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Acute leptin exposure reduces megalin expression and upregulates TGFβ1 in cultured renal proximal tubule cells

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ABSTRACT

Increased leptin concentrations observed in obesity can lead to proteinuria, suggesting that leptin may play a role in obesity-related kidney disease. Obesity reduces activation of AMP-activated protein kinase (AMPK) and increases transforming growth factor-β1 (TGF-β1) expression in the kidney, leading to albuminuria. Thus we investigated if elevated leptin altered AMPK and TGF-β1 signaling in proximal tubule cells (PTCs). In opossum kidney (OK) PTCs Western blot analysis demonstrated that leptin upregulates TGF-β1 secretion (0.50 μg/ml) and phosphorylated AMPKα (at 0.25, and 0.50 μg/ml), and downregulates megalin expression at all concentrations (0.05–0.50 μg/ml). Using the AMPK inhibitor, Compound C, leptin exposure regulated TGF-β1 expression and secretion in PTCs via an AMPK mediated pathway. In addition, elevated leptin exposure (0.50 μg/ml) reduced albumin handling in OK cells independently of megalin expression. This study demonstrates that leptin upregulates TGF-β1, reduces megalin, and reduces albumin handling in PTCs by an AMPK mediated pathway.

1. Introduction

Obesity rates in Western cultures are rising rapidly, with comorbidities including chronic kidney disease (CKD) and end stage renal failure rising concurrently (Eknoyan, 2007). One of the earliest hallmark characteristics of CKD is the presence of albumin in the urine (albuminuria), which is typically the result of both a glomerular and proximal tubule dysfunction (Takeyama et al., 2011; Wohlfarth et al., 2003; Wolf et al., 2004). The hormone leptin is predominately produced by adipocytes (Vázquez-Vela et al., 2008), with plasma leptin concentrations reflecting adiposity (Garibotto et al., 1998), with higher leptin concentrations seen in obese individuals. Leptin binds to two transmembrane receptors, either the leptin receptor (ObRb) or the scavenger receptor megalin, in a cell specific manner. Megalin is responsible for the transportation of leptin in the hypothalamus, placenta, and renal tubules (Briffa et al., 2014; Hama et al., 2004), whereas ObR is responsible for leptin transport and signaling in most other tissues (Ahima, 2008).

Circulating leptin is filtered from the blood by the glomerulus and is reabsorbed by cells of the proximal tubule by the scavenger receptor megalin (Briffa et al., 2014; Hama et al., 2004), resulting in negligible leptin secretion in the urine (Meyer et al., 1997), even in obesity (Cumin et al., 1997; Lönnqvist et al., 1997). The plasma concentration of leptin in non-obese individuals is approximately 5.5 ng/ml, with 9.5% being transferred from the blood to the filtrate, with an estimated renal leptin clearance of 0.0595 μg/ml (Garibotto et al., 1998). The serum concentration of leptin in obese individuals is approximately 5 to 10 times higher than normal individuals (Garibotto et al., 1998). This value is disputed, with others determining that the maximum plasma leptin concentration observed in obesity is likely to be 200 ng/ml (Maffei et al., 1995). However, individuals with CKD demonstrate leptinemia at concentrations up to 490 ng/ml (Dagogo-Jack et al., 1998).

Albuminuria is a hallmark characteristic of renal dysfunction, with studies demonstrating that exposure to elevated leptin can lead to a significant loss of albumin in the urine (Gunduz et al., 2005), suggesting that leptin may provide a link between CKD and obesity (Briffa et al., 2013). Albuminuria is typically the result of an increase in protein filtration through the glomerulus, caused by basement membrane thickening (Wohlfarth et al., 2003; Wolf et al., 2004), which is further compounded by an impairment in protein endocytosis by the proximal tubule cells (PTCs) which are unable to cope with the increased protein load (Takeyama et al., 2011). Importantly, exposure of the renal tubules to elevated concentrations of albumin induces the production of proinflammatory, profibrotic and vasoactive factors *in vitro* (Wolf et al., 2004). For example the

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profibrogenic cytokine transforming growth factor beta (TGF-β) is increased in the glomerular filtrate, which upregulates extracellular matrix production, ultimately resulting in basement membrane thickening (Wolf et al., 2004). Interestingly, exposure to elevated leptin has also been shown to increase TGF-β1 secretion in glomerular endothelial cells *in vitro* (Wolf et al., 1999). Exposure of renal cells to elevated protein and/or leptin also results in an accumulation of collagen in glomerular cells *in vitro*, which would result in tubulointerstitial fibrosis and basement membrane thickening *in vivo* (Wohlfarth et al., 2003). These data taken together suggest that leptin may play a role in obesity-related nephropathy by upregulating the expression of fibrotic mediators. Importantly, research by Gekle et al. (2003) has previously identified in opossum kidney (OK) cells, a well established model of the proximal tubule, exposure to 3 μg/ml of TGF-β1 for 48 h significantly upregulates collagen secretion (types I and IV) and reduces albumin handling by decreasing megalin and cubilin protein expression.

In addition to the fibrotic changes obesity causes in the kidney, obesity is also associated with tubular inflammation (Declèves et al., 2011), which further deteriorates kidney function. AMP-activated protein kinase (AMPK), a key regulatory of energy homeostasis, plays important roles in renal inflammation and fibrosis (Declèves et al., 2011; Sharma et al., 2008). Obesity results in a decrease in renal AMPK expression (Declèves et al., 2011), resulting in an upregulation of inflammatory mediators, as well as fibrotic changes to the kidneys (Declèves et al., 2011; Sharma et al., 2008). Specifically, research by Declèves et al. (2011) identified that one week of high fat feeding (60% fat) in six-week old C57BL/6 mice *in vivo* causes an increase in renal inflammatory markers that preceded albuminuria, with treatment of an AMPK activator preventing the increase in urinary expression of these mediators. Importantly, AMPK activation has been shown to decrease the fibrotic actions of TGF-β1, with an *in vivo* study identifying that AMPK activation, using Metformin, decreased the renal injury, adipokine expression, and macrophage infiltration that is associated with high fat feeding (60% fat) in six-week old C57BL/6 mice (Kim et al., 2013).

In the kidney *in vivo* and *in vitro* (OK, LLC-PK1 and glomerular endothelial cells) leptin has been shown to upregulate fibrotic mediators and causes collagen deposition, which may compound renal function (Wohlfarth et al., 2003; Wolf et al., 1999), ultimately resulting in nephropathy. Therefore the overall aim of the current study was to identify if acute exposure to leptin will alter the expression of fibrotic mediators and, specifically, AMPK signaling in PTCs. We also aim to identify the effect leptin has on albumin handling in PTCs, and characterize changes in megalin expression. We hypothesize that exposure of PTCs to elevated leptin will upregulate fibrotic mediator expression and decrease AMPK expression, in addition to reducing albumin handling in PTCs.

2. Materials and methods

2.1. Cell culture

We utilized OK cells for this study, as they are a widely used model for the renal proximal tubule as they express all proteins of the megalin signaling complex (Amsellem et al., 2010; Birn and Christensen, 2006; Hryciw et al., 2012). OK cells were maintained in Dulbecco's Modified Eagle's Medium and Ham's F-12 (DMEM/F12) media (Life Technologies; Victoria, Australia) supplemented with 10% fetal bovine serum (Life Technologies) with 1% penicillin/streptomycin (Life Technologies), and incubated at 37 °C in 5% CO_2. The cells were seeded at confluence and grown for two days in 25 ml flasks. Forty-eight hours prior to experimentation the cells were incubated in DMEM/F12 with 5 mM glucose medium lacking serum.

2.2. Animal care

Experimental procedures were approved by the Howard Florey Institute Animal Ethics Committee (AEC 09-050). As used in our previous study (Jenkin et al., 2010), six-week old male Sprague Dawley rats (mean initial body weight approximately 178 g) were housed within individual cages in an environmentally controlled laboratory (ambient temperature 22–24 °C) with a 12 h light/dark cycle (7:00–19:00). *Ad libitum* access to food and water was maintained. At ten-weeks, rats were deeply anesthetized with sodium pentabarbitone (100 mg/kg; Virbac, Peakhurst, Australia) then euthanized via cardiac puncture. Kidney and white adipose tissues (WAT) were then removed and tissues were stored at –80 °C for subsequent Western blot analysis.

2.3. Immunoprecipitations

OK cells were seeded onto 175 cm^2 flasks and grown to confluence, and then serum starved for 48 h. The cells were then 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). Immunoprecipitations (IPs) were then performed on 1 mg protein from each treatment. Cells were incubated overnight at 4 °C with no antibody, Normal Rabbit IgG (Merck Millipore; Victoria, Australia), Megalin antibody (ThermoFisher Scientific; Victoria, Australia), or Leptin antibody (Enzo Life Sciences; New York, USA) with end-to-end rotation. The next day 50 μl pre-cleared Protein G Agarose (ThermoFisher Scientific) was added to each treatment and spun 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. Fifty microliters Laemmli Sample Buffer was then added to each treatment, and heated to 100 °C for 10 min. The treatments were then centrifuged and Western blots were performed on the supernatant. Equal aliquots (20 μl) of each treatment were separated on a 7.5% (Leptin IP) or 4–20% SDS-PAGE gel (Megalin IP) (Bio-Rad; NSW, Australia) and transferred onto a nitrocellulose membrane. The membranes were then probed with either Leptin (Megalin IP) or Megalin (Leptin IP) antibodies to identify if leptin and megalin bind.

2.4. 'Real-time' polymerase chain reaction (PCR)

OK PTCs were serum starved for 48 h (Briffa et al., 2014; Hryciw et al., 2006), then treated with human recombinant leptin (ProSpec) for 2 h at 0.05, 0.10, 0.25, and 0.50 μg/ml (Briffa et al., 2014). RNA was then extracted using TRIzol reagent according to the manufacturer's instructions (Life Technologies) as described previously (Jenkin et al., 2010). 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) using random hexamers and oligo dTs as described previously (Jenkin et al., 2010). 'Real-time' PCR was conducted using MyiQ™ single color 'real-time' PCR detection system (Bio-Rad) with iQ™ SYBR Green Supermix (Bio-Rad) as the fluorescent agent. Forward and reverse oligonucleotide primers for megalin (forward *5′*TGC CCC ACC CGT TAT CCT A *3′* and reverse *5′* ACA GAC ATG GTT CTT ACA CTC AAA CAT *3′*; Accession Number XM_007494924.1) and TGF-β1 (forward *5′*CCT GGA CAA CCA GTA CAG CA *3′* and reverse *5′*TTC CGG CCC ACA TAG TAG AC *3′*; Accession Number XM_007491983.1) were designed using the OligoPerfect™ Suite (Life Technologies). Selective gene homology was confirmed using BLAST analysis (National Centre for Biotechnology Information; Maryland, USA). To compensate for