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Calcium channel blockers as potential therapeutics for obesity-associated autophagy defects and fatty liver pathologies

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Nonalcoholic fatty liver disease (NAFLD), typically associated with overnutrition and obesity, is one of the most common liver diseases both in the US and worldwide. During obesity and NAFLD, lipotoxic injuries to hepatocytes can provoke formation of protein inclusions consisting of SQSTM1/p62 and ubiquitinated proteins. It has been suggested that autophagy deregulation during obesity contributes to protein inclusion formation and progression of other liver pathologies including insulin resistance, steatohepatitis, and hepatocellular carcinoma. To examine how lipotoxicity and obesity affect autophagy, we established an in vitro system where cultured HepG2 cells exhibit prominent accumulation of SQSTM1 and ubiquitinated proteins in insoluble inclusion bodies upon treatment with saturated fatty acids. Using this system and a mouse model of obesity, we have determined that obesity induces chronic elevation of cytosolic calcium levels in hepatocytes, which interferes with the fusion between autophagosomes and lysosomes. Intriguingly, pharmacological inhibition of calcium channels using the FDA-approved drug verapamil successfully restores autophagic flux and suppresses protein inclusions, not only in HepG2 cells but also in mouse liver. Verapamil also reduces hepatic lipid droplet accumulation, insulin resistance and steatohepatitis, suggesting that calcium channel blockers can be used for correction of general NAFLD pathologies. Indeed, there have been a number of clinical observations in which beneficial effects of calcium channel blockers against obesity-associated metabolic pathologies are observed in humans and animal models.

Keywords: autophagic flux, calcium channel blocker, high fat diet, lipotoxicity, NAFLD, obesity, protein inclusions, verapamil

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For a long time, it has been observed that obesity and lipotoxicity generally increase the number of autophagosomes in a variety of cultured cells as well as in human and animal tissues. However, it is not known whether this accumulation is due to increased formation or decreased degradation of autophagosomes. HepG2, a human hepatoma cell line, is among the cells that accumulate autophagosomes in response to palmitate-induced lipotoxicity. Using 3 independent autophagic flux assays, including the microtubule-associated protein 1A/1B light chain 3B (MAP1LC3B or LC3)-I to LC3-II conversion assay, an autophagosome-lysosome colocalization assay and a flux indicator mCherry-GFP-LC3 assay, we have shown that palmitate induces autophagosome accumulation in HepG2 cells primarily through blocking the fusion between autophagosomes and lysosomes. Also in mouse liver, high fat diet (HFD)-induced obesity results in substantial dissociation between LC3- and LAMP1 (lysosomal-associated membrane protein 1)-positive vesicles, which respectively correspond to autophagosomes and lysosomes. Thus, we were able to conclude that lipotoxicity and obesity attenuate hepatic autophagic flux at the step where autophagosomes are degraded by fusion with lysosomes. This conclusion is consistent with another well-appreciated observation that obesity and NAFLD induce prominent accumulation of insoluble protein inclusions composed of SQSTM1 and ubiquitinated protein aggregates, which are substrates of autophagy. Thus, lipotoxic inhibition of

autophagic flux could be one of the mechanisms underlying how protein aggregates are formed in hepatocytes during obesity and NAFLD.

Our results suggest a potential mechanism of how lipotoxicity can impair the autophagosome-lysosome fusion process. ATP2A2/SERCA2 is an endoplasmic reticulum (ER) calcium pump that is expressed in liver cells and inhibited by saturated fatty acid accumulation in the ER membrane. Pharmacological ATP2A2 inhibitors, just like the saturated fatty acids, strongly impair autophagosome-lysosome fusion in HepG2 cells. Conversely, overexpression of ATP2A2 partially mitigates the autophagic arrest induced by palmitate treatment, suggesting that ATP2A2, whose function is required to maintain autophagic flux, may be the target of palmitate. Because calcium channel blockers strongly suppress the lipotoxicity-induced autophagy defects, we concluded that chronically increased cytosolic calcium levels seen during obesity are responsible for the observed autophagic arrest.

Based on these findings, we hypothesized that calcium channel blockers might be beneficial in correcting obesity-induced autophagy defects and their pathological sequelae. Indeed, administration of verapamil, an FDA-approved calcium channel blocker, to obese mice restores the fusion between autophagosomes and lysosomes, reduces the accumulation of both protein inclusions and lipid droplets, and ameliorates fatty liver-associated pathologies such as inflammation and insulin resistance. In addition to calcium channel inhibition, ATP2A2 reactivation could provide an alternative approach to reduce cytosolic calcium and restore autophagy. Several former reports support this rationale and show that viral overexpression of ATP2A2 in liver restores obesity-associated hepatosteatosis and insulin resistance. However, because development of ATP2A2 activators has been technically challenging, safe pharmacological methods for activating ATP2A2 *in vivo* have not been established yet. Thus, using calcium channel blockers, rather than ATP2A2 activators, would be a more immediate option as an autophagy-correcting therapeutic method.

As calcium controls many cellular processes, we can speculate that global inhibition of calcium channels can unintentionally block some critical physiological processes other than autophagy regulation, which may result in undesirable side effects. However, many calcium channel blockers including verapamil have been safely used in humans for a long period of time, suggesting that any adverse effects would be largely trivial and that calcium channel blockers may be even beneficial for some co-morbidities of obesity such as cardiovascular diseases. Indeed, we have shown that verapamil administration significantly improves cardiac functionality in obese mice. To minimize any unexpected off-target effects, we propose to deliver calcium channel blockers specifically into the liver where they will function as autophagy promoters, especially considering that many liver-specific drug delivery methods are currently available. Because there has already been ample clinical evidence that calcium channel blockers may improve physiological homeostasis during obesity, which were identified through semantic-based literature mining technology as described in our study, the therapeutic potential of calcium channel blockers against obesity-associated metabolic pathologies is very promising and warrants further mechanistic and clinical investigation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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