-way analysis of variance (ANOVA) (for data that were normally distributed and homogenous), followed by a post-hoc least significant difference (LSD) to determine the group that has a significant difference, and the Kruskal–Wallis test (for data that were not homogenous and normally distributed).

RESULTS

Expression of HIF-1 α mRNA and protein

The relative mRNA expression of HIF-1 α showed a significant increase in the 48- and 72-h groups (Kruskal–Wallis test, $p=0.002$). In contrast, the protein expression analyzed by one-way ANOVA showed a significant increase in the 24-h group ($p=0.017$) compared to the control group and a significant decrease in the 48-h ($p=0.004$) and 72-h ($p=0.007$) groups (Figure 1).

Expression of HIF-2 α mRNA and protein

The relative mRNA expression of HIF-2 α was significantly increased at 24- and 72-h compared to the control group (Kruskal–Wallis test, $p=0.034$). The HIF-2 α protein expression increased significantly in the 72-h group as analyzed by one-way ANOVA ($p=0.036$) (Figure 2).

Nrf2 protein levels and GPx specific enzymatic activity

The Nrf2 protein levels were elevated in the 48-h group. Regarding the GPx specific enzymatic activity, there was a significant increase at 24-h post-immunization (one-way ANOVA, $p=0.002$) and a significant decrease after 48-h ($p=0.000$) and 72-h ($p=0.001$) of immunization (Figure 3).

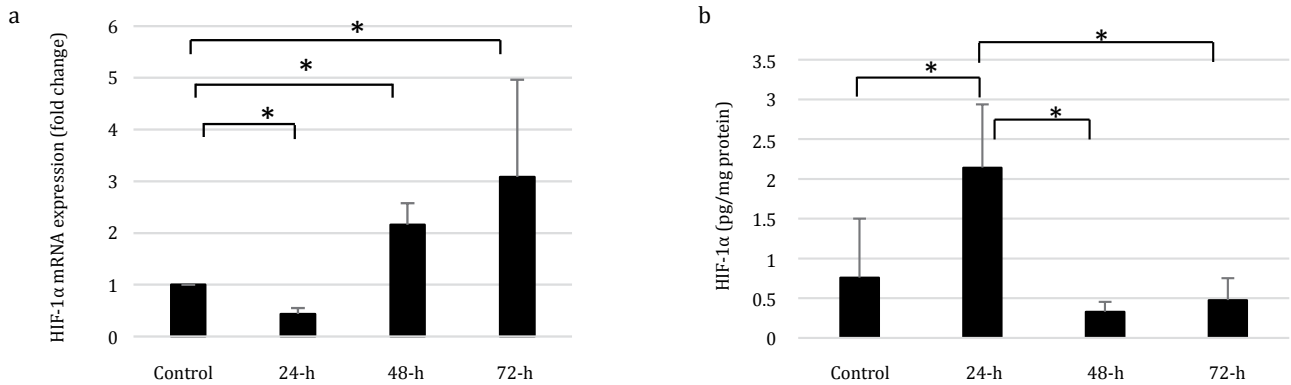


Figure 1. Expression of HIF-1 α in SRBC-immunized mice after 24-, 48-, and 72-h. (A) qRT-PCR was performed to determine the HIF-1 α mRNA relative expression after SRBC exposure. mRNA relative expression was compared to control, whereas the control value was arbitrarily set to 1. HIF-1 α mRNA relative expression was significantly increased two-fold after 48- and three-fold after 72-h of immunization relative to control group. (B) HIF-1 α protein analysis was measured by ELISA. Immunization promotes hypoxia, indicated by the elevated levels of HIF-1 α protein (2.143 pg/mg protein) after 24-h of SRBC exposure compare to control (0.759 pg/mg protein). * $p < 0.05$

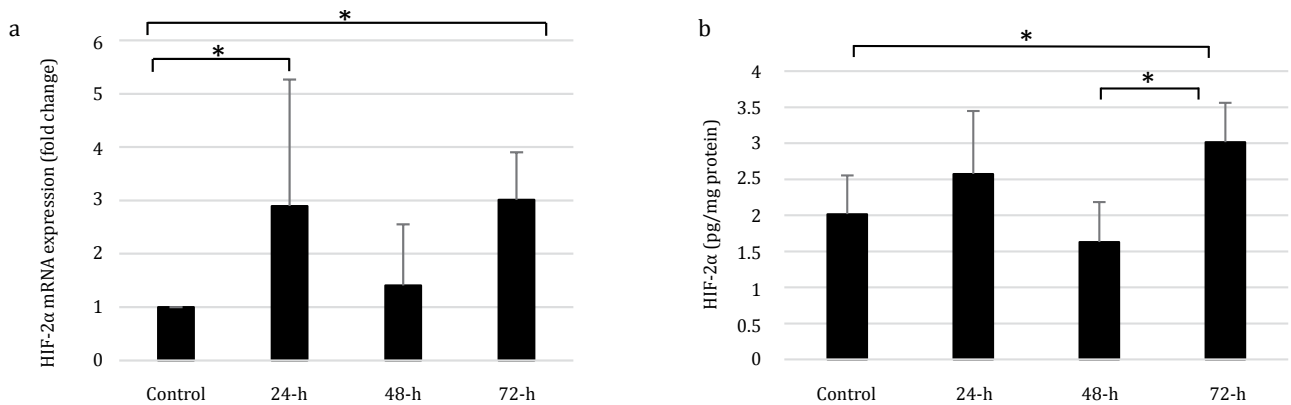


Figure 2. Expression of HIF-2 α in SRBC-immunized mice. (A) mRNA relative expression was determined by qRT-PCR method. Relative mRNA expression was compared to the control, which was set to 1. HIF-2 α relative mRNA expression was increased three-fold both in the 24- and 72-h groups, compare control group. (B) HIF-2 α protein expression was increased to 3.015 pg/mg protein after 72-h of treatment compared to control group (2.016 pg/mg protein) as evaluated by ELISA. * $p < 0.05$

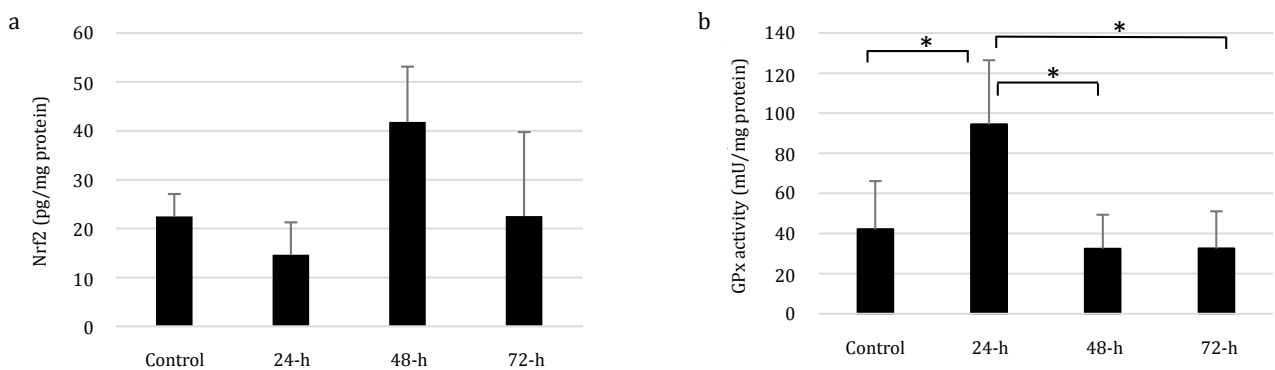


Figure 3. Nrf2 protein expression and GPx activity in SRBC-immunized mice. (A) Nrf2 protein expression was measured by ELISA, which showed an increase (41.8 pg/mg protein) 48-h post-immunization compare to control (22.49 pg/mg protein). (B) Spectrophotometry was used to determine the GPx activity, which showed an increase (94.5 mU/mg protein) after 24-h of immunization compare to control (42.14 mU/mg protein). * $p < 0.05$

DISCUSSION

The results of this study indicated that SRBC immunization induced a state of relative hypoxia in the lymphocytes after 24-h, as indicated by the increase in HIF-1 α protein levels. This phenomenon is caused due to the stabilization of the protein rather than *de novo* synthesis as indicated by the low mRNA levels. On the other hand, the relative expression of mRNA was elevated after 48- and 72-h of antigen exposure, which may be the result of *de novo* compensation of HIF-1 α levels in response to hypoxic conditions.^{3,14}

Based on these results, not all mRNA was translated to its corresponding protein or that it was translated but quickly degraded. Alternatively, report shown that the HIF-1 α protein expression can be regulated by its own downstream target gene.¹⁵ Thus, the elevation of HIF-1 α protein may due to the transcription of its own target gene.

The analysis of HIF-2 α mRNA and protein levels showed that the relative expression of mRNA was elevated 24- and 72-h post-immunization. Meanwhile, the protein levels increased 72-h after immunization. The increase in mRNA levels in the 24-h group is most probably a response to relative hypoxia. However, the mRNA was not translated immediately into protein, which may be due to the high levels of HIF-1 α protein.^{5,14} The HIF-2 α protein levels were increased after 72-h of immunization, which could be attributed to the role of HIF-2 α protein in prolonged hypoxia; thus, the 72-h period after immunization can be categorized as chronic hypoxia.^{14,16}

Previous studies have demonstrated that DNA synthesis in PBMCs reaches its maximum level at 48-h after stimulation with phytohemagglutinin A (PHA).^{17,18} In our study, we applied an immunization procedure using a complex antigen, i.e. SRBC suspension. A complete immune response includes not only the mitosis of both T and B lymphocytes but also the synthesis of immunoglobulins by B lymphocytes after their differentiation into plasmocytes. It is well known that plasmocytes can be easily observed under a microscope 72-h after immunization. At this stage, plasmocytes synthesize and produce antibodies, which require a vast amount of energy. At 72-h after immunization, there is a peak in HIF-2 α

levels, which indicates a state of chronic hypoxia. In addition, HIF-2 α is primarily expressed by differentiated cells.^{2,19}

HIF-2 α protein is known for its role in supporting the progression of the cell cycle, while HIF-1 α protein is known for its role in supporting the cell to enter the G0 phase (arrest). Elevated concentrations of HIF-1 α protein 24-h after immunization may be a response to oxygen scarcity. After 48- and 72-h, HIF-2 α protein initiates angiogenesis, vasodilation, and erythropoiesis to meet the lymphocytes' energy requirement for differentiation.¹⁹

Another study using SRBC-immunized mice was conducted on macrophages, which demonstrated that macrophages had increased levels of ROS as a result of the immune response to SRBCs.^{20,21} Moreover, in our previous study, we demonstrated that rats that were placed under hypoxic condition for an extended period of time experienced oxidative stress as indicated by the increase in malondialdehyde (MDA) levels and a decrease in GSH levels.²² We concluded that this paradoxical condition may be due to mitochondrial leakage and the high level of xanthine dehydrogenase activity. In the present study, we hypothesized that healthy lymphocytes under normoxic conditions, when immunized, will concurrently exhibit hypoxia and oxidative stress. We measured the levels of the transcription factor Nrf2, which regulates various antioxidant proteins such as GPx.

This study also confirmed that there was a peak in free radical production in the 24-h group, which could be due to increased metabolism of activated lymphocytes, exhibited by the elevation of GPx specific enzymatic activity. The Nrf2 protein levels were not increased during the 24-h time interval, presumably because the increase in free radical production can still be counteracted by existing GPx. However, 48-h after immunization, the lymphocytes were predicted to become active and required more energy, thereby leading to oxidative stress and ultimately the increased expression of Nrf2 protein.

The Nrf2 protein regulates the transcription of not only GPx but also other antioxidant enzymes such as catalase (CAT) that has the same substrate as GPx.^{7,8,23} In the 48-h group, the elevated concentrations of Nrf2 might also lead to the

transcription of CAT. Hence, the high concentration of free radicals was neutralized by CAT and the GPx enzyme specific activity also decreased.⁸

Lymphocytes also experienced oxidative stress as indicated by an elevation of Nrf2 protein levels after 48-h of immunization. This condition is a result of increased levels of oxidative respiration required to meet the continuous energy demands under hypoxic conditions. Therefore, the findings of this study suggest that hypoxia occurs before oxidative stress.

In conclusion, immunization of Balb/c mice with SRBC induced relative hypoxia in the lymphocytes 72-h after immunization, as indicated by the increasing levels of HIF-1 α and HIF-2 α mRNA and protein. Actively proliferating and differentiating lymphocytes produce high amounts of ROS after 24-h of immunization. These ROS were neutralized by the GPx enzyme.

Conflict of Interest

Sri W.A. Jusman is one of the editorial board members, but was not involved in the review or decision process of the article.

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