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Sinomenine, theophylline, cysteine, and levamisole: Comparisons of their kinetic effects on mineral formation induced by matrix vesicles

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ABSTRACT

The effects of sinomenine (SIN, an alkaloid extracted from the Chinese medicinal plant *Sinomenium acutum* used for centuries to treat rheumatic disease, including rheumatoid arthritis) on apatitic nucleation and matrix vesicle (MV)-induced mineral formation were compared with those of cysteine, levamisole, and theophylline. We found that SIN was not an inhibitor of tissue non-specific alkaline phosphatase (TNAP), a marker of biological mineralization, but confirmed that cysteine, levamisole, and theophylline were. Further, none of these four molecules directly affected the nucleation of hydroxyapatite (HA) formation, in contrast to pyrophosphate (PP_i) which did. Incubation of 0.25–1.0 mM cysteine, theophylline, or levamisole with MVs in synthetic cartilage lymph (SCL) containing AMP and Ca^{2+}, but not inorganic phosphate (P_i), prolonged the induction time of mineral formation, apparently by inhibiting TNAP activity. SIN at the same levels neither inhibited TNAP activity nor affected the induction time of MV mineral formation. However, SIN did markedly delay MV-induced mineral formation in SCL containing P_i (instead of AMP) in a manner similar to theophylline, but to a lesser extent than levamisole. Cysteine did not delay, in fact it slightly accelerated MV-induced mineral formation in Pi-containing SCL. These findings suggest that levamisole, SIN and theophylline may directly affect Ca^{2+} and/or P_i accretion during mineral formation; however, TNAP was not directly involved. The possible roles of annexins and other ion transporters, such as proteins of the solute carrier family implicated in Ca^{2+} and P_i influx are discussed.

1. Introduction

Sinomenine (SIN; 7,8-didehydro-4-hydroxy-3,7-dimethoxy-17-methylmorphinan-6-one) is an alkaloid belonging to the morphinan family, and extracted from the Chinese medicinal plant *Sinomenium acutum*. SIN has been used for many centuries to treat patients with rheumatic diseases, including rheumatoid arthritis (RA) [1,2]. SIN is known to have a broad pharmacological profile; therefore several molecular mechanisms may be implicated in its beneficial therapeutic effects. For example, the anti-arthritic mechanism of SIN may be related to its anti-proliferative effects on synovial fibroblasts [3]; it is also known to cause reduction of mRNA expression of pro-inflammatory cytokines including tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β), based on findings in rats with adjuvant arthritis [3,4]. The effects of SIN also may be due to the suppression of both Th1-type cytokine (Th1) and Th2-type cytokine (Th2) immune responses, as observed in collagen-induced arthritis in mice [5]. SIN is thought to reduce the degree of articular degeneration in rabbits with osteoarthritis through decreasing the content of IL-1β in synovial fluid and serum [6]; it is a potent anti-inflammatory and neuroprotective agent acting through inhibition of microglial NADPH oxidase [7]. SIN also reduces the production of prostaglandin E2 and nitric oxide by macrophages [8]. However, so far, SIN has not been evaluated for any direct effect on mineral formation. In this report, the effects of SIN on DMSO-induced hydroxyapatite (HA) formation, which mimics simple nucleation, as well as its effects on matrix vesicle (MV)-induced mineral formation, which mimics biological mineral formation, were studied at physiological pH and temperature (37 °C). The effects of SIN were compared with the effects of cysteine, levamisole, and theophylline. Cysteine [9–12], levamisole [13–16], and theophylline [17–22] are known inhibitors of tissue non-specific alkaline phosphatase (TNAP). Levamisole has been used for treating RA [23,24]. It has been proposed that cysteine could serve as a therapeutic option for calcium pyrophosphate dihydrate crystal deposition disease [12]. Theophylline, as well as other methyl xanthines (caffeine, 2-methoxyxanthines) are

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adenosine antagonists [25,26] and can be used to block adenosine receptors. The reported comparison of the effects of these molecules on TNAP activity, as opposed to inorganic phosphate (P_i) transport or on simple nucleation, reveal several aspects of the mechanism of MV mineralization. To address the effect of cysteine, levamisole, SIN and theophylline on the calcification process, DMSO-induced HA formation [27] was used to monitor their effects during simple nucleation of HA. Isolated MVs were then used to provide an easily quantifiable model for analysis of the initiation of biological HA using a synthetic extracellular lymph [28,29].

2. Experimental procedures

2.1. Chemicals

Cysteine, levamisole, and AMP were purchased from Sigma. Theophylline was purchased from China National Pharmaceutical Group Corporation. SIN was obtained from Chengdu Mansite Pharmaceutical Co., Ltd. (China). The purity of theophylline and SIN was further confirmed by ^{1}H NMR and mass spectrum analysis.

2.2. Preparation of synthetic cartilage lymph

Standard synthetic cartilage lymph (SCL) was prepared as a stock solution and frozen at −20 °C until used. The solution contained 100 mM NaCl, 63.5 mM sucrose, 16.5 mM *N*-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES), 12.7 mM KCl, 5.55 mM D-glucose, 1.83 mM $NaHCO_3$, 0.57 mM $MgCl_2{\cdot}6H_2O$, and 0.57 mM Na_2SO_4, pH 7.6 [30]. Added to the solution were 0–2 mM Ca^{2+}, 0–3.42 mM P_i, or 0–3.42 mM AMP, as indicated.

2.3. Extraction and characterization of matrix vesicles (MVs)

Collagenase-released MVs were isolated from bone and epiphyseal cartilage slices from 17-day-old chicken embryos according to Balcerzak et al. [31]. Bone tissue slices were digested at 37 °C for 3 h in SCL containing 2 mM Ca^{2+} and collagenase type IA (ICN Biomedicals Inc., 200 units/g of tissue using a volume of 4 mL g^{-1} of tissue). The partially digested tissue was vortexed, and the suspension was centrifuged at 13,000*g* for 20 min at 4 °C (Beckman J32B centrifuge, rotor JA20). The pellet was discarded, and the suspension was centrifuged again at 80,000*g* for 1 h at 4 °C (Kontron TGA centrifuge, rotor 6538). The MV pellet was resuspended in SCL omitting Ca^{2+} and P_i to prevent mineral formation. The MV suspension was prepared as a stock solution containing approximately 1–2 mg of vesicle protein/mL in SCL and stored at 4 °C for further use. The protein concentration in the MVs was determined using the Bradford method [32]. Electron-microscopy (Philips CM120 at 80 kV accelerating voltage, Centre Technologique des Microstructures, Lyon 1) was performed by diluting a drop of the MV suspension (25 μg MV protein/mL) and transferring the solution to carbon-coated grids. Prior to the complete drying of the MV samples, the grids were covered with 2% uranyl acetate solution according to the negative staining method. Electrophoresis was performed in 10% (w/v) SDS–polyacrylamide gel after protein denaturation at 100 °C for 3 min in Laemmli buffer with 5% (w/v) β-mercaptoethanol [33]. Proteins were stained with Coomassie Brilliant Blue R-250.

2.4. Alkaline phosphatase activity

To detect the differential effects of the soluble tissue non-specific alkaline phosphatase (TNAP) inhibitors cysteine, levamisole, theophylline, and SIN, the TNAP activity of MVs was determined under both alkaline and physiological conditions using a pH 10.4 buffer containing 25 mM piperazine, 25 mM glycylglycine, 5 mM $MgCl_2$, and 5 μM $ZnCl_2$, or a pH 7.5 buffer containing 0.1 M Tris–HCl with 5 mM $MgCl_2$ and 5 μM $ZnCl_2$, respectively. Mixtures containing the buffer, 13 μg mL^{-1} MV protein, and different concentrations (0–4 mM) of cysteine, levamisole, theophylline, or SIN were incubated at 37 °C for 10 min, and 0.1 mM *p*-nitrophenyl phosphate (*p*NPP) was added in the final seconds to initiate the reaction [34]. The activity was quantified at 420 nm using a molar absorption coefficient of 18.6 cm^{-1} mM^{-1} at pH 10.4 and 9.2 cm^{-1} mM^{-1} at pH 7.5. One unit of alkaline phosphatase activity was defined as the amount of enzyme required to hydrolyze 1 μmol *p*NPP per min at 37 °C. The specific activity of the MVs in the stock solution for mineralization was 15 ± 1 U mg^{-1} when averaged from five different experiments.

2.5. Mineralization assay

The light scattering method [35] was employed for the real-time measurement of MV-induced mineral formation (15–20 μg of MV protein) in SCL buffer with different ion (Ca^{2+}, P_i) or substrate (AMP) concentrations at pH 7.6 to a final volume of 1 mL. SIN, theophylline, cysteine, and levamisole were prepared as 10 mM stock solutions in SCL buffer. Neither SIN nor any of the three TNAP inhibitors up to 4 mM affected the pH of the SCL buffer (pH 7.6) by more than 0.04. The concentrations of ions, substrate, and inhibitors are indicated in the figure legends for each experiment. All samples were mixed vigorously and then incubated in the cuvettes at 37 °C without stirring. The real-time light scattering at 340 nm was automatically read at 15 min intervals. The amplitude of light scattering reflects the level of mineral formed in the SCL buffer. Each experiment was repeated at least three times. Formation of HA mineral was confirmed by FTIR and X-ray diffraction as previously reported [27].

2.6. DMSO-induced HA formation assay

The DMSO-induced HA assay was prepared as previously described [27]. HA formation was induced by 4% (v/v) DMSO in SCL medium containing 3.42 mM P_i and 2 mM Ca^{2+} within one hour [27]. To determine the direct inhibitory effect on HA formation, 1–4 mM cysteine, levamisole, theophylline, or SIN was employed in this system, PP_i being a negative control. The process of HA formation was monitored using the light scattering method as described above [35]. Formation of HA mineral was confirmed by FTIR and X-ray diffraction as reported [27].

3. Results

3.1. Characterization of matrix vesicles

The MVs isolated from the femurs of 17-day-old chick embryos were observed by transmission electron microscopy to be round-shaped organelles having diameters of 100–200 nm (Fig. 1A). Gel electrophoresis revealed three major protein bands with apparent molecular masses of 45 kDa, 37 kDa, and 31 KDa (Fig. 1B), which is typical of collagenase-released MVs [36–39]. MVs were able to mineralize and their apparent TNAP activities were around 15 U mg^{-1} MV protein, indicating TNAP enrichment, a landmark of MVs. Taken together, these findings indicated that the isolated MVs were functional and relatively pure.

3.2. Comparison of the effects of cysteine, levamisole, sinomenine and theophylline on alkaline phosphatase activity and on HA formation

SIN did not inhibit *p*NPP hydrolysis by TNAP at 37 °C and pH 10.4 (Fig. 2A) or pH 7.5 (Fig. 2B), which is in contrast to known