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IRISIN-IMMUNOREACTIVITY IN NEURAL AND NON-NEURAL CELLS OF THE RODENT

S. L. DUN,[a] R.-M. LYU,[b] Y.-H. CHEN,[a†] J.-K. CHANG,[b] J. J. LUO[a,c] AND N. J. DUN[a*]

[a] *Department of Pharmacology, Temple University School of Medicine, Philadelphia, PA 19140, USA*

[b] *Phoenix Pharmaceuticals, Inc., Burlingame, CA 94010, USA*

[c] *Department of Neurology, Temple University School of Medicine, Philadelphia, PA 19140, USA*

Abstract—Irisin is a recently identified myokine secreted from the muscle in response to exercise. In the rats and mice, immunohistochemical studies with an antiserum against irisin peptide fragment (42–112), revealed that irisin-immunoreactivity (irIRN) was detected in three types of cells; namely, skeletal muscle cells, cardiomyocytes, and Purkinje cells of the cerebellum. Tissue sections processed with irisin antiserum pre-absorbed with the irisin peptide (42–112) (1 μg/ml) showed no immunoreactivity. Cerebellar Purkinje cells were also immunolabeled with an antiserum against fibronectin type II domain containing 5 (FNDC5), the precursor protein of irisin. Double-labeling of cerebellar sections with irisin antiserum and glutamate decarboxylase (GAD) antibody showed that nearly all irIRN Purkinje cells were GAD-positive. Injection of the fluorescence tracer Fluorogold into the vestibular nucleus of the rat medulla retrogradely labeled a population of Purkinje cells, some of which were also irIRN. Our results provide the first evidence of expression of irIRN in the rodent skeletal and cardiac muscle, and in the brain where it is present in GAD-positive Purkinje cells of the cerebellum. Our findings together with reports by others led us to hypothesize a novel neural pathway, which originates from cerebellum Purkinje cells, via several intermediary synapses in the medulla and spinal cord, and regulates adipocyte metabolism. **© 2013 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: Purkinje cells, vestibular nucleus, thermogenesis, cardiomyocyte, glutamate decarboxylase, rostral ventrolateral medulla.

INTRODUCTION

Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) is a transcriptional coactivator regulating gene in response to metabolic and/or neuroendocrine signals (Finck and Kelly, 2006). Exercise, particularly chronic, is associated with increased expression of PGC-1α in skeletal muscle; whereas, type 2 diabetes or a sedentary lifestyle is correlated with a reduced expression (Handschin and Spiegelman, 2008). Increased PGC-1α expression protects against weight gain, inflammation, oxidative stress, muscle wasting and bone loss (Wenz et al., 2009). The molecule(s) that conveys the signal from PGC-1α to the target cell, however, has yet to be firmly identified.

A recent report by Boström et al. (2012) shows in mice that over-expression of PGC-1α induced a brown-like adipose tissue gene program, including uncoupling protein 1 (UCP1) expression, in white adipose tissue, which can also be induced by free wheel running exercise. Moreover, the observation that subcutaneous adipocytes treated with medium from muscle cells expressing PGC-1α, had a similar response, raises the possibility that a secreted substance from muscle cells over-expressing PGC-1α may be responsible for browning of white adipocyte tissues (Boström et al., 2012). Profiling of muscle genes activated by PGC-1α identified a factor termed fibronectin type III domain containing 5 (FNDC5) with predicted structural features of a type I membrane protein that could be proteolytically cleaved to release a smaller protein, which was subsequently named irisin, into the blood stream (Boström et al., 2012). Several observations support the contention that irisin may be the signaling molecule. First, exercise-induced *FNDC5* gene expression in muscles was accompanied by a parallel increase in the concentration of circulating irisin. Second, irisin activated oxygen consumption and thermogenesis of the fat cells in culture. Third, injection of an adenoviral vector over-expressing irisin into mice resulted in browning of subcutaneous white fat and increased total body energy expenditure. These results support the model that exercise induces muscle *FNDC5* expression. The latter increases the amount of circulating irisin, which in turn, activates adipocyte

*Corresponding author. Address: Department of Pharmacology, Temple University School of Medicine, 3400 N, Broad Street, Philadelphia, PA 19140, USA. Tel: +1-215-707-3498; fax: +1-215-707-7068.

E-mail address: ndun@temple.edu (N. J. Dun).

† Present address: Graduate Institute of Acupuncture Science, China Medical University, Taichung, Taiwan.

Abbreviations: CVLM, caudal ventrolateral medulla; EIA, enzyme immunoassay; FNDC5, fibronectin type III domain containing 5; FITC, fluorescein isothiocyanate; GAD, glutamate decarboxylase; irFNDC5, FNDC5 immunoreactivity; irIRN, irisin-immunoreactivity; IVe, inferior vestibular nucleus; LVe, lateral vestibular nucleus; MVe, medial vestibular nucleus; PBS, phosphate-buffered saline; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; RVLM, rostral ventrolateral medulla; SVe, superior vestibular nucleus; UCP1, uncoupling protein 1.

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thermogenic programs, leading to mitochondrial heat production and energy expenditure (Boström et al., 2012).

While *FNDC5* is detected in skeletal muscle (Boström et al., 2012), the cellular distribution of irisin has not been established. Further, *FNDC5* is expressed in the adult rodent heart and brain (Ferrer-Martínez et al., 2002). Immunohistochemical studies were undertaken with the aim to identify the type(s) of cells expressing irisin in the rat and mouse brain and musculature.

EXPERIMENTAL PROCEDURES

Experimental animals

Male Sprague–Dawley rats (250–300 g) and ICR mice (25–30 g) were used in this study. Experimental protocols were reviewed and approved by the Temple University Institutional Animal Care and Use Committee.

Design of irisin antigen

The selection of the fragment irisin (42–112) as the antigen to immunize the rabbits was based on the following considerations. First, the region of irisin (1–44) is absent from the human FNDC5 isoform 3 (Q8NAU1-3) and 4 (Q8NAU1-4); but, both are protein transcripts that begin with irisin (45–112) to the C-terminal end of FNDC5. Second, when modeling the protein using the Hidden Markov Models (HMMs) bioinformatics, the residues of irisin (39–42), QKKD, are the transition sequences between the protein domain 1 and protein domain 2 (Krogh et al., 1994), raising the possibility that irisin (42–112) being the functional domain capable of turning on the UCP1 gene. Lastly, both the isoform 4 of FNDC5 and the 112 residues of irisin have the same bioactivity in activating the CD137-positive beige precursor cells which, in turn, trigger UCP1 gene expression (see Fig. 4C of (Wu et al., 2012). The overlapping region of these two proteins is that of irisin (45–112), which may serve a key function in turning on the UCP1 gene expression. From the MASS Spectrometry analysis, we have detected the presence of irisin-derived peptide that was possibly cleaved from irisin between K^{41} and residue D^{42}. Therefore, our design for an antibody specifically recognized the antigen containing three residues of corresponding sequences, DVR, at the N-terminal side, in addition to irisin (45–112). The specificity of the antibody against the entire sequence of irisin and irisin (42–112) was later confirmed by the enzyme immunoassay (EIA) and radioimmunoassay. For these reasons, the fragment irisin (42–112) was selected as the surrogate of irisin in our study.

Immunohistochemistry

Rats and mice anesthetized with 4% isofurane were intracardially perfused with 0.1 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS. Brains, hearts and gastrocnemius were removed, postfixed for 2 h, and stored in 30% sucrose/PBS overnight.

In a single staining, tissues were processed for irisin-immunoreactivity (irIRN) by the avidin–biotin complex procedure (Dun et al., 2006, 2010). Tissues were first treated with 3% H_2O_2 to quench endogenous peroxidase, washed several times, blocked with 10% normal goat serum, and incubated in irisin-antiserum (1:700 dilution; a rabbit polyclonal against the human C-terminus irisin fragment (42–112); Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA). The amino acid sequence of 112 residues portion of irisin is identical in human (uniprot: Q8NAU1-1 and Q8NAU1-2), rat (uniprot: Q8K3V5), and mouse (uniprot: Q8K4Z2) (Ota et al., 2004). Cross-reactivity of irisin-antiserum against several peptides was evaluated by EIA. Irisin-antiserum showed 100% cross-reactivity with the following peptides: irisin (human, rat, and mouse), irisin (42–112) (human, rat, and mouse); 9% cross-reactivity was noted with FNDC5 isoforms 4 (Q8NAU1-4) (human), and no cross-reactivity against FNDC5 (162–209) (rat, mouse) and irisin (42–95) (human, rat, and mouse).

Heart and gastrocnemius were sectioned horizontally or transversely. Coronal sections of 50 μm were prepared from the brain. After thorough rinsing, sections were incubated in biotinylated anti-rabbit IgG (1:100 dilution; Vector Laboratories, Burlingame, CA, USA) for 2 h and rinsed with PBS and incubated in avidin–biotin complex solution for 1.5 h (1:100 dilution; Vector Laboratories). After several washes in Tris-buffered saline, sections were developed in 0.05% diaminobenzidine/0.001% H_2O_2 solution and washed for at least 2 h with Tris-buffered saline. Sections were mounted on slides with 0.25% gel alcohol, air dried, dehydrated with absolute alcohol followed by xylene, and coverslipped with Permount.

With respect to FNDC5 immunoreactivity (irFNDC5), cerebellar sections from rats and mice were incubated in a rabbit polyclonal antiserum directed against FNDC5 C-terminal fragment (162–209) (1:2000, Phoenix Pharmaceuticals, Inc.). The immunostaining procedure was similar to that described for irisin. The FNDC5 antibody exhibits no cross-reactivity with irisin, but recognizes the intact recombinant protein FNDC5 (Phoenix Pharmaceuticals, Inc.).

For control experiments, cerebellar and muscle sections were processed with irisin- or FNDC5-antiserum pre-absorbed with the irisin peptide fragment (42–112) (1 μg/ml) or FNDC5 peptide fragment (162–209) (mouse, rat) (1 μg/ml) overnight.

Immunofluorescence procedures

In the case of double-labeling experiments, immunofluorescent techniques were applied (Dun et al., 2010). Cerebellar sections were first incubated with irisin antiserum (1:500 dilution) and then with glutamate decarboxylase 65 (GAD65) antibody (1:300 dilution, a mouse monoclonal, Chemicon International, Inc., Temecula, CA, USA). The GAD65 monoclonal antibody has been shown to label Purkinje cells of the rat cerebellum, albeit cell bodies were less intensely labeled as compared to that labeled with GAD67 antibody (Esciapez et al., 1994). Sections were incubated with appropriate secondary antiserum conjugated to either fluorescein isothiocyanate (FITC) or Texas Red, and examined under a confocal scanning laser microscope (Leica TCS SP5, Heidelberg, Germany) with excitation wavelengths set to 488 nm for FITC and 561 nm for Texas Red in the sequential mode.

Microinjection of Fluorogold to the vestibular nucleus of rat medulla

Purkinje cells in the cerebellum project some of their axons to the vestibular nucleus of the medulla in several species (Carleton and Carpenter, 1983; De Zeeuw and Berrebi, 1995). Retrograde tract-tracing experiments were performed to determine whether irIRN Purkinje cells project their axons to the vestibular nucleus. Rats (n = 6) anesthetized with 4% isofurane were placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA) and under a heat lamp. Aseptic conditions were maintained during the surgical procedure. The stereotaxic coordinates for microinjection to the vestibular nuclei were as follows: 10.8–11.5 mm caudal from the bregma, 1.5–2.0 mm lateral from the midline and 6.6–8.0 mm deep from the surface of cerebellum (Paxinos and Watson, 1998). Each rat received an injection of 15 nl 4% Fluorogold solution along the rostro-caudal axis of the

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