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Vanadium(IV)-chlorodipicolinate alleviates hepatic lipid accumulation by inducing autophagy *via* the LKB1/AMPK signaling pathway *in vitro* and *in vivo*

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ABSTRACT

Numerous studies have demonstrated that vanadium compounds are able to improve lipemia and triglyceridemia in both humans and animals. However, the molecular mechanism remains elusive. The present study was conducted to investigate the anti-hyperlipidemic effect of vanadium(IV) complex with 4-chlorodipicolinic acid (VOdipic-Cl)-induced autophagy on hepatic lipid accumulation. To explore the possible underlying mechanisms, primary rat hepatocytes, human hepatoma cell line HepG2, and liver tissue from C57BL/6 mice fed a high-fat diet (HFD) were used. *In vitro*, cultured primary rat hepatocytes were treated with palmitate (0.25, 0.5 and 0.75 mM) prior to VOdipic-Cl (50, 100, and 200 μM) for 24 h, respectively. *In vivo*, C57BL/6 mice were fed with high-fat diet for 16 weeks. VOdipic-Cl (10 mg V/kg body weight) was given by daily gavage for 4 weeks. *In vitro* results showed that VOdipic-Cl significantly inhibited lipid droplet formation by increasing the level of conversion and punctuation of microtubule-associated proteins light chain 3 (LC3) in a dose-dependent manner, and activated liver kinase B-1 (LKB1) and adenosine monophosphate-activated protein kinase (AMPK) phosphorylation. Confocal microscopy images also showed that VOdipic-Cl induced sequestration of lipid droplets (LDs) by autophagy. *In vivo*, VOdipic-Cl attenuated the increase in serum and liver triglyceride levels in the mice fed with high-fat diet, while significantly increased autophagy induction and activated LKB1 and AMPK phosphorylation in the liver. Taken together, these results suggest that VOdipic-Cl reduces hepatic lipid accumulation by inducing autophagy *via* the activation of LKB1/AMPK-dependent signaling pathway.

1. Introduction

Metabolic syndrome is a common nutritional problem that is often associated with clinical features of obesity, glucose intolerance, dyslipidemia, and fatty liver (excess fat deposition in liver) [1]. Obesity arises from metabolic changes in the tissues and organs of the human body [2]. In mammals, liver plays a central role in the regulation of fatty acid metabolism [3]. The amount of lipid that can be exported from the liver is dependent on synthesis as well as the availability of triglycerides (TGs) that are stored within the hepatocyte in the lipid droplets (LDs) organelle [3]. Hepatocytes has the greatest capacity to store TGs in the form of small LDs [4]. Excessive LDs accumulation that occurs in hepatocytes can result in lipotoxicity with consequences of inflammation and subsequent cell death [5,6]. The accumulation of LDs in hepatosteatosis is likely due to disruption in LDs packaging and/or secretory [5,6]. Recent study has reported that autophagy plays an important role in regulating hepatic lipid homeostasis and contributes lipoprotein assembly [7].

Autophagy is an intracellular catabolic process with an essential function in the maintenance of cellular and energy homeostasis [8]. Autophagy clears the misfolded proteins and the damaged organelles, as well as recycles the cytosolic components during starvation to compensate for nutrient deprivation [9]. Autophagy has been considered to be an adaptive response to stress and to be activated during

Abbreviations: 3-MA, 3-methyladenine; ALT, alanine aminotransferase; AMPK, adenosine monophosphate-activated protein kinase; AST, aspartate aminotransferase; BSOV, bis((5-hydroxy-4-oxo-4H-pyran-2-yl)methyl-2-hydroxy-benzoatato) oxovanadium (IV); DMEM, Dulbecco's modified Eagle's medium; FFA, free fatty acids; H&E, hematoxylin and eosin; HBSS, Hanks' balanced salt solution; HCC, human hepatocellular carcinoma; HEPES, *N*-2-hydroxyethylpiperazine-*N*-ethane-sulphonicacid; HFD, high-fat diet; HRP, horseradish peroxidase; LC3-I, protein I light chain 3; LC3-II, protein II light chain 3; LDs, lipid droplets; LKB1, liver kinase B1; MDC, monodansylcadaverine; PLIN, perilipin; PMSF, phenylymethanesulfonyl fluoride; PNS, postnuclear supernatant; P-VO_2, nano-sized paramontrodeite VO_2 nanocrystals; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SOV, sodium orthovanadate; TBST, tris/buffered saline/Tween-20; TGs, triglycerides; VOdipic-Cl, diaqua-(4-chloro-2,5-dipicolinato)oxidovanadium(IV) complex; $VO(dmpp)_2$, bis(1,2-dimethyl-3-hydroxy-4-pyridinonato)oxovanadium(IV)

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several pathogenic conditions and diseases [10]. An impairment in hepatocyte autophagy may not only promote steatosis but also lead to progression to liver injury and steatohepatitis due to loss of autophagy's protective function against cell death [11]. Thus, autophagy has been considered as a new cellular target for abnormalities in lipid metabolism and accumulation [12].

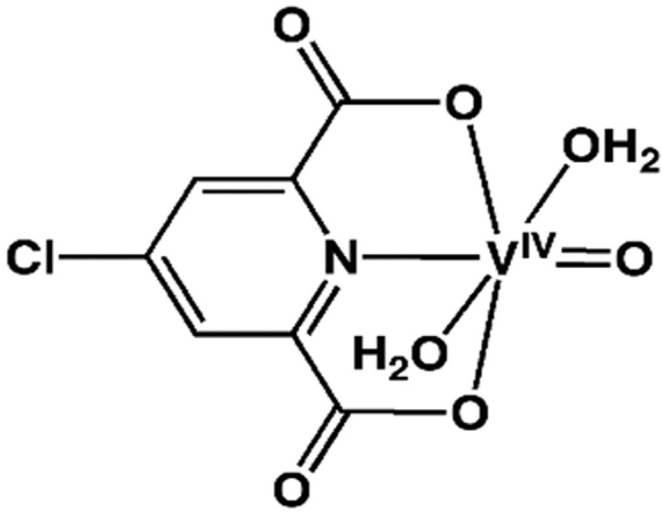
Added to the well-characterized turnover of proteins by autophagy, lipids can also undergo degradation by macroautophagy, by sequestration into autophagosomes that then fuse with lysosomes [13]. Autophagy-mediated lipolysis (macrolipophagy) selectively targets LDs, intracellular lipid stores that serve as energy source through hydrolysis of TGs into free fatty acids (FFA). LDs are surrounded by structural proteins of the perilipin (PLIN) family, with PLIN1 being primarily an adipocyte protein and PLIN2 and PLIN3 expressed ubiquitously [14]. On the other hand, lipid can be selectively degraded by the lysomal pathway of macroautphagy through a process termed lipophagy [15,16]. Lipophagy is another form of selective organelle removal by autophagy, which functions to regulate intracellular lipid stores, cellular levels of free lipids such as fatty acids and energy homeostasis [15]. Accumulating evidences have shown that adenosine monophosphate-activated protein kinase (AMPK) plays critical roles in regulating hepatic lipid metabolism [17,18]. Activation of AMPK down-regulates fatty acid biosynthesis and switches on fatty acid oxidation [19]. AMPK is phosphorylated at Thr^{172} by upstream kinases, among which LKB1 has been identified as a major AMPK kinase in the liver [17,20]. Moreover, AMPK has been linked to cellular processes including autophagy [21]. It has been reported that loss of AMPK results in aberrant accumulation of the autophagy adaptor p62 [22].

Vanadium compounds are generally regarded to have insulin-mimetic and anti-diabetic effects both *in vivo* and *in vitro* [23–25]. In addition to their action on glucose metabolism, vanadium compounds can modulate lipid metabolism both *in vitro* and *in vivo* [26,27]. In isolated hepatocytes [28] and adipocytes [29,30], sodium orthovanadate and diaqua-(4-chloro-2,5-dipicolinato)oxidovanadium(IV) (VOdipic-Cl, $[V^{IV}O(dipic\text{-}Cl)(H_2O)_2]$) complex modulates lipid metabolism by stimulating lipogenesis and suppressing lipolytic activity. Furthermore, $VO(dmpp)_2$, [bis(1,2-dimethyl-3-hydroxy-4-pyridinonato)oxovanadium(IV)] treatment of insulin-resistant, obese pre-diabetic Zucker rats significantly decreases hepatic triglyceride content [31]. VOdipic-Cl has also shown to alleviate hepatic lipid abnormalities [32,33] and ameliorate liver function in diabetic rats [34]. However, the precis mechanism by which vanadium compounds elicits anti-hyperlipidemic effects remain poorly characterized. Recently, the novel ability of vanadium to modulate the process of autophagy has been documented [35]. Nano-sized paramontrodeite VO_2 nanocrystals (P-VO_2) has been shown to induce cyto-protective autophagy in cultured HeLa cells [36]. Sodium orthovanadate (SOV) can effectively suppress the growth of human hepatocellular carcinoma (HCC) cells through the regulation of autophagy [37]. Therefore, the aims of this current study were to further investigate the effect of VOdipic-Cl-induced autophagy on lipid metabolism through regulation of LKB1-AMPK signaling pathway in palmitate-treated primary hepatocytes isolated from Sprague-Dawley rats and the liver tissue of high-fat diet (HFD)-fed C57BL/6 mice.

2. Materials and methods

2.1. Chemicals

The vanadium(IV)-chlorodipicolinate ($V^{IV}O(dipic\text{-}Cl)(H_2O)_2$, VOdipic-Cl) was prepared according to a previously reported procedure [30,33]. The schematic structure of VOdipic-Cl compound used in this study is shown in Fig. 1. Anti-LC3 antibody, 3-methyladenine (3-MA), palmitate, rapamycin, monodansylcadaverine (MDC), Oil Red O, tricine, sucrose, William's medium E and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma-Aldrich (MO, UAS). Anti-



$[V^{IV}O(dipic\text{-}Cl)(H_2O)_2](VOdipi\text{-}Cl)$

Fig. 1. Schematic structure of $V^{IV}O(dipic\text{-}Cl)(H_2O)_2$.

p62/SQSTM1 antibody was obtained from MBL (Nagoya, Japan). Anti-AMPKα antibody, anti-phospho-AMPKα (Thr^{172}), anti-LKB1 antibody and anti-phospho-LKB1 were obtained from Cell Signaling Technology (MA, USA). Anti-PLIN2 (anti-ADFP) was obtained from Abcam (MA, USA). 24-well plates and cell culture dishes were purchased from Costar Cambridge (MA, USA). Phenylymethanesulfonyl fluoride (PMSF) and *N*-2-hydroxyethylpiperazine-*N*-ethane-sulphonicacid (HEPES) were purchased from Ameresco (MA, USA). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum were detected using standard kits from Randox (Antrim, UK). The tissue triglyceride assay kit was obtained from Applygen (Beijing, China). Dorsomorphin was purchased from Selleck Chemicals (TX, UAS). Lipid dye BODIPY493/503 was purchased from Invitrogen (WI, USA). Water in experiments was ultra-pure deionized water (≥18.2 MΩ) obtained from a Milli-Q system (OH, UAS). All other chemicals used were of analytical grade.

2.2. Cell culture and treatment

Isolation of primary rat hepatocytes were performed by non-recirculating perfusion with collagenase as described previously [38]. In brief, the liver of male Sprague-Dawley rats (100–120 g, 6-week-old) was perfused with Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution containing 0.5 mM EDTA, 10 mM HEPES, 4.2 mM $NaHCO_3$, 0.5% type IV collagenase and 50 μg/ml trypsin inhibitor (pH 7.5). The liver was then removed and isolated cells were purified with 40% Percoll (GE Healthcare Bio-Sciences, USA). The cells were washed with Hanks' balanced salt solution (HBSS), followed by added to DMEM medium containing 10% fetal calf serum, 1% penicillin-streptomycin and 1% non-essential amino acids and re-suspended. Hepatocytes were cultured William's E medium containing 10% (W/V) fetal bovine serum (Wisent, Canada), 2 mM L-glutamate and penicillin-streptomycin (10,000 U/ml, Gibco, NY, USA) at 37 °C in a 5% CO_2 atmosphere. The cells were seeded at a density of 1×10^6 cells per well and cultured for another 24 h. And then, the cells were pretreated with 0.25, 0.5 and 0.75 mM palmitate for 24 h, respectively. Subsequently, the cells were treated with 50, 100 and 200 μM of VOdipic-Cl for 24 h, respectively.

2.3. Oil Red O staining

To visualize lipid accumulation in primary rat hepatocytes and liver, intracellular lipid accumulation was determined by Oil Red O staining [16]. The cells and frozen liver tissue sections were washed twice with