the ion-sensitive electrode (corrected for changes in ionic strength) revealed a very weak interaction between thiosulfate and Ca\(^{2+}\) \((K_a = 10.9 \pm 1.0 \times 10^{-6} \text{ M}^{-1})\) that resulted in a 4% decrease in ionized Ca\(^{2+}\) in culture medium at 5 mM thiosulfate and a corresponding 5% increase in the solubility product for calcium–phosphate. Adjustment of the total Ca\(^{2+}\) concentration to account for this did not prevent the inhibition of aortic calcification by thiosulfate. Thiosulfate did not inhibit hydroxyapatite formation from seed crystals or the calcification of purified elastin and did not alter medium pH.

**Conclusions.** Thiosulfate inhibits vascular calcification at millimolar concentrations through a direct extracellular effect that does not require intact smooth muscle cells but is related to cellular injury. This effect is not specific for thiosulfate since sulfate has similar properties. Inhibition
cannot be explained by effects on ionized calcium, calcium–phosphate solubility, pH, oxidative stress or hydroxyapatite formation.

Introduction

Calcification of the medial (smooth muscle) layer of arteries is common in advanced renal failure and almost certainly contributes to the accelerated cardiovascular disease in this condition. Treatment is primarily preventive and consists of controlling hyperphosphatemia and calcium intake. Other treatments that may directly inhibit the calcification processes have been proposed but have not been rigorously tested. One such therapy is sodium thiosulfate, which has been successfully used to control calcium nephrolithiasis [1,2] and which has been reported to reverse calcific uremic arteriolopathy (CUA) [3–5] and uremic soft tissue calcification [6] in case reports. Recently, thiosulfate was shown to inhibit aortic calcification in rats with renal failure [7], providing the first scientific evidence of efficacy in vascular calcification. The mechanism by which thiosulfate affects medial calcification is unknown and has been attributed to binding of calcium ions, increased solubility of calcium thiosulfate, acidosis and antioxidant properties [1,7] with little supporting data. It is also not clear whether thiosulfate acts directly on calcium deposits, via effects on vascular smooth muscle cells, or indirectly through systemic effects. Despite the uncertainties about its efficacy, mechanism of action and safety, thiosulfate is widely used to treat CUA and its effect on large vessel calcification is the subject of several clinical trials.

In this study, physicochemical measurements are combined with studies in an ex vivo model of medial vascular calcification in an attempt to elucidate the mechanism of action of thiosulfate.

Materials and methods

Aortic calcification in vitro

Aortas were cultured from adult male Sprague–Dawley rats as previously described [8]. Briefly, aortas were dissected from surrounding tissue and perfused with physiologic saline under sterile conditions. After most of the adventitia was removed, the aortas were cut into 2–3 mm rings and placed in high-phosphate Dulbecco’s modified Eagle’s medium (DMEM) containing 1.8 mM calcium, 3.8 mM phosphate and 45Ca, with medium changes every 3 days. Calcification was induced either by adding calf intestinal alkaline phosphatase (3.75 U/mL) to the medium or by injuring the aorta by rubbing with a cotton swab prior to culture. After 9 days, the elastin was pelleted, washed three times with buffered saline and extracted with 1 M HCl to remove 45Ca for counting by liquid scintillation.

Elastin calcification

Purified elastin (Elastin Products Co., Owensville, MO) was suspended in DMEM containing 3.8 mM phosphate and 45Ca. At specified times, the elastin was pelleted, washed three times with buffered saline and extracted with 1 M HCl to remove 45Ca for counting by liquid scintillation.

Results

Direct effects of thiosulfate on medial vascular calcification were examined in an ex vivo model of rat aortas cultured in high-phosphate medium [8]. Normal aortas do not calcify under these conditions but do calcify when alkaline phosphatase is added to the medium to remove inhibitory pyrophosphate or when aortas are mechanically injured prior to culture. As shown in Figure 1A, sodium thiosulfate (Na2S2O3) inhibited calcification of injured aortas but only at concentrations that exceeded 1 mM. Inhibition of calcification was also observed in aortas that had been devitalized by freezing and thawing prior to culture (Figure 1B). However, there was no inhibition of calcification in uninjured aortas cultured with alkaline phosphatase (Figure 1C). Sulfate, a related divalent anion, also inhibited calcification of cultured aortas at a similar concentration (Figure 2A). Calcification was also inhibited by NaCl at the same ionic strength, but to a much smaller degree than Na2S2O3 (Figure 2B). To determine whether thiosulfate can reverse calcification, release of 45Ca from calcified aortas was measured as previously described [9]. This release follows a biexponential decline with a small pool showing a half-life of less than a day and the bulk of the calcium having a half-life of >100 days [9]. Thiosulfate did not alter the half-life of either pool (15.7 ± 0.4 versus 17.0 ± 0.8 and 3382 ± 313 versus 3642 ± 350 h).

Additional studies were performed to determine whether thiosulfate directly interferes with calcium crystallization. First, the interaction between thiosulfate and calcium ions was investigated by measuring free calcium in tissue culture medium with an ion-selective electrode at a fixed total CaCl2 concentration and varying amounts of Na2S2O3. As shown in Figure 3A, thiosulfate had a very weak effect on ionized calcium, lowering it <4% at 5 mM. The kinetics were consistent with a simple first-order reaction with an association constant of 10.9 ± 1.0 × 10⁶ M⁻¹ (Figure 3B). A similar value was obtained in the high-phosphate DMEM (10.0 ± 0.68 × 10⁶ M⁻¹). The association constant for sulfate was 58% greater than for

Hydroxyapatite formation

This was performed as previously described [10] with some modifications. Hydroxyapatite (final concentration of 0.112 mg/mL) was added to a solution of 1.67 mM CaCl₂, 0.2 mM KH₂PO₄ and 0.8 mM K₂HPO₄ in 150 mM NaCl at room temperature. The pH was adjusted to 7.4 and the amount of NaOH required to maintain that pH was recorded. The rate of hydroxyapatite formation was calculated based on 8 mol of protons released per mole of hydroxyapatite formed.

Statistics

Results are presented as means ± standard errors. Significance was determined by two-tailed t-tests.

Materials and methods

Aortic calcification in vitro

Aortas were cultured from adult male Sprague–Dawley rats as previously described [8]. Briefly, aortas were dissected from surrounding tissue and perfused with physiologic saline under sterile conditions. After most of the adventitia was removed, the aortas were cut into 2–3 mm rings and placed in high-phosphate Dulbecco’s modified Eagle’s medium (DMEM) containing 1.8 mM calcium, 3.8 mM phosphate and 45Ca, with medium changes every 3 days. Calcification was induced either by adding calf intestinal alkaline phosphatase (3.75 U/mL) to the medium or by injuring the aorta by rubbing with a cotton swab prior to culture. After 9 days, the rings were washed, extracted with 1M HCl, dried and weighed. Radioactivity in the extract was counted by liquid scintillation. Release of calcium from calcified aortas containing 45Ca was measured as previously described [9].

Ionized calcium

Ionized calcium was measured with a Ca-selective electrode (radiometer) in DMEM maintained at 37°C in a 5% CO₂ environment. Na2S2O3 was added in increments from 1 M solutions. The electrode was calibrated with different CaCl₂ concentrations in 150 mM NaCl and 10 mM HEPES, pH 7.4.
Thiosulfate and vascular calcification

To test whether this small decrease in ionized calcium could explain the inhibition of vascular calcification, aortas were cultured with increased calcium concentration to compensate for the effect of thiosulfate. As shown in Figure 3C, calcification was still inhibited by thiosulfate. Addition of 5 mM sodium thiosulfate to culture medium in 5% CO₂ did not alter the pH (not shown).

Next, the effect of thiosulfate on the interaction of calcium and phosphate was investigated. Solubility of calcium phosphate was determined by gradually increasing the concentration of phosphate (added as a balanced mixture of NaH₂PO₄ and Na₂HPO₄ to maintain pH at pH 7.4) in a 1.8 mM solution of CaCl₂ in 150 mM NaCl and 10 mM HEPES until a precipitate formed. Na₂S₂O₃ at 5 mM increased the solubility product just 5% from 19.6 × 10⁻³ to 20.6 × 10⁻³, consistent with the decrease in ionized calcium observed above. The effect on hydroxyapatite formation was assessed by measuring proton production after adding seed crystals of hydroxyapatite to a solution of calcium and phosphate. Each mole of hydroxyapatite formed yields 8 moles of protons. As shown in Figure 4, neither 5 mM Na₂S₂O₃ or 15 mM NaCl (control for ionic strength) altered the rate of hydroxyapatite formation. By contrast,

**Fig. 1.** Effect of sodium thiosulfate on calcification of rat aortas in culture. (A) Injured aortas. *P = 0.005, **P < 0.0001 versus no thiosulfate. (B) Frozen and thawed aortas. *P = 0.029, **P = 0.014, ***P < 0.001 versus no thiosulfate. (C) Uninjured aortas cultured with alkaline phosphatase. Control aortas were incubated in the same medium but without injury, freezing or alkaline phosphatase. Results are the means ± SE of at least 14 separate aortic rings.

**Fig. 2.** Effect of sodium sulfate and sodium chloride on calcification of rat aortas in culture. (A) Injured aortas cultured with 5 mM sodium thiosulfate or 5 mM sodium sulfate. *P < 0.0001 versus control. (B) Injured aortas cultured with 5 mM sodium thiosulfate or 15 mM of additional sodium chloride. *P < 0.001 versus control or additional NaCl, **P = 0.005 versus control. Control aortas were injured and incubated in the same medium without Na₂SO₄, Na₂SO₃ or additional NaCl. Results are the means ± SE of at least 14 separate aortic rings.
2 μM pyrophosphate reduced the rate by about half, and this was also not affected by thiosulfate. Sulfate produced an 8% increase in hydroxyapatite formation that was not significant (not shown).

The possibility that thiosulfate alters the binding of calcium to the vessel wall was explored by incubating freshly isolated aortas from normal rats with 45Ca in a physiologic salt solution containing 1 mM calcium followed by rapid washing in ice-cold buffer without 45Ca. To minimize cellular uptake, the incubations were performed at room temperature for only 15 min. The amount of Ca incorporated into the vessels was 11.5 ± 0.6 nmol/mg in the presence of 5 mM Na2S2O3 compared with 11.9 ± 0.3 nmol/mg in the absence of thiosulfate. Since elastin is the initial site of medial vascular calcification, the calcification of purified elastin was also examined. As shown in Figure 5, there was a progressive increase in calcium incorporation into elastin that was not altered by 5 mM Na2S2O3.

Discussion

Thiosulfate has been used to treat nephrolithiasis [1], tumoral calcification [6] and vascular calcification [3–5] without definitive evidence of efficacy. However, two recent studies in animals have shown that thiosulfate reduces uremic vascular calcification [7] and calcium nephrolithiasis [2]. Thiosulfate was also found to prevent accumulation of calcium in uremic kidneys and heart
Thiosulfate and vascular calcification

[7]. While studies in vivo have suggested a beneficial effect on vascular calcification, they have provided very little information on the mechanism and have left investigators to speculate among a myriad of potential actions. This is the first in vitro examination of thiosulfate and vascular calcification, and although the precise mechanism by which thiosulfate inhibits vascular calcification was not identified, there were several important findings that narrow the possibilities and disprove some popular hypotheses.

The fact that thiosulfate inhibited calcification of aortas in culture indicates a direct effect on vessels independent of systemic actions although additional systemic actions are not ruled out. In particular, Na$_2$S$_2$O$_3$ increases urinary calcium excretion in rats [2,7] but this was not observed in humans [1] and would not occur in patients with end-stage kidney disease. Concentrations of several millimolar were required to inhibit aortic calcification in vitro, but this is within the range of serum concentrations achieved in patients with end-stage renal disease (ESRD) [3] and rats with renal failure [7] treated with Na$_2$S$_2$O$_3$. The inhibition of calcification in devitalized aortas demonstrates that it is not occurring through an intracellular action in vascular smooth muscle cells. Although the findings point to a direct extracellular action of thiosulfate on the calcification process, there was no inhibition of calcification in uninjured aortas cultured with alkaline phosphatase. This was not explained by an interaction of thiosulfate with pyrophosphate since inhibition of hydroxyapatite formation by pyrophosphate was not altered by thiosulfate and thiosulfate did not alter PPI synthesis or hydrolysis in aortas (data not shown). These results suggest that thiosulfate inhibits calcification initiated by cell injury.

Sodium sulfate (Na$_2$SO$_4$) inhibited aortic calcification at similar concentrations, indicating that the effect is not specific for thiosulfate. NaCl at the same ionic strength produced much less inhibition, indicating that an increase in ionic strength was not the sole explanation. However, there was a significant inhibition by NaCl, suggesting that some of the inhibition of calcification by Na$_2$S$_2$O$_3$ was due to an increase in ionic strength. The finding with Na$_2$SO$_4$ has other important implications. Firstly, it disproves the hypothesis that the action of thiosulfate is related to the high solubility of calcium thiosulfate compared to other calcium salts [1] since the solubility of calcium sulfate is much lower. Secondly, it also eliminates the possibility that the effect of thiosulfate on calcification is related to its property as an antioxidant since sulfate is the terminal oxidation state of sulfur. It is unlikely that thiosulfate is acting through conversion to sulfate since it is stable at physiologic pH and it is oxidized to sulfite and sulfur rather than sulfate. Serum concentrations of sulfate are elevated in advanced kidney disease and ESRD, up to ~2 mM [11], suggesting that sulfate could be an endogenous inhibitor of vascular calcification in these patients.

Although thiosulfate is widely categorized as a calcium chelator, this has not been quantified. The term ‘chelator’ is not appropriate since it applies to compounds that bind ions at more than one site. Any interaction is best described as ion pairing. Such pairing was apparent in measurements of ionized calcium and in calcium phosphate solubility but was too weak to have a meaningful effect on ionized calcium concentrations in vivo, consistent with a previous study [2]. Furthermore, there was no inhibition of hydroxyapatite formation, and an increase in total calcium concentration to compensate for the small decrease in ionized calcium did not prevent the inhibition of aortic calcification by thiosulfate. Thiosulfate also did not alter the binding of calcium to aortas or the calcification of purified elastin. This large body of evidence indicates that the mechanism by which thiosulfate inhibits vascular calcification is independent of interactions with calcium or hydroxyapatite.

Hydroxyapatite formation is sensitive to pH and vascular calcification in vitro [12] and in vivo [13] is reduced by acidosis. Therefore, the expansion acidosis produced by sodium thiosulfate in vivo has been proposed as a mechanism for inhibition of vascular calcification [7,14]. However, the inhibition of calcification of cultured aortas by sodium thiosulfate occurred in the absence of any change in medium pH, indicating that acidification cannot be the mechanism.

The results of this study point to a direct extracellular action of Na$_2$S$_2$O$_3$ to inhibit vascular calcification that is shared by Na$_2$SO$_4$ and is only partly explained by an increase in ionic strength. This inhibition appears to be independent of any interaction with Ca$^{2+}$, effect on pH or interference with hydroxyapatite formation. The fact that thiosulfate only inhibited calcification of injured or devitalized aortas and not uninjured aortas or purified elastin also argues against a generalized effect on calcification and suggests inhibition specifically of calcification initiated by cellular injury. This could involve cellular fragments or breakdown products, such as apoptotic bodies or matrix vesicles. It is likely that this effect occurs in other tissues since thiosulfate inhibits uremic calcification in kidneys and heart [7] as well as kidney stones [2]. Although the precise target of thiosulfate remains to be elucidated, the results of this study substantially clarify the potential mechanisms.

Acknowledgements. Funding. This work is supported by NIH grant DK069681.

Conflict of interest statement. None declared.

References


Received for publication: 4.2.11; Accepted in revised form: 6.6.11

doi: 10.1093/ndt/gfr302
Advance Access publication 9 June 2011

Atrial natriuretic peptide ameliorates peritoneal fibrosis in rat peritonitis model

Hiroshi Kato1,2,*, Tomohiro Mizuno1,2,*, Masashi Mizuno3, Akiho Sawai3, Yasuhiro Suzuki3, Hiroshi Kinashi3, Fumiko Nagura3, Shoichi Maruyama3, Yukihiro Noda1, Kiyofumi Yamada2, Seiichi Matsumo3 and Yasuhiko Ito3

1Department of Neuropsychopharmacology, Faculty of Pharmacy, Meijo University, Nagoya, Japan, 2Department of Neuropsychopharmacology and Hospital Pharmacy and 3Department of Nephrology and Renal Replacement Therapy, Nagoya University Graduate School of Medicine, Nagoya, Japan

Correspondence and offprint requests to: Yasuhiko Ito; E-mail: yasuit@med.nagoya-u.ac.jp
*These authors contributed equally to this work.

Abstract

Background. Atrial natriuretic peptide (ANP) was recently reported to ameliorate fibrosis in the heart and experimental renal diseases and vascular thickening after balloon injury. Peritoneal fibrosis is an important complication of long-term peritoneal dialysis, and peritonitis is a factor in its onset. In the present study, we investigated the effects of ANP in a rat peritonitis-induced peritoneal fibrosis model.

Methods. As pretreatment, an osmotic pump containing vehicle (saline) or ANP (0.15 or 0.3 μg/min) was inserted through the carotid vein in male Sprague–Dawley rats. ANP or saline was continuously infused using the osmotic pump. Three days after administration of ANP or saline, rats underwent peritoneal scraping in a blind manner and were sacrificed on Day 14. The effects of ANP were evaluated based on peritoneal thickness, immunohistochemistry and real-time polymerase chain reaction. In each experiment, we evaluated messenger RNA (mRNA) expression of the ANP receptor natriuretic peptide receptor A (NPR-A) in the peritoneum after scraping. The effects of ANP were also studied in cultured peritoneal fibroblasts and mesothelial cells.

Results. We observed a significant increase in NPR-A mRNA in the peritoneum. Peritoneal thickness increased with time and peaked on Day 14, but ANP significantly reduced peritoneal thickness. Parameters such as number of macrophages and CD-31-positive vessels and expression of type III collagen/transforming growth factor-β1/plasminogen activator inhibitor-1 (PAI-1)/connective tissue growth factor (CTGF) were significantly suppressed by ANP. In cultured peritoneal fibroblasts and mesothelial cells, ANP suppressed angiotensin II-induced upregulation of CTGF and PAI-1.

Conclusions. Our results suggest that ANP is useful in preventing inflammation-induced peritoneal fibrosis.

Keywords: ANP; CTGF; peritoneal fibrosis; renin-angiotensin system; TGF-β

Introduction

The characteristic feature of chronic peritoneal damage in peritoneal dialysis (PD) treatment is decreased ultrafiltration capacity associated with submesothelial fibrosis,