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FNIP-1 Expression in Mice Muscle Negatively Correlated with Myoglobin Level and Endurance Performance on Treadmill Run Test

La expresión de FNIP-1 en el músculo de ratones se correlaciona negativamente con el nivel de mioglobina y el rendimiento de resistencia en la prueba de carrera en cinta rodante

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Abstract. Previously, FNIP-1 was found to play a critical role in the coupling of mitochondria function to restore ATP. Mitochondria was need to be supplied sufficient oxygen by myoglobin in the myocyte. We proposed that FNIP-1 also regulated myoglobin levels to support endurance performance. This study aimed to investigate whether FNIP-1 expression in muscle was correlated with myoglobin level and endurance performance on the treadmill run test. Male adult balb/c mice performed a run on the treadmill for as long as possible. The duration of running was measured as endurance performance. Its calf muscles were analyzed for myoglobin level and FNIP-1 expression. Lower FNIP-1 expression in calf muscle was found on higher levels of myoglobin and the longer duration of running on a treadmill. The Pearson test showed that the P value was <0.05, which indicated that there was a correlation between FNIP-1 and myoglobin with a score of -0.77, which indicated that there was a negative correlation. The Spearman test also showed that the P value was <0.05, which indicated that there was a correlation between FNIP-1 and endurance performance with a score of -0.70, which indicated that there was a negative correlation. So, FNIP-1 expression negatively correlated with myoglobin level and endurance performance of mice. Further study needs to investigate the FNIP-1 expression in human muscle to look at its correlation with the level of lactate, oxygen saturation (SaO_2), oxygen fraction (FiO_2), and maximum volume of oxygen uptake (VO_2 max).
Keywords: FNIP-1, muscle, endurance, myoglobin, healthy lifestyle.

Resumen. Anteriormente, se descubrió que FNIP-1 desempeñaba un papel fundamental en el acoplamiento de la función de las mitocondrias para restaurar el ATP. Las mitocondrias necesitaban recibir suficiente oxígeno a través de la mioglobina en el miocito. Propusimos que FNIP-1 también regulara los niveles de mioglobina para apoyar el rendimiento de resistencia. Este estudio tuvo como objetivo investigar si la expresión de FNIP-1 en el músculo se correlacionaba con el nivel de mioglobina y el rendimiento de resistencia en la prueba de carrera en cinta rodante. Los ratones machos adultos balb/c corrieron en la cinta durante el mayor tiempo posible. La duración de la carrera se midió como rendimiento de resistencia. Se analizaron los músculos de la pantorrilla para determinar el nivel de mioglobina y la expresión de FNIP-1. Se encontró una expresión más baja de FNIP-1 en el músculo de la pantorrilla en niveles más altos de mioglobina y en una carrera más prolongada en cinta rodante. La prueba de Pearson mostró que el valor de P fue <0,05, lo que indicó que existía una correlación entre FNIP-1 y mioglobina con una puntuación de -0,77, lo que indicó que existía una correlación negativa. La prueba de Spearman también mostró que el valor de P fue <0,05, lo que indicó que había una correlación entre FNIP-1 y el rendimiento de resistencia con una puntuación de -0,70, lo que indicó que había una correlación negativa. Entonces, la expresión de FNIP-1 se correlacionó negativamente con el nivel de mioglobina y el rendimiento de resistencia de los ratones. Es necesario realizar más estudios para investigar la expresión de FNIP-1 en el músculo humano para observar su correlación con el nivel de lactato, la saturación de oxígeno (SaO2), la fracción de oxígeno (FiO2) y el volumen máximo de consumo de oxígeno (VO2 máx).
Palabras clave: FNIP-1, músculo, resistencia, mioglobina, estilo de vida saludable.

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Introduction

Mitochondria is an essential organelle responsible to regenerate the metabolic fuel of ATP. Mitochondria perform sequential metabolic reactions with the final result of ATP coupling (Hargreaves & Spriet, 2020; Sherwood, 2016). Previously, folliculin Interacting protein-1 (FNIP-1) played a critical role in the ATP coupling of mitochondria (Liu et al., 2016). The FNIP-1 expression regulated mitochondria biogenesis and aerobic performance of oxidative phosphorylation enzymes (Liu et al., 2016; Xiao et al., 2021).

Mitochondria need to be supplied continuously with sufficient oxygen to maintain the aerobic performance of oxidative phosphorylation enzymes (Costill, 2012; Wilson, 2017). Myoglobin is an iron carrier protein in muscle, who responsible to bind and transport oxygen into mitochondria (Kamga et al., 2012; Ordway & Garry, 2004). Since FNIP-1 expression regulated the mitochondrial coupling of ATP, we proposed that FNIP-1 expression in myocytes also correlated with the myoglobin level and oxygen supplied.

FNIP-1 was an inhibitory protein of AMPK signals for mitochondrial biogenesis and oxidative phosphorylation enzyme expression (Reyes et al., 2015; Wilson, 2017). High FNIP-1 expression was associated with the type-2 fast glycolytic muscle fiber development. Type-2 fast glycolytic muscle is characterized by a low level of myoglobin and insufficient oxygen availability in low endurance outcome individuals. Endurance primarily revolves around cardiorespiratory fitness (Suhadi et al., 2023). Since FNIP-1 expression was found constitutively in muscle, the development of type-2 fast glycolytic muscle set as default (Liu et al., 2016; Xiao et al., 2021).

On the contrary, aerobic performance and myoglobin level were associated with type-1 slow oxidative muscle fiber (Nemeth & Lowry, 1991). Muscle fiber type-1 myosin heavy chain (Myh7/Myh7b) gene expression regulated its development. Myosin heavy chain (Myh7/Myh7b) gene

expression resulted in intronic miRNAs i.e.: Myh7/miR-208b and Myh7b/ miR-499 (Liu et al., 2016). Recently, miR-499 was found to dis-inhibited FNIP-1 expression to release AMPK and improve mitochondrial function (Xiao et al., 2021). The presence of miR-499 downregulated the FNIP-1 expression to stimulate type 1 muscle fiber development (Smrkolj & Škof, 2013; Talbot & Maves, 2016; Xiao et al., 2021).

Since FNIP-1 under-expression was associated with the development of type 1 muscle fiber, the correlation of FNIP-1 expression with myoglobin level was proposed. Myoglobin was essential for the characteristic of type-1 muscle fiber but it was still unclear whether myoglobin was included in targets of Myh7b/ miR-499 actions. Endurance performance is an important basic component and must be considered (Contreras et al., 2023; Diaz-Ochoa et al., 2023; Kul et al., 2022). Discussion between FNIP-1 expression and endurance performance is still minimal and requires detailed discussion. This study aimed to investigate the correlation between FNIP-1 expression in muscle with myoglobin level and endurance performance among mice.

Materials and methods

Participants

Animals

This was an analytic observational study with a cross-sectional design using 36 (*Mus musculus*) balb/c mice obtained from the animal unit of physiology and biochemistry laboratory, Faculty of Medicine, Airlangga University, Indonesia. The inclusion criteria were healthy mice, male, 8 weeks old, and 25 grams of body weight. The sample size was determined using (Lemeshow et al., 1991). The equation is as follows:

$Z_{1-\alpha/2} = 1.96$
The proportion of positive FNIP-1 expression (p) = 75% (Xiao et al., 2021)
Limit of error (d) = 0.05
The population of mice in the laboratory in time (N) = 40

$$n = \frac{Z^2_{1-\alpha/2}\, p\,(1-p)\, N}{d^2(N-1) + Z^2_{1-\alpha/2}\, p\,(1-p)}$$

Mice were used as an experimental unit because it is similar to human genetic, physiology, and biochemical characteristics (Herrmann et al., 2019). Mice were housed under standard conditions – ambient temperature 24 ± 2 °C, humidity 55 ± 10%, 12 h light/dark cycle (lights on from 7:00 to 19:00) (Ruanpang et al., 2018; Sari et al., 2024). Exclusion criteria were given to mice that were found deformed, injured, or inflamed on the limbs (front and back). The dropout criterion is applied to mice that are unable to complete running activities on a treadmill with minimum time. Mice were acclimatized individually in a 10x10x10 cm cage for 7 days before the treadmill endurance test and were given food and water ad libitum. All protocols have been ethically approved by the Animal Care and Utilization Committee of the Faculty of Veterinary Medicine, Airlangga University in letter no. 3.KE.132.11.2021. This research was carried out fully in accordance to the ethical standards of the International Journal of Exercise Science (Navalta et al., 2019).

Protocol

Treadmill test

Columbus Treadmill was used for an endurance treadmill test among mice. Partition was used to build 5 rows of running grids which were 5 cm wide each. The mice run in their row and were prevented to cross another row by a temporary partition. The treadmill was set at 0 inclination in 4 steps of speed. The maximal running test was performed after 5 minutes of warming up, i.e.: 1 minute in 0 cm/s of speed, 2 minutes in 11 cm/s of speed, 2 minutes in 14 cm/s of speed, and 2 minutes in 21 cm/s of speed. Mice had to run as long as possible at 30 cm/s of speed on a treadmill. The test was finished whenever any mice failed to continue running and moved to the back grid for at least 5 seconds. The duration of the run on the treadmill was determined in minutes using a digital stopwatch (Dougherty et al., 2016; Petrosino et al., 2016).

Tissue preparation

Post treadmill test, mice were anesthetized to collect right calf muscles. Part of the muscle was ground in cold poly buffer saline (PBS) to collect its homogenate from the supernatant. The muscle protein level was determined from the homogenate by spectrophotometry using Biuret and the myoglobin level was measured from the homogenate using an ELISA kit. The level of myoglobin was in nmol/mg muscle protein. Another part was preserved in a 10% buffer formalin solution and embedded into a paraffin block. The embedded muscle tissues were sliced in a cross-sectional direction for the immunohistochemistry process.

Myoglobin and FNIP-1 evaluation

Myoglobin level was determined using an ELISA kit of FineTest® EM1223 for mice myoglobin determination, obtained from Wuhan Fine Biotech Co., Ltd. Embedded muscles were sliced in a cross-sectional direction for the immunohistochemistry process. FNIP-1 expression was determined using mice Abcam® anti-FNIP-1 antibody ab61395 on sliced mice calf muscles. The positive cell of FNIP-1 expression was characterized as a brownish myocyte with 3,3' Diaminobenzidine (DAB) chromogen. The expression of FNIP-1 was determined using microscope observation with 400x magnification in the percent of a positive myocyte. The FNIP-1 expressions were the average of two expert observations.

Statistical Analysis

All data were presented as mean ± standard deviation (SD), minimum, and maximum in the table. We performed the normality test for data distribution. Pearson correlation test was used for the result of the

normality test $p > 0.05$ and the Spearman association test was used for the opposite. The correlations were determined from the p-value ($p < 0.05$) and the correlation score. The statistical analysis in this research utilized the SPSS version 25 statistical package program for all computations and data interpretation.

Results

The data results of all variable measurements were seen in table 1. The duration of mice running was measured between 7.39 – 53.33 minutes. The level of myoglobin was found between 0.28 – 36.28 nmol/mg muscle protein. FNIP-1 expressions were found between 14.72 – 82.67 % positive myocyte. Data of FNIP-1 expression and myoglobin level were in a normal distribution, but not for the data of running duration. The association between variables involved in running duration was performed using the Spearman test. Pearson test was performed to analyze the correlation between FNIP-1 expression and myoglobin level.

The muscle FNIP-1 expressions were seen in figure 1. Brownish dots in the myocyte represented the FNIP-1 protein. Myocyte with a brownish dot was positively expressed FNIP-1 protein. The observers counted several brownish myocytes who expressed FNIP-1 per 100 myocytes in ten visual fields. Three pictures represented a different expression of FNIP-1; (A) 14%, (B) 45%, and (C) 82%.

Table 1.
Data description of variables measurement and distribution

Variables	Mean ± SD	Min	Max	p-value
FNIP-1 expression (%)	46.37 ± 20.00	14.72	82.67	0.39*
Myoglobin level (nmol/ mg muscle protein)	14.78 ± 10.73	0.28	36.28	0.22*
Duration of the run (minutes)	25.60 ± 16.62	7.39	53.33	0.02

*. Data distribution was normal due to p-value > 0.05

Figure 1. The expression of FNIP-1 in myocyte (A) was 14%, (B) 45%, and (C) 82%. Brownish dots in the myocyte showed FNIP-1 expression. Myocyte with the brownish dot was counted as a positive expressed cell among 100 myocytes found in ten visual fields of 400x microscope observation.

Table 2.
The Spearman association tests

Associations	Scores	p values
FNIP-1 – duration of the run	- 0.70	0.001*
Myoglobin – duration of the run	0.77	0.001*

*. The variables were associated at $p < 0.05$

The Spearman tests confirmed associations between the duration of running with FNIP-1 expression and myoglobin level. The duration of the run was closely associated with FNIP-1 expression and myoglobin level (Spearman scores > - 0.7). The association between FNIP expression and the duration of the run was negative. Longer duration of mice running was found a lower percentage of muscle FNIP-1 expression. It was different from the association between myoglobin with the duration of the run. Longer duration of mice running was found at a higher level of muscle myoglobin.

Table 3.
The Pearson correlation test

Correlation	Score	p-value
FNIP-1 – myoglobin	- 0.77	0.001*

*. The variables were correlated at $p < 0.05$

The Pearson test confirmed that FNIP-1 expression correlated with myoglobin level in the mice calf muscle. The correlation was also found in negative. A higher level of myoglobin was found in mice muscle with a lower level of FNIP-1 expression. This was similar to the association between FNIP-1 expression with the duration of running. The Myoglobin level and duration of running were negatively associated with FNIP-1 expression in the calf muscle of mice.

Discussion

FNIP-1 expression in the calf muscle

Folliculin Interacting Protein-1 (FNIP-1) was a protein found regulated 5' AMP-activated protein kinase (AMPK) signaling pathways (Reyes et al., 2015). Mitochondrial biogenesis and oxidative enzyme synthesis were regulated by AMPK signaling. The presence of FNIP-1 inhibited PGC-1α to activate the AMPK signaling. Higher expression of FNIP-1 resulted lower in several mitochondria and oxidative enzyme levels (Liu et al., 2016; Xiao et al., 2021).

FNIP-1 is expressed constitutively in the cytosolic myocyte. It was part of the default system of metabolic function of muscle to regenerate ATP in mitochondria (Liu et al., 2016; Xiao et al., 2021). Myosin heavy chain (Myh7/Myh7b) gene expression resulted in intronic miRNAs i.e.: Myh7/miR-208b and Myh7b/ miR-499. The Myh7b/ miR-499 inhibited FNIP-1 expression and released PGC-1α/ AMPK signaling from the FNIP-1 inhibition. As long as the Myh7/Myh7b gene was active, the FNIP-1 would be in low expression in muscle (Liu et al., 2016).

The Myh7/Myh7b gene expression was active during myoblast development to differentiate myocytes (Cech & Martin, 2012). It determined the fiber typing just before fusion at the end stage of skeletal muscle differentiation. The Myh7/Myh7b gene expression resulted in the activity of intronic miR-499 which downregulated the FNIP-1 expression (Liu et al., 2016)(Talbot & Maves, 2016). The activity of Myh7b/ miR-499 is associated with the type-1 slow oxidative muscle fiber. The type-1 myocyte was characterized by low expression of FNIP-1. On the contrary, FNIP-1 was expressed higher in type-2 myocytes (Xiao et al., 2021).

The protein of FNIP-1 was presented as a brownish dot

inside the myocyte cytosol. Myocyte with brownish dots was positively expressed FNIP-1 and characterized as a type-2 myocyte (Xiao et al., 2021). It was seen in (B) and (C) of figure 1. A brownish dot was found at (A) of figure 1 which represented a type 1 myocyte. The figures were captured with a microscopic camera under 400 x magnification.

FNIP-1 and the myoglobin level correlation

In this study, it was found that Fnip 1 was strongly correlated with myoglobin, with a negative correlation. This means that the lower the expression of Fnip 1, the higher the myoglobin levels. High myoglobin levels are associated with red or ST muscle fiber types, which usually have a high oxygen content in the muscles. Muscle endurance performance is reflected in the oxidative capacity of muscle fibers, oxygen supply and myoglobin concentration (Van Der Zwaard et al., 2018). In experiments conducted by (Hasumi et al., 2014) and (Baba et al., 2012) mice conditionally deficient in Fnip1 or Flcn in muscle displayed a red fiber phenotype in skeletal muscle caused by increased mitochondrial biogenesis. This is in line with the theory that Fnip 1 exerts control on the oxidative program of muscle mitochondria through AMPK signaling (Ramírez et al., 2019). Fnip1 represents a previously unknown negative regulator of muscle mitochondrial function and AMPK is essential for Fnip1's action in skeletal muscle as well as a dominant that coordinates the programming of muscle fiber types and mitochondria and regulates muscle fitness (Xiao et al., 2021). The expression level of Fnip 1/FLCN is negatively correlated with AMPK activity (Mi et al., 2017). AMPK activity is higher in oxidative fibers, and activation of AMPK in skeletal muscle induces expression of its downstream transcriptional regulator, peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), thereby directly activating the genetic program of slow-twitch fiber specification, metabolism. oxidative, and mitochondrial biogenesis. Folliculin (FLCN) and folliculin-interacting protein 1 (Fnip1) can regulate skeletal muscle fiber type specification via the AMPK/PGC-1α pathway (Liu et al., 2016).

Myoglobin is haem-protein binding oxygen in the myocyte. Myoglobin determined the level of oxygen availability in the muscle. It transferred oxygen from the cell membrane to the mitochondrial membrane and is also able to store the oxygen inside the cytosol (Postnikova & Shekhovtsova, 2018). Under oxidative stress, myoglobin played a role as an antioxidant to reduce the oxidant molecules (Ordway & Garry, 2004). Myoglobin affinity to oxygen was higher compared to hemoglobin. This affinity gradient between myoglobin and hemoglobin resulted in oxygen uptake in the muscle being higher than others (Postnikova & Shekhovtsova, 2018).

Myoglobin was not part of oxidative enzymes or involved in mitochondria biogenesis. The stimulation of the Myh7b/ miR-499 signal increased mitochondria biogenesis and oxidative enzymes (Liu et al., 2016). It was proposed to be excluded from the Myh7b/ miR-499 action on FNIP-1 expression. On the contrary, myoglobin was the main characteristic found in type-1 slow oxidative myocytes which more in mitochondria number and oxidative enzymes level (Masuda et al., 2013; Nemeth & Lowry, 1991). It was still uncertain whether myoglobin level correlated with FNIP-1 expression or not.

Our finding confirmed that myoglobin level was correlated with FNIP expression in the mice calf muscle. It was a negative correlation (as seen in table 3). Higher levels of myoglobin were found in lower expression of FNIP-1. Since FNIP-1 expression inhibited the determination of myocyte fiber typing to be slow oxidative type-1, FNIP-1 expression was proposed to be involved in myoglobin level down-regulation. Our findings reconfirmed (Liu et al., 2016) study results on rats. The FNIP-1 -/- rats showed three-fold higher myoglobin mRNA expression compared to the wild type. The absence of FNIP-1 gene expression activated PGC1α/ AMPK signaling pathway on mitochondrial function. The characteristics of type-1 slow oxidative myocytes found in the FNIP-1 -/- rat muscles.

Since FNIP-1 expression diminished myoglobin level in the calf muscle of mice, it might also reduce oxygen saturation (SaO_2), oxygen fraction (FiO_2), and maximum volume of oxygen uptake (VO_2 max) of muscle. It would explain more about the exact role of FNIP-1. FNIP-1 not only determines muscle fiber typing but also contributed to muscle oxygenation, oxygen uptake, and oxygen use. Unfortunately, this study was not performed SaO_2, FiO_2, and VO_2 max measurements. Future it is purposed to perform human study.

FNIP-1 and the duration of running association among mice

Our finding confirmed the idea that the presence of FNIP-1 in the cytosol was associated with myoglobin level as the main characteristic of the type-1 slow oxidative myocyte. Higher myoglobin levels resulted in better endurance capacity (Van Der Zwaard et al., 2021). Subjects with higher endurance capacity performed any activities for a longer duration (Kenney et al., 2012). We evaluated this through the maximum run test on a treadmill. Calf muscles were essential for mice during running, so FNIP-1 expression during running needed to be associated.

The test resulted in a negative association between FNIP-1 expression in mice calf muscle with the duration of running on a treadmill. Longer duration of running was found at lower FNIP-1 expression in the mice calf muscle. The presence of FNIP-1 in cytosol inhibited the development of type-1 slow oxidative myocyte characteristics, such as mitochondria number and oxidative enzymes (Xiao et al., 2021). FNIP-1 inhibited PGC1α/ AMPK signaling pathway on mitochondrial function in muscle (Liu et al., 2016). Low levels of myoglobin resulted in low availability of oxygen supply for mitochondria (Postnikova & Shekhovtsova, 2018). Lack of oxygen availability did not support oxidative enzymes of

mitochondria to couple ATP. It impaired the ATP regeneration to support activity performance for as long as possible (Glancy et al., 2021; Hargreaves & Spriet, 2020). In another word, FNIP-1 expression diminished the endurance performance of mice on maximum running tests on the treadmill.

Since the expression of FNIP-1 reduced oxygen supply for mitochondrial coupling of energy, it also proposed a contribution to lactate released from muscle. Lactate was the residue of anaerobic glycolysis in the cytosol. It was a fast alternative solution to recover ATP during mitochondrial coupling of energy muted (Glancy et al., 2021). Lactate suddenly accumulated in the bloodstream 15 minutes after a break (Goodwin et al., 2007; Theofilidis et al., 2018). Unfortunately, we did not perform lactate level measurement in serial time to evaluate its raise.

Conclusion

This study revealed a novel role of FNIP-1 in muscle development and performance. The expression of FNIP-1 correlated with myoglobin level in the mice calf muscle and was also associated with the duration of running. The connections were negative so the presence of FNIP-1 in the muscle impaired the oxygen supply for mitochondrial coupling and limited the endurance performance. Further study needs to investigate the FNIP-1 expression in human muscle to look at its correlation with the level of lactate, oxygen saturation (SaO_2), oxygen fraction (FiO_2), and maximum volume of oxygen uptake (VO_2 max).

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