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Phosphate-induced autophagy counteracts vascular calcification by reducing matrix vesicle release

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Autophagy is a dynamic and highly regulated process of self-digestion responsible for cell survival and reaction to oxidative stress. As oxidative stress is increased in uremia and is associated with vascular calcification, we studied the role of autophagy in vascular calcification induced by phosphate. In an *in vitro* phosphate-induced calcification model of vascular smooth muscle cells (VSMCs) and in an *in vivo* model of chronic renal failure, autophagy was inhibited by the superoxide dismutase mimic MnTMPyP, superoxide dismutase-2 overexpression, and by knockdown of the sodium-dependent phosphate cotransporter Pit1. Although phosphate-induced VSMC apoptosis was reduced by an inhibitor of autophagy (3-methyladenine) and knockdown of autophagy protein 5, calcium deposition in VSMCs was increased during inhibition of autophagy, even with the apoptosis inhibitor Z-VAD-FMK. An inducer of autophagy, valproic acid, decreased calcification. Furthermore, 3-methyladenine significantly promoted phosphate-induced matrix vesicle release with increased alkaline phosphatase activity. Thus, autophagy may be an endogenous protective mechanism counteracting phosphate-induced vascular calcification by reducing matrix vesicle release. Therapeutic agents influencing the autophagic response may be of benefit to treat aging or disease-related vascular calcification and osteoporosis.

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Macroautophagy (hereafter referred to as autophagy) is a dynamic and highly regulated process of self-digestion. It is a highly conserved cellular process responsible for removal or recycling of long-lived proteins and organelles, and provides cells with an alternative source of nutrients from the reuse of cellular proteins and organelles.[1,2] This lysosomal degradation pathway is essential for cell survival, differentiation, and development, as well as the cellular response to stress, and thus is associated with neurodegenerative diseases, cancer, heart disease,[3,4] and arteriosclerosis.[5,6] Limited autophagy in response to nutrient starvation has a survival function, and specific removal of damaged mitochondria by autophagy can prevent the activation of apoptotic pathways.[7] However, in some systems, induced autophagy can contribute to or enhance the apoptotic response.[8]

Vascular calcification is a major risk factor of cardiovascular mortality, particularly in patients with end-stage renal disease.[9] Hyperphosphatemia, manifested during chronic renal failure (CRF) and subsequent dialysis, is highly associated with the extent of vascular calcification and contributes directly to high morbidity and mortality in vascular disease.[10] Phosphate (Pi) level was found to be important in vascular calcification in clinical trials[10] and *in vitro*[11] and *in vivo*[12] experimental models. The potential mechanisms mediating Pi-induced matrix calcification of vascular smooth muscle cells (VSMCs) include osteogenic/chondrogenic conversion, apoptosis, matrix vesicle (MV) release, and matrix remodeling.[13,14] Recently, we found that a high Pi level induced the production of mitochondrial $O_2^{\cdot -}$ and activated NF-κB to induce vascular calcification in both bovine aortic smooth muscle cells (BASMCs) and a rat model of CRF.[15,16]

Many studies have highlighted the important contribution of mitochondrial reactive oxygen species (ROS), especially superoxide ($O_2^{\cdot -}$), to inducing autophagy.[17–19] ROS-mediated basal autophagy has an important role in maintaining chondrocyte and osteoblast/osteocyte survival and terminal differentiation, as well as regulating bone growth.[20] However, whether autophagy is involved in Pi-induced vascular calcification remains to be elucidated.

Here, we examined the induction of autophagy by Pi-increased ROS levels, and the role of autophagy in Pi-induced calcification in VSMCs and a rat model of CRF.

RESULTS

High Pi promotes autophagy in VSMCs

To determine whether autophagy is involved in Pi-induced vascular calcification, we first examined the effect of Pi on autophagosome formation in VSMCs. Pi dose dependently (1.5–3 mmol/l) increased the level of the lipid-conjugated form of the autophagosome marker light-chain 3-II (LC3II) in BASMCs (Figure 1a). With lysosome-dependent degradation of LC3II blocked by chlorquinol (25 μmol/l), Pi further increased the LC3II level (Figure 1b). Increased accumulation of LC3 puncta tagged with green fluorescent protein (GFP) indicated Pi induction of autophagosomes (Figure 1c), and the proportion of cells with GFP–LC3 puncta was sustained from 0.5 to 7 days with Pi treatment (Figure 1d). Electron microscopy of typical autophagic structures in BASMCs gave direct evidence of the autophagy activation, and increased autophagic structures were observed in cells exposed to Pi for 12 h (Figure 1e). Increased autophagy was observed in parallel in human aortic VSMCs (HA-VSMCs; Figure 1f and Supplementary Figure S1 online). In addition, immunofluorescence analysis of LC3 puncta revealed autophagosome formation in renal artery walls of a patient with CRF (Figure 1g). Thus, high Pi promoted autophagy in both animal and HA-VSMCs.

ROS mediate Pi-induced autophagy in VSMCs

Mitochondrial ROS production contributes to autophagosome formation in many conditions, and the superoxide dismutase mimic MnTMPyP reduces Pi-induced calcification by eliminating mitochondrial ROS.[15] Here, Pi significantly increased mitochondrial ROS level in BASMCs (Figure 2a). MnTMPyP (25 μmol/l), as well as SOD2 overexpression, significantly decreased Pi-increased LC3II level in BASMCs (Figure 2b and c). These results were further confirmed by MnTMPyP, decreasing the Pi-increased formation of GFP–LC3 puncta from 47.2 to 20.3% in BASMCs (Figure 2d). Therefore, ROS generation mediated Pi-induced autophagy *in vitro*. In addition, MnTMPyP reduced LC3 level (Figure 2e) and mitochondrial ROS level (Figure 2f) in abdominal aortas of CRF rats with high serum Pi level (2.73 ± 0.10 mmol/l in control group vs. 5.50 ± 0.36 mmol/l in CRF group, $P < 0.05$). Indirect immunofluorescence analysis of LC3 puncta in both calcified and non-calcified regions of the aorta (Supplementary Figure S2 online) further showed that MnTMPyP decreased autophagosome formation in the abdominal aortic wall of CRF rats (Figure 2f).

Type III sodium-dependent Pi cotransporter Pit1 mediates Pi-induced autophagy

HA-VSMCs were transfected with scramble small interfering RNA (siRNA) or Pit1 siRNA for 48 h; knockdown of Pit1 expression was confirmed by reverse transcriptase PCR (Figure 3a) and western blot analyses (Figure 3b). Pi increased LC3II level in scramble siRNA–treated HA-VSMCs, and Pit1 knockdown significantly reduced Pi-increased LC3II level (Figure 3c). Similarly, Pit1 knockdown significantly decreased Pi-induced calcium deposition (Figure 3d). Therefore, Pit1 mediated Pi-induced autophagy and calcification in VSMCs.

Inhibition of Pi-induced autophagy aggravates calcification

To address the potential role of autophagy in Pi-induced calcification in VSMCs, we first used 3-MA (5 mmol/l), a pharmacological inhibitor of autophagy. 3-MA attenuated the Pi-increased LC3II level (Figure 4a) and formation of LC3 puncta (43% reduction) (Figure 4b). To confirm the pharmacological results of 3-MA, siRNA knockdown of autophagy protein 5 (Atg5) expression in rat VSMCs (Figure 4c and Supplementary Figure S3 online) resulted in a significant reduction of LC3 puncta in Pi-treated rat VSMCs (Figure 4d).

Alizarin red S staining (Figure 5a) and calcium content assay (Figure 5b) revealed that 3-MA increased calcium deposition in BASMCs pretreated with Pi (3 mmol/l) at 3, 7, and 10 days. Similarly, in HA-VSMCs (Figure 5c) and rat VSMCs (Figure 5d), 3-MA significantly increased Pi-induced calcium deposition. These findings were further confirmed by siRNA knockdown of Atg5, which significantly augmented Pi-induced calcium deposition in both primary rat VSMCs (Figure 5e) and the rat SMC cell line A7r5 (Figure 5f). Furthermore, *ex vivo* experiments of rat aortic rings showed that 3-MA aggravated Pi-induced calcification (Figure 5g). In contrast, valproic acid (1 mmol/l), a pharmacological inducer of autophagy, significantly ameliorated Pi-induced calcium deposition in both cultured rat aortic rings (Figure 6a) and BASMCs (Figure 6b). Therefore, autophagy ameliorated Pi-induced vascular calcification.

The pro-calcification effect of autophagy inhibition does not involve ROS-induced apoptosis

Autophagy induction under cellular stress may contribute to cell apoptosis or be a mechanism for cell survival, and VSMC apoptosis is one of the mechanisms of Pi-promoted vascular calcification.[21,22] As expected, Pi increased the activity of apoptosis marker–cleaved caspase-3 (Figure 7a) and caspase-3/7 (Figure 7b). These increases were reversed by siRNA knockdown of Atg5 and MnTMPyP in rat VSMCs. Consistently, both 3-MA and MnTMPyP reduced the Pi-increased apoptosis (indicated by terminal transferase dUTP nick-end labeling staining) (Figure 7c and Supplementary Figure S4 online) and caspase-3/7 activity (Figure 7d) in BASMCs. Interestingly, MnTMPyP and autophagy inhibition by Atg5 siRNA knockdown or 3-MA had a synergistic effect on reducing caspase 3/7 activation and apoptosis (Figure 7b–d). However, the apoptosis inhibitor Z-VAD-FMK (20 μmol/l) significantly ameliorated Pi-induced calcification; 3-MA still significantly aggravated Pi-induced calcification, even in the presence of Z-VAD-FMK (Figure 7e).