Protein carbamylation exacerbates vascular calcification

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Protein carbamylation is a posttranslational modification that can occur non-enzymatically in the presence of high concentrations of urea. Although carbamylation is recognized as a prognostic biomarker, the contribution of protein carbamylation to organ dysfunction remains uncertain. Because vascular calcification is common under carbamylation-prone situations, we investigated the effects of carbamylation on this pathologic condition. Protein carbamylation exacerbated the calcification of human vascular smooth muscle cells (hVSMCs) by suppressing the expression of ectonucleotide pyrophosphate/phosphodiesterase 1 (ENPP1), a key enzyme in the generation of pyrophosphate, which is a potent inhibitor of ectopic calcification. Several mitochondrial proteins were carbamylated, although ENPP1 itself was not identified as a carbamylated protein. Rather, protein carbamylation reduced mitochondrial membrane potential and exaggerated mitochondria-derived oxidative stress, which down-regulated ENPP1. The effects of carbamylation on ectopic calcification were abolished in hVSMCs by ENPP1 knockdown, in mitochondrial-DNA-depleted hVSMCs, and in hVSMCs treated with a mitochondria-targeted superoxide scavenger. We also evaluated the carbamylation effects using *ex vivo* and *in vivo* models. The tunica media of a patient with end-stage renal disease was carbamylated. Thus, our findings have uncovered a previously unrecognized aspect of uremia-related vascular pathology.

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Vascular calcification, a pathologic condition that erodes the compliance and elastance of the aorta, is common in aging and in chronic kidney disease (CKD).[1] Because vascular calcification is an independent predictor of mortality,[2,3] numerous studies in both animals and humans have investigated its underlying pathologic mechanisms.[4–6] However, several key aspects of vascular calcification remain unresolved.

Posttranslational protein modifications are involved in the mechanisms of various human diseases. Carbamylation comprises an irreversible posttranslational protein modification that can occur nonenzymatically in the presence of urea.[7,8] In the human body, urea exists in equilibrium with cyanate and its reactive form isocyanate, which can add the carbamoyl moiety [-$CONH_2$] to the amino groups of free amino acids and the N^{ε}-amino group of protein lysine residues (homocitrulline).[9] Myeloperoxidase-dependent oxidation of thiocyanate has been recently revealed as an alternative pathway that induces cyanate in the body.[10] Notably, carbamylation is recognized as a prognostic biomarker in humans. Positive association with mortality has been reported for the serum level of protein-bound homocitrulline in patients undergoing maintenance hemodialysis, the plasma level of carbamylated low-density lipoprotein in predialysis patients with CKD, and the plasma level of protein-bound homocitrulline in non-CKD study participants.[10–12] These reports suggested that protein carbamylation is associated with mortality regardless of CKD stage. However, the mechanism by which protein carbamylation affects mortality remains uncertain.

Here, we investigated whether protein carbamylation exacerbates medial vascular calcification (VC). We hypothesized that protein carbamylation would exaggerate medial VC based on several points. First, medial calcification is common in patients with CKD whose protein carbamylation ratios are elevated.[13] Second, medial calcification is also common in elderly individuals,[1] and Gorisse *et al.*[14] recently reported that the protein carbamylation ratio is elevated in the elderly. Third, a low-protein diet exacerbates medial VC in uremic rats through unknown mechanisms,[15] and Berg *et al.*[13] have reported that mice with low-protein-diet–induced amino acid deficiencies showed increased susceptibility to carbamylation.

Accordingly, we performed various assays to examine carbamylation effects using *in vitro*, *ex vivo*, and *in vivo* models of medial calcification. Our results provide novel insights into the biology of protein carbamylation.

RESULTS

Protein carbamylation promotes calcification of hVSMCs by suppressing ENPP1

We used cultured human vascular smooth muscle cells (hVSMCs) to assess the effects of protein carbamylation on medial VC. hVSMCs were first treated with urea at 250 mmol/l for 3 days and then cultured in normal or calcifying medium for 20 days without urea. Urea was not included during the 20-day culture because we aimed to examine effects of carbamylation, not the direct effects of urea. Equimolar mannitol-pretreated cells served as control cells. Both quantification of cellular minerals and alizarin red staining revealed that urea pretreatment exaggerated calcification under calcifying conditions (Figure 1a and b). To more directly evaluate the effects of carbamylation, we performed similar experiments using 10 mmol/l of cyanate as a carbamylation inducer. Mineral quantification revealed that cyanate pretreatment also exaggerated hVSMC calcification in the calcifying medium (Figure 1a and b).

To investigate the molecular mechanisms underlying the aforementioned process, we performed real-time polymerase chain reaction (PCR) analyses, which revealed that culturing hVSMCs in the calcifying medium resulted in up-regulated mRNA expression of ectonucleotide pyrophosphate/phosphodiesterase 1 (ENPP1) (Figure 1c). ENPP1 constitutes the key enzyme that hydrolyzes extracellular adenosine triphosphate (ATP) into adenosine monophosphate and pyrophosphate (PPi). Because PPi is a potent inhibitor of ectopic mineralization, the up-regulation of ENPP1 in the calcifying medium serves as a counterregulatory response that suppresses calcification.[16–18] Here, both urea pretreatment and cyanate pretreatment suppressed the counterregulatory elevation of ENPP1 under calcifying conditions (Figure 1c). Pretreatment with cyanate but not urea suppressed runt-related transcription factor 2 (RUNX2) expression under calcifying conditions. Because RUNX2 is an inducer of VC, down-regulated RUNX2 did not contribute to the pathogenesis in this study.[19,20] Moreover, protein carbamylation did not affect the levels of other calcification-related molecules, including bone morphogenic protein 2, tissue-nonspecific alkaline phosphatase (TNAP), osteoprotegerin, and human homolog of progressive ankyloses (Figure 1c). Western blotting analyses further confirmed that protein carbamylation suppressed the counterregulatory elevation of ENPP1 under calcifying conditions (Figure 1d). Consequently, PPi levels in the calcifying culture medium decreased when cells were pretreated with either urea or cyanate (Figure 1e). Cyanate pretreatment also suppressed PPi levels in the normal culture medium (Figure 1e). Exogenous PPi added into the culture media suppressed calcification of urea- or cyanate-pretreated hVSMCs in a dose-dependent manner (Figure 2a). Calcification was exacerbated by protein carbamylation in nontargeted short hairpin RNA (shRNA)-transfected control hVSMCs, but not in ENPP1-knockdown cells (Figure 2b and c). All these data indicated that the suppression of ENPP1 played essential roles in the effects of carbamylation on ectopic calcification.

We also examined whether inflammation, a key factor that exacerbates VC, contributed to the pathogenesis of protein carbamylation. Levels of interleukin-6 and -1β were not elevated by carbamylation (Supplementary Figure S1).

Glycylglycine, an inhibitor of carbamylation, suppresses ectopic calcification in hVSMCs

Western blotting analyses of proteins isolated from urea- or cyanate-treated hVSMCs revealed that multiple proteins were carbamylated (Figure 3a and b). We also analyzed protein carbamylation levels in rat aortic tissues. Samples were obtained from 6-week-old male Sprague Dawley (SD) rats that had been randomly divided into 2 groups and injected for 3 days with either isotonic saline ("vehicle" group) or cyanate ("cyanate" group). In agreement with the *in vitro* results, multiple aortic proteins were carbamylated in the tissue from the cyanate group (Figure 3c). To analyze effects of carbamylation on ectopic calcification more precisely, we inhibited carbamylation by treating hVSMCs with glycylglycine, a compound that has been reported to inhibit carbamylation.[13] Western blotting analysis showed that glycylglycine inhibited protein carbamylation (Figure 3d). The extent of calcification was attenuated in glycylglycine-pretreated hVSMCs (Figure 3e). We confirmed the specificity of the anti-carbamylated-lysine antibody used in this study, which detected carbamylated bovine serum albumin (BSA) but not unmodified or glycated BSA in Western blots (Supplementary Figure S2).

Mitochondria play essential roles in carbamylation effects

We used immunoprecipitation in combination with mass spectrometry (MS) and identified carbamylated proteins in hVSMCs and rat aortae (Tables 1 and 2). The results showed that several of the same mitochondrial and cytoskeletal proteins were carbamylated both *in vitro* and *in vivo* (underlined molecules in Tables 1 and 2). We confirmed the carbamylation of vimentin, a molecule listed in Tables 1 and 2, by an immunoprecipitation assay (Supplementary Figure S3). Notably, ENPP1 was not detected as a carbamylated protein (Tables 1 and 2). In addition, we confirmed that ENPP1 was not carbamylated through an immunoprecipitation assay (Supplementary Figure S3). Because ENPP1 expression was inhibited at the transcriptional level (Figure 1c), our results suggested that the carbamylation of proteins other than ENPP1 caused the suppression of ENPP1 expression.

To investigate whether the carbamylation of mitochondrial proteins, such as ATP synthase subunits α and β (Tables 1 and 2), plays essential roles in exacerbating calcification, we used hVSMCs in which the mitochondrial DNA had been depleted but the nuclear DNA had been retained: ρ0-hVSMCs. Although ATP synthase subunits α and β are encoded by nuclear DNA, these subunits cannot fully function as ATP synthase (complex V of the oxidative phosphorylation pathway) without 2 subunits encoded by mitochondrial DNA, ATPase 6 and ATPase 8.[21–23] Depletion of mitochondrial DNA in ρ0-hVSMCs was