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Research article

LXR activation increases the expression of GnRH AND αMSH in the rat hypothalamus *in vivo*

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A B S T R A C T

Liver X receptors (LXR) are important transcription factors involved in the regulation of carbohydrate and lipid metabolism. Recently, we described LXR receptors expression in the hypothalamus but their function in this brain area remains unknown. Here, we evaluated the function of LXR on the expression of factors produced in the hypothalamus *in vitro* and *in vivo* by Western blotting and immunocytochemistry. More precisely we studied the expression of GnRH and GHRH, αMSH and NPY in male Sprague-Dawley rats. The effects of two synthetic LXR agonists, T0901317 and GW3965, were first tested *in vitro*. Hypothalamic explants were treated with either T0901317 or GW3965 (10 μM) for 2, 4, 6 and 8 h. As a positive control the cholesterol ABCA1 and glucose GLUT2 transporters were used. No changes were observed in the expression of the factors evaluated *in vitro*.

The effects of the LXR agonists were then tested *in vivo*. Rats were injected ICV into the third ventricle with either T0901317 or GW3965 (2.5 μg/5 μL ICV) and after 3.5 h or 24 h the hypothalami were dissected out and rapidly frozen for analysis. αMSH and GnRH expression was significantly increased after 3.5 h of T0901317 treatment. Anterior/posterior hypothalamic ratio increases for αMSH expression and decreases for GnRH expression after 24 h of LXR activation. Altogether these results show that LXR activation affects the expression of GnRH and αMSH, suggesting that LXR in the hypothalamus is capable of modulating hypothalamic responses related to appetite, sexual behavior and reproductive functions.

1. Introduction

Liver X receptors (LXR), LXRα and LXRβ, are nuclear receptors involved in the regulation of carbohydrate and lipid metabolism that are now emerging as new drug targets. In the brain LXR activation facilitates cellular cholesterol excretion, reduces the deposition of amyloid plaques and improves cognitive deficits [1,2]. Therefore LXR are being considered to treat diseases such as atherosclerosis and Alzheimer's. Despite the growing importance of LXR in the brain, little is known about their function and location in the CNS. In the hypothalamus, in the supraoptic and paraventricular nuclei, LXRβ regulates arginine vasopressin (AVP) expression and it is implicated in the control of water balance in both brain and kidney [3]. The expression of thyrotropin releasing hormone (TRH) and melanocortin receptor type 4 (MC4R) by thyroid hormone (TH) is repressed by activation of LXR in the hypothalamus [4,5]. Recent studies from our laboratory show that LXR are expressed in different brain areas but only the expression of these receptors in the hypothalamus is sensitive to serum glucose, insulin and triglycerides [6,7] indicating a link between hypothalamic LXR and the intermediate metabolism. In the hypothalamus we found LXRα expressed in the paraventricular (PVN) and the ventromedial (VMN) nuclei while LXRβ are present in the arcuate nucleus (ARC). Both isoforms are expressed in the medial preoptic area (mPOA). Moreover, altered levels of hypothalamic LXR were found in two animal models with metabolic abnormalities [6,7]. The presence of glucosensing mechanisms dependent on LXR in the hypothalamus has also been described [8–10]. Altogether these studies show a close relationship between the carbohydrate and lipid homeostasis and the expression of LXR in the hypothalamus. However, whether LXR could trigger neuronal responses related to the control of food intake and energy expenditure is still unknown. In this project we examined the role of LXR in the hypothalamus through the characterization of some of the neuropeptides regulated by LXR and its activation products *in vitro* and *in vivo*.

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2. Materials and methods

2.1. Animals and ex vivo cultures

Animal procedures were approved by the Animal Care and Use Ethical Committee of the School of Medicine, University of Buenos Aires, in accordance with guidelines defined by the European Communities Council Directive of November 24, 1986 (86/609/EEC), and the National Institutes of Health Guide for the Care and Use of Laboratory Animals procedures. Animals were kept under standard laboratory conditions at 24 °C, with 12 h light:12 h darkness cycles and food and water ad libitum. 7-week-old Sprague–Dawley rats (n = 6) were killed by decapitation and the hypothalami were rapidly dissected out and placed in oxygenated fresh aCSF solution for 30 min. Explants of hypothalamus were used as *in vitro* model as described elsewhere [11,12]. Hypothalami were separated by transverse section to increase exposure to metabolites treatments. The tissues were then cultured in a six-well culture dish containing 1 mL of DMEM, 1% FBS, 10 μg/mL streptomycin and glutamine pH 7,4 at 37 °C/5% CO_2 [8,13], with the addition of T0901317 (10 μM) or GW3965 (10 μM) for 2, 4, 6 and 8 h. Control hemisections were incubated with vehicle for the same times. When incubation time was finished the explants were quickly frozen on dry ice and stored at −80 °C.

2.2. LDH measurement

Measurement of the enzyme lactate dehydrogenase (LDH) activity in the extracellular medium was performed as a quantitative method for assessing cell injury by using a cytotoxicity kit assay (Cyto Tox 96 nonradiactive, Promega) [14]. Explants treated with H_2O_2 (7% v/v) for 2 h were used as positive control to determine the viability of the explants. H_2O_2 exposed explants presented greater LDH levels (approximately 650% more) compared to the LDH values obtained from the explants used for the study (0.005–0.012 mg/mL).

2.3. ICV drug injection

9 to 10-week-old Sprague–Dawley male rats were anesthetized by ketamine-xylazine (80:4 mg/Kg Holliday/Richmond, Argentina) and placed in a stereotaxic apparatus. A 10 μL-Hamilton syringe was placed in the 3 V, 2.3 mm posterior to Bregma, on the midline, and 8.5 mm ventral to the brain surface of the rat brain atlas of Paxinos and Watson (1998). 5 μL (1 μL per minute) solution containing 2.5 mg of GW3965 or T0901317 was injected in aCSF with methylene blue (1 mg/mL) as indicator of the injected zone. After 5 min the needle was slowly withdrawn and animals were removed from the stereotaxic apparatus and sutured. Tramadol (0.05%) was offered to the animals in the drinking water as a painkiller. Control animals were injected ICV with vehicle alone (< 1% DMSO in aCSF + 1 mg/mL of blue methylene). After 3.5 h or 24 h one set of the animals were sacrificed by decapitation and hypothalami were rapidly dissected out and frozen on dry ice. Another set of animals were deeply anesthetized and perfused for immunocytochemical analysis.

2.4. Western blotting

Homogenates were prepared by sonication in ice-cold lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 1 mM Na_3VO_4, and 1% Triton 100, pH 7.4) containing a protease inhibitor cocktail (Roche Diagnostics) as previously described [14,15]. A total of 20 mg of protein was separated by 10% SDS–PAGE in Tris–glycine electrophoresis buffer at 120 V for 90 min. Proteins from gels were transferred onto PVDF membranes (Bio-Rad), and the membranes were blocked with TBS-T (20 mmol/l Tris, pH 7.5; 150 mmol/l NaCl; and 0.1% Tween-20) containing 5% fat-free milk for 1 h. Blocked membranes were incubated with the primary antibody in TBS-T containing 5% fat-free milk at 4 °C overnight. The primary antibodies used were GHRH (1:1000, Abcam), GnRH (1:500, kindly provided by Dr. Damasia Becu, IByME-CONICET) [16,17], αMSH (1:1000, SIGMA) (García-Tornadú 2009), NPY (1:2000, Peninsula Laboratories) [18], ABCA1 (1/700, Abcam), GLUT2 (1/500, Abcam) and F-actin (1:1000, Santa Cruz Biotechnology) [7]. Immunoblots were then washed with TBS-T three times and incubated at room temperature for 1 h with the respective HRP-conjugated secondary antibodies (1:5000, GE Healthcare Life Sciences, Buenos Aires, Argentina). Chemiluminescence was detected with the ECL system (GE Healthcare Life Sciences) and exposure to hyperfilm (GE Healthcare Life Sciences). All membranes were then stripped and reprobed for F-actin as a loading control. Signals in the immunoblots were scanned and analyzed by Scion Image Software (National Institutes of Health, Washington DC, USA). The amount of target protein was indexed to F-actin in all cases to ensure correction for the amount of total protein on the membrane. The results were reported as percentages of values obtained from expression of target proteins compared to controls.

2.5. Immunocytochemistry and fluorescent microscopy

The animals were deeply anesthetized by i.p. injection with chloral hydrate 28% (w/v, 0.1 mL/100 g of body weight) and the animals were fixed by intracardiac perfusion using 600 mL of 4% cold paraformaldehyde (PFH) in PBS, pH 7.4 [15]. The brains were removed immediately and left in 4% PFH overnight. They were then washed with PBS and the hypothalamus was sectioned with a vibratome. Coronal sections (70 mm thick; Bregma K0.26 to K3.20 mm) were collected and incubated in PBS containing 0.1% Tween 20 and 7% normal donkey serum for 1 h at room temperature. The tissue samples were then incubated with a rabbit anti-GnRH (1:500) and mouse anti-αMSH (1:1000, SIGMA) in PBS containing 2% donkey serum and 0.1% Tween 20 overnight at 4 °C. Subsequently, they were rinsed in PBS for 30 min and then incubated with donkey anti-mouse Cy3 and donkey anti-rabbit Cy3 (Millipore, all 1:300) for 2 h at RT. Nuclei were counterstained with DAPI (1 μg/mL). Finally, after washing, sections were mounted on glass slides and examined with a Olympus IX81-DSU Spinning Disk Confocal Microscope. All pictures were obtained under the same conditions; exposition time, lamp intensity and filters were kept constant throughout the experiment. The primary antibody was omitted in some sections as control; those were further processed under the same protocol described earlier. The fluorescence staining intensity from those sections was used as a marker to identify positive staining.

2.6. Quantification of the immunoreactive area

The immunoreactive area in each image was measured using the Scion Image Software (National Institutes of Health, Washington DC, USA). The Cy3 red staining was first filtered to prevent parameter overestimation. The immunopositive area was calculated as the ratio between the positive area and the corresponding subfield area, using a user-defined threshold as it was performed elsewhere [19].

2.7. Statistical analysis

Values are expressed as mean ± SEM. At least two similar but separate experiments were evaluated in all cases containing samples from three to four different animals per treatment. The significances among variables were evaluated using ANOVA followed by Fisher's post-hoc test or Student's *t*-test for two-group comparisons. In all cases, the Statview Statistical Software (SAS Institute, Inc., Cary, NC, USA; v5.0.1) was used. Differences were considered significant at $p < 0.05$.