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Research Article

FNDC5 Gene Expression in the Smooth Muscles of Dromedary Camels (*Camelus dromedarius*)

Doaa Kirat[1]*, Taku Miyasho[2], Tahany Amin[1], Amira Moustafa[1] and Mohamed Hamada[1]

[1]Department of Physiology, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt; [2]Laboratory of Animal Biological Responses, Department of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido, Japan
***Corresponding author:** doaakirat@hotmail.com

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ABSTRACT

Irisin is a recently reported adipo-myokine. It is synthesized after the proteolytic cleavage of its precursor, fibronectin type III domain-containing protein 5 (FNDC5) prior to its release into the circulation. Currently, it is unknown whether irisin/FNDC5 exists in the tissues of camel species. Our findings demonstrated for the first time the existence of mRNA transcripts for FNDC5 gene in the dromedary camel smooth muscles, as determined by RT-PCR analysis and showed predominant localization of irisin/FNDC5 protein in the visceral and vascular smooth muscle cells of dromedary camels, as assessed by immunohistochemical analysis. The present study suggests that irisin/FNDC5 possibly has physiological role(s) in the contractility and motility of the camel intestinal smooth muscle cells as well as it might be implicated in the maintenance and control of blood pressure in camels. These important factors could contribute to the distinct biological characteristics of dromedary camels for adaptation to harsh environmental conditions.

Key words: FNDC5, Camels, Blood vessels, Blood pressure, Cellular localization, Small intestine

INTRODUCTION

The smooth muscle cells have unique structure, arrangement, innervation, and regulation that vary from that of skeletal and cardiac muscles (Hall, 2016). It is located around various organs, vessels, and tracts and implicated in maintaining survival (Hafen and Burns, 2018). Smooth muscles are organized into two broad types; single- and multi-unit smooth muscles that have different positions and features (Hafen and Burns, 2018). Single-unit smooth muscle fibers are found in the walls of all visceral hollow organs. Whereas, the multi-unit fibers are present in the respiratory airways and blood vessels (Hafen and Burns, 2018).

Smooth muscles are responsible for the harmonization of physiological processes that are essential for maintaining the body homeostasis, ranged from the control of blood vessels tone to the gastrointestinal functions (Bowens and Parmacek, 2012). Smooth muscles are non-striated and involuntary. Although skeletal muscle fibers are stimulated exclusively by the nervous system, numerous signals such as nervous, neurotransmitters, hormones, chemical agents, as well as autocrine/paracrine signals regulate the smooth muscles and trigger their contraction (Hall, 2016). The wide distribution of smooth muscle throughout the body and its unique properties make it imperative topic for researchers to have an in-depth understanding of its physiology.

Myokines and adipokines are cytokines which are synthesized, produced, and released by muscle as well as adipose tissues, respectively (Pedersen *et al.*, 2007; Raschke and Eckel, 2013). These cytokines are displayed to communicate with cells in an autocrine/paracrine mode, locally within the tissue, or in an endocrine manner to distant tissues (Raschke and Eckel, 2013). Irisin is considered as a new adipo-myokine which is created by the cleavage of its cellular precursor form; fibronectin type III domain-containing protein 5 (FNDC5) prior to its release into the circulation (Roca-Rivada *et al.*, 2013). Various studies on human beings and rodents reviewed that irisin plays important roles in the homeostasis of glucose and lipid metabolism as well as in the functions of endothelial, cardiovascular and central nervous systems (Boström *et al.*, 2012; Perakakis *et al.*, 2017; Mahgoub *et al.*, 2018). Irisin has vital physiological functions such as inducing the browning of white adipocytes and thermogenesis, increasing energy expenditure, improving glucose utilization, reducing fasting insulin and insulin resistance, neurogenesis, protecting effect on endothelial function, and treating several diseases (Gizaw *et al.*, 2017; Mahgoub *et al.*, 2018). Despite the importance of irisin/FNDC5,

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there is limited literature about the expression and distribution of irisin/FNDC5 in various tissues and cells of many species.

The distinctive characteristics of camel genome as well as their exceptional anatomical and physiological features allow them to live and thrive in severe environmental conditions and also to be less susceptible to many diseases (Gebreyohanes and Assen 2017; Ali *et al.,* 2019). Investigation of key genes involved in the camel adaptive homeostatic mechanisms to the desert environment might contribute to realize their vital physiological mechanisms that enable them to withstand, produce, and reproduce.

Until recently, studies on the existence of FNDC5 gene in camel species are lacking. Therefore, the principal aim of this work was to study the gene expression of FNDC5 in the smooth muscles of dromedary camels. We have also intended to explore the precise cellular localization pattern of irisin/ FNDC5 protein in the visceral and vascular smooth muscles of dromedary camels.

MATERIALS AND METHODS

Ethical approval

This study was approved by the Ethics of Animal Use in Research Committee of Zagazig University, Egypt.

Animals and tissue samples

A total of three male dromedary camels (4-5 years old; 400-500 kg body weight) were used in the present experiments. Immediately after slaughter of camels, visceral smooth muscle samples were excised from the small intestine. Strips of camel small intestinal smooth muscle were cut into segments, snap frozen in liquid nitrogen, and stored at -40°C to be used for RT-PCR analysis. For the immunohistochemical study, Strips of camel small intestinal smooth muscle were directly placed in neutral-buffered formalin (10%) overnight at room temperature.

Reverse transcription-polymerase chain reaction

Messenger RNAs, cDNAs, and PCR were prepared from the examined small intestinal smooth muscles of camels as demonstrated earlier by Kirat *et al*. (2009). Primers for FNDC5 were specifically designed from the predicted Camelus dromedarius FNDC5 (GenBank accession no. XM_010976059) (sense: 5′-CACTGTCAGGCATCTCAAGGCCA -3′; antisense: 5′-TCATATCTTGCTGCGGAGAAGACC -3′). Primers for Glyceraldehyde-3-phosphate dehydrogenase were constructed from the predicted Camelus dromedarius GAPDH (GenBank accession no. XM_010990867.1) (sense: 5′-ATGGTGAAGGTCGGAGTGAACGG-3′; antisense: 5′- GCAGAGATGATGACCCTCTTGGC -3′) to be used as standard control for PCR. PCR was done using an iCycler^{TM} thermocycler (Bio-Rad, USA) that adjusted to perform a denaturation step for two minutes at 94 °C followed by thirty five amplification cycles (one minute at 94 °C, one minute at 58°C, and two minutes at 72 °C) and subsequent final extension for five minutes at 72 °C). For the negative control study, PCR analysis was made without the addition of cDNAs. Mouse heart was used as positive control for the expression of FNCD5 mRNA (Kim *et al.,* 2017). The PCR yields were electrophoresed by using agarose gel (1.5 %) and seen under ultraviolet lamp after staining with ethidium bromide.

Immunohistochemical analysis

After tissue samples fixation, specimens were put in graded ethanol and xylene for dehydration and clearance, respectively and then paraffinized. The immunohisto-chemical localization of irisin/FNDC5 protein was detected using the Thermo Fisher Scientific UltraVision LP kit (Cat. no. TL-125-HL; USA) as described by the manufacturer. The deparafinized 4 µm tissue sections were then boiled for 15 minutes in (0.01 M) sodium citrate solution for unmaking the antigen. Endogenous peroxidase was blocked by incubation in H_2O_2 block for 10 min. After that, Ultra V Block was used for 10 min to block the nonspecific binding. Subsequently, tissue sections were incubated at room temperature in diluted (1:100) rabbit anti- human irisin/FNDC5 antibody (Cat. no. NBP2-59680; Novus Biologicals, USA). After washing, sections were treated with horseradish peroxidase polymer for 15 min and then immersed in diaminobenzidine (0.5% DAB) and H_2O_2 (0.01%) in PBS. Negative control slides were involved in every immunostaining run. PBS was used instead of the irisin/FNDC5 primary antibody for negative controls. Each slide was lastly counterstained using Mayer's haematoxylin solution. For histological examination, sections were subjected to hematoxylin and eosin staining. All slides were scanned and observed by Olympus light microscope.

RESULTS AND DISCUSSION

The proteolytic cleavage of FNDC5 resulted in the production of a novel adipo-myokine, named irisin which released into the blood (Boström *et al.,* 2012; Roca-Rivada *et al.,* 2013) to exert various physiological functions and protect against several diseases (Gizaw *et al.,* 2017; Mahgoub *et al.,* 2018).

Smooth muscles have essential roles in controlling the functions of various hollow organs (Wilson 2011). The smooth muscle fibers vitally participate in the tasks of both visceral systems as gastrointestinal peristalsis and the vascular system such as the regulation of blood pressure (Hafen and Burns 2018). The blood vessel endothelial cell lining layer servers as a selective permeability barrier between surrounding tissues and blood, therefore their integrity is critical for the maintenance of blood vessel homeostasis (Triggle *et al.,* 2012). Endothelial dysfunction has been implicated in cardiovascular diseases (Libby 2002).

Studies by Xie *et al.* (2015) revealed that irisin increases the expression of certain genes that linked with the differentiation and growth of muscles. These genes such as myocardin, the master regulator for smooth muscle (Yoshida *et al.,* 2003), follistatin which promotes the muscle growth via inhibiting myostatin (Lee and McPherron 2001), and also the dominant actin isoform in the smooth muscle cells of blood vessels, α-smooth muscle actin gene, which is essential for fibrogenesis (Cherng *et al.,* 2008), regulates the fibroblast contractile activity (Hinz *et al.,* 2001), and initiates the cardiomyocyte differentiation and growth (Clement *et al.,* 2007).

Furthermore, irisin plays an essential role in maintaining the endothelium homeostasis in humans and rodents. Irisin has been shown to promote the endothelial cell proliferation and angiogenesis of umbilical vein endothelial cells in humans (Song *et al.,* 2014). Also, in diabetic mice, it could alleviate the endothelial dysfunction (Zhu *et al.,* 2015).

It has been shown that blood irisin levels are positively linked with the endothelium-dependent vasodilation in type 2 diabetic patients who do not have clinical angiopathy (Xiang *et al.,* 2014). Moreover, it has been reported that irisin decreases the blood pressure through improvement of endothelial dysfunction of the mesenteric artery of spontaneously hypertensive rats (Fu *et al.,* 2016). Recently, irisin/FNDC5 was demonstrated to inhibit atherosclerosis through the enhancement of the proliferation of endothelia or the inhibition of endothelial dysfunction and inflammation of blood vessels (Zhang *et al.,* 2016).

Nonetheless, up to date the presence, significance, and role(s) of irisin/FNDC5 in the physiological systems of camels are not discovered yet. This led us to examine and detect the gene expression and cellular localization of irisin/FNDC5 in camels in order to investigate the physiological context of irisin/FNDC5 action in camels. Here we have defined the basal gene expression of FNDC5 in smooth muscles of dromedary camels and identified its prominent localization in the visceral and vascular smooth muscle cells of camels which support an important role for irisin/FNDC5 in gastrointestinal and vascular tissues of camels.

Our RT-PCR analysis demonstrated the expression of FNDC5 mRNA transcripts in the camel smooth muscles (Figure 1A). Remarkably, FNDC5 is highly expressed in camel smooth muscles (Figure 1). A single specific band encoding FNDC5 was observed in the small intestinal smooth muscle of the three examined camels (Figure 1A). The transcript size properly matched the predicted length of the amplified FNDC5 fragment (500 bp) and has the same size as the band observed for the positive control (Figure 1A). PCR product of the expected size (362 bp) of GAPDH was detected in all examined tissues, which indicate that the amount of cDNA was uniform in the samples used for PCR analysis (Figure 1B). In the negative study, no amplicons were seen when the reverse transcriptase enzyme was excluded from the step of cDNAs synthesis during RTPCR analysis (Figure 1A and B).

In humans and rodents, FNDC5 mRNA has been identified in several organs other than skeletal muscle, such as adipose, cardiac, brain, pancreatic, hepatic, and renal tissues (Huh *et al.,* 2012; Roca-Rivada *et al.,* 2013; Kurdiova *et al.,* 2014; Kim *et al.,* 2017).

The smooth muscle has many distinct characteristics and a precise histological structure. The hematoxylin and eosin staining displayed the smooth muscle cell as elongated spindle, or fusiform-shaped that has tapered ends with single oval nucleus that is stained purple (Figs. 2A, B and 3A). The smooth cells are non-striated, but their eosinophilic cytoplasm is filled with actin and myosin (Figures. 2A, B and 3A).

Figures (2 and 3) display cross sections in the smooth muscle layers of the small intestinal wall and the blood vessels of dromedary camels, respectively. Histologically, the muscularis externa of the small intestine consists of smooth muscle fibers arranged into two layers (Figure 2A). In a cross-section of smooth muscle in the wall of the small intestine, cells of the inner (circular) layer are cut longitudinally and appear as linear bundles that can be recognized by the long, thin central nuclei and lack of striations (Figure 2A and B). Whereas the cells of the outer (longitudinal) layer of the smooth muscle are cut transversely (cross section) and have polygonal profiles, which are identified by central nuclei and a small ring of surrounding cytoplasm (Figure 2A).

Under the microscope, the blood vessel histologically consists of three concentric layers which composed of an endothelial cell layer (tunicae intima) facing the vascular lumen, a thick layer of smooth muscle cells (tunicae media), and an outer layer of connective tissues (tunicae adventitia) (Figure 3A).

Immunohistochemical analysis was performed on paraffin-embedded camel tissue sections using irisin/ FNDC5 polyclonal antibody to immunolocalize the irisin/ FNDC5 protein in the visceral and vascular smooth muscles (Figures 2 and 3). The irisin/FNDC5 antibody positively stains the smooth muscle cells in the tunica muscularis (muscularis externa) of the camel small intestine with brown color (Figure 2D-F). Irisin/FNDC5 signals were detected in the smooth muscle layers throughout the camel intestinal tract (Figure 2D-F). Strong immunoreactive signals of irisin/FNDC5 were observed in the circular and longitudinal smooth muscle layers of camel small intestine (Figure 2D-F). Our present findings agreed and confirmed the immunohistochemical data of Gür *et al.* (2018) who detected irisin immunoreactivity in the smooth muscle cells in lamina muscularis and tunica muscularis in stomach and small intestine of adult dwarf hamster.

In vasculature, smooth muscle cells are also important constituent of the blood vessels, in which they are surrounded every blood vessel to regulate the blood flow. We further showed that irisin/FNDC5 positivity was predominantly localized in the smooth muscle layers of camel blood vessels (Figure 3B). The staining intensity of irisin/FNDC5 in the endothelial cells lining the blood vessel lumen was much slighter than that in the vascular smooth muscle cells (Figure 3B). In the negative control study, there was no immunoreactions could be detected which proved the specificity of the staining (Figs. 2C and 3C, D). Our observations are consistent with the previous immunohistochemical results of Gür *et al.* (2017) who observed the irisin positive reactions in the endothelium and smooth muscle cells of blood vessels in lung tissues of porcupine.

To tolerate and adapt to the harsh climate, thirst, and poor grazing of desert for long duration, camels acquire several amazing abilities, anatomical characteristics, and varied physiological mechanisms.

Ali *et al.* (2019) and Jirimutu *et al.* (2012) revealed that camels have the ability to consume high dietary salt; they can intake eight times salt more than other ruminants, and the really impressive thing is that camels don't develop hypertension. In humans, Zhao *et al.* (2003) showed that CYP2J gene controls the hypertension and its suppression leads to increase the blood pressure. However, it has been reported that camels have several isoforms of CYP2J genes that allow the camel to keep their blood pressure low even when they consume a lot of salt (Ali *et al.,* 2019; Jirimutu

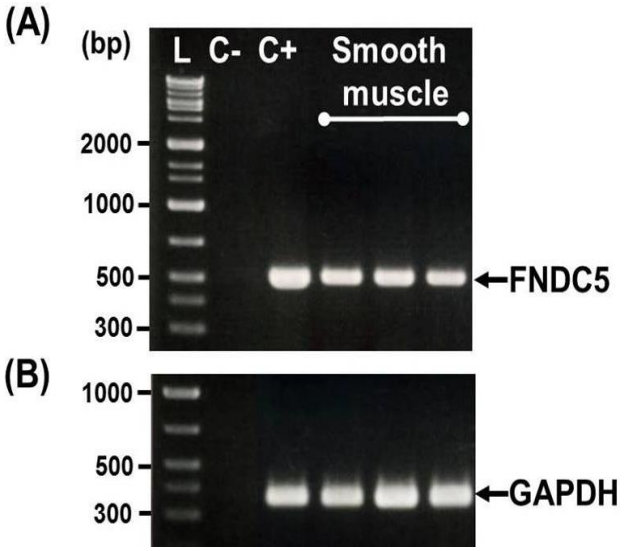

Fig. 1: Expression of FNDC5 gene in the camel smooth muscles. RT-PCR was performed with specific oligonucleotide primers for FNDC5 and mRNAs collected from camel small intestinal smooth muscles. 4 µl PCR samples prepared from dromedary camels (n=3) were electrophoresed on agarose gel (1.5%). A 100 bp DNA ladder was involved as a marker for band size. L, ladder; C-, negative control; C+, positive control (mouse heart). Bands of the expected size for FNDC5 (500 bp) and GAPDH (~360 bp) were identified in all the examined samples. Notice the absence of FNDC5 and GAPDH fragments in the negative control study.

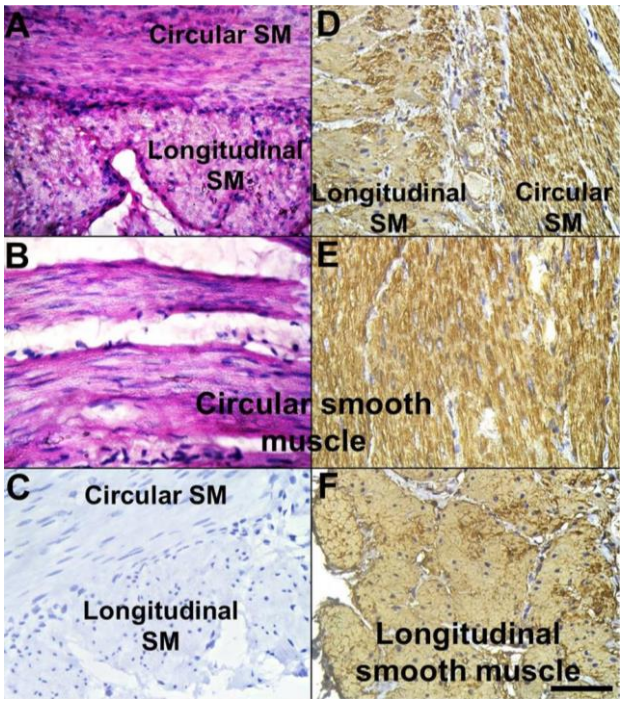

Fig. 2: Cellular localization of irisin/FNDC5 protein in visceral smooth muscle cells of camels. (A and B) Histological images of cross-sectioned small intestinal smooth muscles of camels stained with H&E. Notice the inner circular smooth muscle layer of the muscularis externa is cut longitudinally, while the outer longitudinal layer is cut in cross-section. (D-F) Representative immunohistochemical image shows the brown positive staining of irisin/FNDC5 in the circular and longitudinal smooth muscle fibers of camels. (C) Negative control section shows no reaction for irisin/FNDC5. Scale bar, 20 µm (A-F).

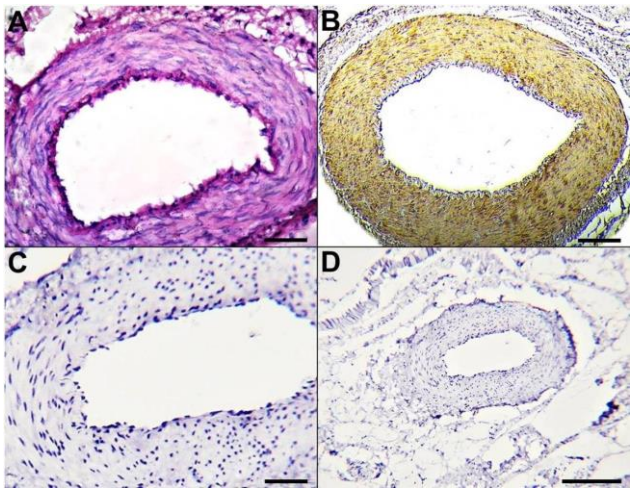

Fig. 3: Cellular localization of irisin/FNDC5 protein in the vascular smooth muscle cells and endothelial cells of camels. (A) Histological cross-section of a blood vessel through the smooth muscle of the camel stained with H&E. (B) Representative immunohistochemical image shows the brown positive staining of irisin/FNDC5 in the endothelial lining and smooth muscle cells of the blood vessels. (C and D) Representative negative control slides with different magnification, displaying no immuno-reactivity for irisin/FNDC5. Scale bar, 50 µm (D) and 20 µm (A-C).

et al., 2012). Therefore, we can speculate that locally synthesized irisin in the endothelial and smooth muscle cells of blood vessels will possibly prevent the increase in the blood pressure of camels. In this regard, Zhang *et al.* (2015) demonstrated that peripherally administrated irisin lowered the blood pressure in spontaneously hypertensive and control rats and verified that both smooth muscle and endothelial cells were involved in the irisin-induced blood vessel dilation.

Our novel data provided that irisin protein and its precursor; FNDC5 gene are expressed and cellularly localized in the visceral and vascular smooth muscles of camel species. Thus, our study suggests that irisin/FNDC5 has physiological role(s) in the mobility and contractility of the camel intestinal smooth muscle cells as well as it might be encountered in maintaining and regulating the pressure and flow of camel blood. Collectively, these new findings extend the knowledge on irisin/FNDC5. Therefore, further studies should be performed to elucidate its roles and mechanisms of action in the camel smooth muscle cells.

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