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Short communication

Uptake of leptin and albumin via separate pathways in proximal tubule cells

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ABSTRACT

The adipokine leptin and oncotic protein albumin are endocytosed in the proximal tubule via the scavenger receptor megalin. Leptin reduces megalin expression and activates cell signalling pathways that upregulate fibrotic protein expression. The aim of this study was to investigate if leptin uptake in proximal tubule cells was via the albumin-megalin endocytic complex. In immortalised proximal tubule Opossum kidney cells (OK) fluorescent leptin and albumin co-localised following 5 min exposure, however there was no co-localisation at 10, 20 and 30 min exposure. In OK cells, acute exposure to leptin for 2 h did not alter NHE3, ClC-5, NHERF1 and NHERF2 mRNA. However, acute leptin exposure increased NHERF2 protein expression in proximal tubule cells. In OK cells, immunoprecipitation experimentation indicated leptin did not bind to ClC-5. Leptin uptake in OK cells was enhanced by bafilomycin and ammonium chloride treatment, demonstrating that uptake was not dependent on lysosomal pH. Thus, it is likely that two pools of megalin exist in proximal tubule cells to facilitate separate uptake of leptin and albumin by endocytosis.

1. Introduction

Obesity is a major risk factor for the development of chronic kidney disease, which may lead to dialysis and ultimately end stage renal failure (Li et al., 2007). In animal models and humans, obesity alters the kidney leading to glomerular hypertrophy, thickening of the glomerular basement membrane, mesangial matrix expansion (Santo et al., 2004), renal inflammation and tubular fibrosis (Nagai et al., 2005).

Current studies investigating the link between kidney disease and obesity have focused on the adipokine, leptin (Johnson et al., 1992), with plasma leptin concentrations reflecting the degree of adiposity. In healthy individuals, the concentration of leptin in the plasma in vivo is approximately 5.48 ng/ml, with pathophysiological levels between 5 and 40 times higher (Maffei et al., 1995; Oka et al., 2007). Leptin is completely cleared from the filtrate by the scavenger receptor megalin in the proximal tubule (Cumin et al., 1997; Johnson et al., 1992). Megalin is responsible for the endocytosis of a number of low molecular weight proteins from the glomerular filtrate, including albumin. Megalin facilitates albumin endocytosis via a complex containing the Na^+–H^+ exchanger (NHE) 3 (Biemesderfer et al., 2001), the Na^+/H^+ Exchange Regulator Factor (NHERF) 1 (Eknoyan, 2007), the chloride channel (ClC)-5 and NHERF2 (Campbell and White, 2008). Once internalised, albumin is believed to be degraded by the lysosomal pathway (Christensen and Birn, 2002). Recent research has also established that albumin can be transcytosed via the neonatal Fc receptor (FcRn), which sorts albumin from the early endosomes to the transcytotic pathway (Tenten et al., 2013).

Recently, we have demonstrated that acute exposure to leptin decreases megalin expression in Opossum Kidney (OK) proximal tubule cells via a 5′ adenosine monophosphate-activated protein kinase (AMPK) mediated pathway (Briffa et al., 2015b). Despite the reduction in megalin, albumin endocytosis is unaltered until cells are exposed to supraphysiological concentrations of leptin (Briffa et al., 2015b). This suggests that albumin endocytosis in the presence of leptin is not limited by the availability of megalin.

Abbreviations: AMPK-5′, adenosine monophosphate-activated protein kinase; ClC-5, chloride channel-5; NHE3, sodium hydrogen exchanger 3; NHERF, sodium hydrogen exchanger regulatory factor; OK, opossum kidney; TGFβ1, transforming growth factor β1.

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Therefore, the aim of this study is to investigate if leptin and albumin are internalised into the tubular cells via the same megalin-mediated macromolecular complex. Our hypothesis is that leptin will be associated with a complex containing albumin, ClC-5, NHERF2 and megalin in proximal tubule cells.

2. Materials and methods

2.1. Cell culture

The opossum kidney (OK) cells were maintained in DMEM/Ham's F-12 (DMEM/F-12) media supplemented with 10% fetal bovine serum, penicillin/streptomycin and incubated at 37 °C in 5% CO_2. For experimental protocols, OK cells were seeded at confluence and grown for 5 days. Two days prior to experimentation, cells were incubated in DMEM/F-12 with 5 mM glucose medium lacking serum (containing no albumin: minus media). For each experiment $n \geq 3$ independent experiments.

2.2. Confocal

Previous research has demonstrated that megalin binds to both leptin (Briffa et al., 2015c) and albumin in the proximal tubule (Cui et al., 1996) and that albumin is internalised into vesicles (Zhai et al., 2000) following 2 min exposure (Devuyst et al., 1999). We therefore investigated if albumin and leptin co-localise. OK proximal tubule cells were serum starved for 48 h in minus media, then treated with 0.05 μg/ml human recombinant leptin (ProSpec; New Jersey, USA) and 50 μg/ml Texas Red albumin (TR-albumin: Molecular Probes; Oregon, USA) for 5, 10, 20 and 30 min. Following treatment cells were fixed with 4% paraformaldehyde (Sigma-Aldrich; Sydney, Australia) and blocked with goat serum (Sigma-Aldrich; Sydney, Australia). Cells were then treated with leptin antibody, followed by goat anti-rabbit conjugated to fluorescein isothiocyanate (FITC) (Sigma-Aldrich; Sydney, Australia). Cells were then mounted using FluroSave (Merck Millipore; Victoria, Australia) and analyzed by confocal microscopy using the Zeiss LSM Pascal (NSW, Australia).

2.3. 'Real – time' polymerase chain reaction (PCR)

OK cells were serum starved for 2 days in minus media (Hryciw et al., 2004a), then treated with human recombinant leptin (ProSpec; New Jersey, USA) for 2 h at 0.05, 0.10, 0.25 and 0.50 μg/ml concentrations (Briffa et al., 2015b). RNA was isolated from OK cells using the TRIzol method (Cornall et al., 2011). Briefly, the RNA was DNase treated, first strand cDNA was then generated from 0.5 μg of template RNA using the commercially available iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Hercules, USA) using random hexamers and oligo dTs as described previously (Ryberg et al., 2007). 'Real – time' PCR was conducted using MyiQ™ single colour 'real-time' PCR detection system (Bio-Rad Laboratories, Hercules, CA) with iQ™ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, USA) as the fluorescent agent. Forward and reverse oligonucleotide primers for the genes of interest were designed using OligoPerfect™ Suite (Invitrogen, Melbourne, Australia). Primer sequences: NHE3 F: 5′ GGAGGCCACCAACTATGAAG 3′ and R: 5′ TCAGGGGAGAACACAGGATT 3′, ClC-5 F: 5′ TGTCCCGAGAGTCACAAAGA 3′ and R: 5′ ATGATGGACGTGCTCACAAC 3′, NHERF1 F: 5′ AGATCTGCCTCCAGCGATAC 3′ and R: 5′ CCAGGGAGATGTTGAAGTCC 3′, NHERF2 F: 5′ TGGCTCTCCTGCTTCTCTCT 3′ and R: 5′ TCCTCCTGTGCCTTGATTCT 3′. Selective gene homology was confirmed using BLAST analysis (National Centre for Biotechnology Information, Bethesda, USA). To compensate for variations in RNA input amounts and reverse transcriptase efficiency mRNA abundance of the genes of interest were normalised to housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). 'Real – time' PCR reactions were run for 50 cycles of 95 °C for 15 s and 60 °C for 60 s. Relative changes in mRNA abundance was quantified using the $2^{-\Delta\Delta CT}$ method as previously detailed (Ryberg et al., 2007) and reported in arbitrary units (normalised to GAPDH). C_T values for GAPDH were not altered by leptin treatment (data not shown).

2.4. Protein extraction and western blot analysis

OK proximal tubule cells were serum starved for 2 days in minus media (Hryciw et al., 2006, 2004a), then treated with human recombinant leptin (ProSpec; New Jersey, USA) for 2 h at 0.05, 0.10, 0.25 and 0.50 μg/ml concentrations. Protein was isolated as described previously (Briffa et al., 2015b). Briefly, following treatment, the cells were lysed with IP lysis buffer (10 mM Tris–HCl, 150 mM NaCl, 1% NP-40; with the pH adjusted to 7.5) supplemented with a Complete Mini Protease Inhibitor Cocktail (Roche; NSW, Australia) and Halt Phosphatase Inhibitor Cocktail (ThermoScientific; Victoria, Australia). Equal aliquots (50 μg of protein) from each treatment then underwent Western blotting. Aliquots (50 μg) of the protein samples were separated on a 4–15% SDS-PAGE gel and transferred onto a nitrocellulose membrane. Membranes were probed with antibodies against ClC-5, NHE3, NHERF1 and NHERF2 (Santa Cruz Biotechnology, Texas, USA) as described (He et al., 2008). All Western blot data was quantified densitometrically using Image J software.

2.5. Immunoprecipitation

Immunoprecipitation was performed as described previously (Briffa et al., 2015b). OK cells were seeded onto 175 cm^2 flasks and grown to confluence, and then serum starved for 48 h. The cells were treated with either control (phosphate buffered saline: PBS) or human recombinant leptin (ProSpec; New Jersey, USA) for 15 min at 0.05 or 0.50 μg/ml. Protein was then isolated from OK cells using immunoprecipitation (IP) lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 5% NP-40 with the pH adjusted to 7.5) supplemented with a Complete Mini Protease Inhibitor Cocktail (Roche; NSW, Australia). IPs were performed on one mg protein from each treatment. Cells were incubated overnight at 4 °C with no antibody, Normal Rabbit IgG (Merck Millipore; Victoria, Australia) or Leptin antibody (ThermoScientific; Victoria, Australia) with end-to-end rotation. The next day 50 μl pre-cleared Protein G Agarose (ThermoFisher Scientific; Victoria, Australia) was added to each treatment and inverted with end-to-end rotation at 4 °C for 5 h. The eppendorf tubes were then centrifuged and the supernatant was discarded, and the beads were washed with IP lysis buffer. 50 μl Laemmli Sample Buffer was then added to each treatment, and heated to 100 °C for 10 min. The treatments were then centrifuged and equal aliquots (10 μl) of each treatment were analyzed by Western blot, as described previously, and probed for ClC-5.

2.6. FITC-leptin endocytosis

Confluent monolayers of OK cells in 48 well plates were incubated in minus media for 48 h. Cells were pre-treated for 1 h with 0.1 μM bafilomycin, 20 mM ammonium choloride or 2 μM latrunculin (Gekle et al., 1995). Monolayers were washed in HEPES-Ringer buffer (122.5 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 0.8 mM $MgCl_2$, 0.8 mM Na_2HPO_4, 0.2 mM NaH_2PO_4, 5.5 mM glucose, 10 mM HEPES; pH 7.4) and incubated with 0.05 μg/ml FITC leptin plus the inhibitors, for 30 min. Cells were washed in HEPES-Ringer buffer (pH 7.4) and lysed with MOPS buffer (20 mM MOPS with 0.1% v/v Triton X-100) (Hryciw et al., 2004b). Fluorescence was determined