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# Short term exposure to elevated levels of leptin reduces proximal tubule cell metabolic activity

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ABSTRACT

Leptin plays a pathophysiological role in the kidney, however, its acute effects on the proximal tubule cells (PTCs) are unknown. In opossum kidney (OK) cells *in vitro*, Western blot analysis identified that exposure to leptin increases the phosphorylation of the mitogen-activated protein kinase (MAPK) p44/42and the mammalian target of rapamycin (mTOR). Importantly leptin (0.05, 0.10, 0.25 and 0.50 μg/ml) significantly reduced the metabolic activity of PTCs, and significantly decreased protein content per cell. Investigation of the role of p44/42 and mTOR on metabolic activity and protein content per cell, demonstrated that in the presence of MAPK inhibitor U0126 and mTOR inhibitor Ku-63794, that the mTOR pathway is responsible for the reduction in PTC metabolic activity in response to leptin. However, p44/42 and mTOR play no role the reduced protein content per cell in OKs exposed to leptin. Therefore, leptin modulates metabolic activity in PTCs via an mTOR regulated pathway.



## 1. Introduction

Obesity is associated with an increased risk of a number of diseases including end stage renal failure (Eknoyan, 2007). Adipose tissue secretes a number of immunomodulators and bioactive molecules including the small peptide adipokine leptin (Vazquez-Vela et al., 2008). The circulating leptin concentration directly reflects the amount of body fat, with elevated levels of total body fat increasing serum leptin (Garibotto et al., 1998). Leptin binds to either the leptin receptor (ObR) or the scavenger receptor megalin in a cell specific manner. Specifically, megalin binds to leptin in the kidney, placenta and the hypothalamus (Hama et al., 2004), and ObR is responsible for leptin transport and signaling in most other regions of the body (Ahima et al., 1996). Activation of ObR by leptin results in an upregulation of a number of signal transduction pathways including the Janus Kinase/Signal transducers and activators of transcription (JAK/STAT), as well as the mitogen-activated protein kinase (MAPK) pathway (Banks et al., 2000).

Serum leptin is processed by the kidney following filtration across the glomerulus, and taken up by the scavenger receptor megalin in the proximal convoluted tubules (Hama et al., 2004). Megalin processing of leptin from the filtrate results in negligible leptin secretion in the urine (Meyer et al., 1997), even in situations when serum leptin is elevated, such as obesity (Lönnqvist et al., 1997; Cumin et al., 1997). In non-obese individuals, the serum level of leptin is approximately 5.5 ng/ml, with 9.5% being transferred from the blood to the filtrate (Garibotto et al., 1998). In non-obese individuals the renal clearance of leptin is approximately 0.0595 μg/ml (59.5 ng/ml) (Garibotto et al., 1998). The renal clearance in obese individuals has not been measured, however obese individuals have approximately 5–10 times higher serum levels of leptin than in normal individuals (Garibotto et al., 1998), with another group determining that the maximum plasma leptin level observed in the obese is likely to be 200 ng/ml (Maffei et al., 1995). Interestingly, individuals with chronic kidney disease also present with leptinemia, at levels up to 490 ng/ml (Dagogo-Jack et al., 1998). Thus, assuming the transfer of 9.5% of leptin from the plasma to the filtrate (Garibotto et al., 1998), with normal glomerular filtration, the maximum level of leptin in the filtrate is likely to be ~47 ng/ml, however this may be an underestimate if glomerular damage has occurred.

Importantly, studies have shown that exposure to elevated leptin can lead to a significant loss of albumin in the urine (albuminuria) (Gunduz et al., 2005). Albuminuria is a hallmark indicator of renal dysfunction, suggesting that leptin may provide a link between chronic kidney disease and obesity. Albuminuria is typically the result of both an increase in protein filtration through the glomerulus, caused by basement membrane thickening, and an

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impairment of protein endocytosis by the proximal tubule cells (PTCs) (Takeyama et al., 2011). Leptin may play a role in this, as previously it has been shown that exposure to 60 nM leptin for 24 h can induce glomerular mesangial cell hypertrophy via Phosphoinositide (PI) 3-kinase and p44/42 MAPK (also known as extracellular-signal-regulated kinase 1/2 (ERK1/2)) pathways (Lee et al., 2005). Mesangial cell hypertrophy increases the protein content in the filtrate, with elevated levels *in vivo* resulting in tubulointerstitial inflammation, fibrosis, and tubular atrophy leading to progressive kidney disease via apoptosis of PTCs (Koral and Erkan, 2012). In addition, studies have shown that leptin acts on PTCs, with exposure to 100 ng/ml leptin for 48 h activating apoptotic pathways (Hsu et al., 2012). Interestingly, leptin exposure (10–100 ng/ml) for 24 h protects PTCs from apoptosis (Chen et al., 2011). In order to be able to intervene early in the onset of renal complications in response to elevated leptin levels, it is important to understand how increases in leptin can start the pathophysiological changes associated with disease. Importantly, the consequence of short term exposure to leptin is unknown.

In a variety of cell types, leptin-mediated signaling can regulate cell proliferation and viability (Rodríguez et al., 2010; Silva et al., 2008). This may be achieved via the activation of signaling pathways that can regulate cellular metabolic activity, ultimately affecting the ability of the cell to provide energy for metabolic function and growth (Mickuviene et al., 2004). Therefore, the overall aim of the current study was to identify if short term exposure of PTCs to leptin activates signaling pathways that may modulate the metabolic balance of these cells. Our hypothesis is that elevated leptin levels will alter metabolic activity in PTCs.

## 2. Materials and methods

### 2.1. Cell culture

The opossum kidney (OK) cell line was maintained in Dulbecco's Modified Eagle's Medium and Ham's F-12 (DMEM/F12) media (Invitrogen; Mulgrave, Australia) supplemented with 10% fetal bovine serum (Invitrogen; Mulgrave, Australia) with 1% penicillin/streptomycin (Invitrogen; Mulgrave, Australia), and incubated at 37 °C in 5% $CO_2$. The cells were seeded at confluence and grown for 2 days in 25 ml flasks. Two days 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 (standard chow rat diet) and water was maintained. After 12 weeks, rats were deeply anaesthetized with sodium pentabarbitone (100 mg/kg; Virbac, Peakhurst, Australia) then euthanized via cardiac puncture. Kidney tissues were then removed and tissues were stored at −80 °C for subsequent Western blot analysis to characterize the expression of megalin and ObR in renal tissues.

### 2.3. Protein extraction and western blotting

Membrane fractions from kidney lysate and OK cell lysate were harvested for Western blot analysis to determine the expression of ObR in OK cells (Jenkin et al., 2010). Cell lysate from homogenized kidneys and OK cells were centrifuged at 100,000*g* for 30 min at 4 °C.

In addition, OK PTCs were serum starved for 48 h in minus media (Hryciw et al., 2006), then treated with human recombinant leptin (ProSpec; East Brunswick, USA) for 2 h at 0.05, 0.10, 0.25 and 0.50 μg/ml leptin. Protein was isolated from OK cells using 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; Castle Hill, Australia) and Halt Phosphatase Inhibitor Cocktail (Pierce, Scoresby, Australia) as described previously (Hryciw et al., 2006). Equal aliquots (50 μg) of the protein samples were separated on a 10% SDS–PAGE gel (Bio-Rad; Gladesville, Australia) and transferred to a nitrocellulose membrane. ObR (N-20) and megalin antibodies were purchased from Santa Cruz Biotechnology (California, USA) and p44/42 MAPK, phosphorylated p44/42 MAPK (p-p44/42 MAPK: Thr202/Tyr204), p38 MAPK, phosphorylated p38 MAPK (p-p38: Thr180/Tyr182), JAK2, phosphorylated JAK2 (pJAK2: Tyr 1007/1008), STAT3, phosphorylated STAT3 (pSTAT3: Tyr690 and Ser727), STAT1, phosphorylated STAT1 (pSTAT1: Tyr 701), suppressor of cytokine signaling 3 (SOCS3), mammalian target of rapamycin (mTOR), phosphorylated mTOR (p-mTOR: Ser2448 and Ser2481), protein kinase B (Akt) and phosphorylated Akt (pAkt (Ser 473)) antibodies were purchased from Genesearch (Arundel, Australia), and were used following protocols described previously (Hryciw et al., 2006; Slattery et al., 2011). Densitometric analysis of the banding was performed using ImageJ software.

### 2.4. Metabolic activity assay

To assess metabolic activity, OK cells were trypsinized, and an equal amount aliquoted to a 96-well dish for exposure to 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) (Sigma–Aldrich; Castle Hill, Australia) (Jenkin et al., 2010). Specifically, OK cells were treated with a phosphate buffer solution (PBS) control or altering concentrations of recombinant human leptin (0.05–0.50 μg/ml) in 5 mM or 17.5 mM glucose. In addition cells were pre-treated with 10 μM of the MEK inhibitor U0126 for 30 min (Genesearch; Arundel, Australia) or 1 μM of the mTOR inhibitor Ku-63794 for 1 h. U0126 inhibits activation of p44/42 MAPK via inhibition of the kinase activity of MAP Kinase Kinase (MAPKK or MEK 1/2) and Ku-63794 inhibits the mammalian target of raptomyocin complexes 1 and 2 (mTORC1 and mTORC2) via inhibition of p-mTOR (Ser 2448) and p-mTOR (Ser2481). MTT uptake was measured as described previously (Jenkin et al., 2010).

### 2.5. Protein content per cell

Following treatment, the cells were washed in PBS, and then lysed utilizing 100 μl 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; Castle Hill, Australia). To determine the amount of protein per cell, a Bicinchoninic Acid (BCA) assay (Pierce; Scoresby, Australia) was performed following the manufacturer's instructions as previously described (Jenkin et al., 2010). In parallel, cell number was assessed by cell counting. A ratio was calculated from the protein content (BCA assay) to cell number (cell count). The ratio is calculated from each treatment well, and compared to the PBS control (equivalent to 100%).

### 2.6. Caspase 3 assay

To determine the effect short term exposure of leptin has on PTC apoptosis, a Caspase 3 Colorimetric Assay (Sapphire Bioscience; Redfern, Australia) was performed on harvested cell lysates.