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INHIBITION OF CALCIUM PHOSPHATE-DNA COPRECIPITATES INDUCED CELL DEATH BY PHOSPHOCITRATES

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1. ABSTRACT

Phosphocitrate [PC] is a powerful inhibitor of biological crystallization and a potential disease modifying drug for crystal associated diseases such as crystals associated osteoarthritis [OA]. Recently, it has been reported that a new PC complex salt, calcium sodium PC [CaNaPC], is much more powerful than its precursor, sodium PC [NaPC], in reducing the size of chemically-induced calcified plaques in rat when examined using a calcergy assay (1). The molecular mechanisms underlying such a superior activity as a calcification inhibitor over its precursor NaPC are currently unknown. In order to evaluate the potential of CaNaPC as a disease modifying drug for crystals associated OA, we examined and compared CaNaPC and its precusor NaPC using several cell- based assays. CaNaPC was found to have an inhibitory potency similar to that of NaPC toward preventing the stimulating effects of basic calcium phosphate [BCP] crystals on the induction of MMP1, thymidine uptake and endocytosis. However, CaNaPC proved much more powerful than NaPC in the inhibition of amorphous calcium phosphate-DNA coprecipitates-induced cell death. These results suggest that the superior anti-calcification activity of NaCaPC over NaPC observed in rat is probably due to its superior activity in the inhibition of the effects associated with amorphous calcium phosphate clusters/aggregates/precipitates but not the effects associated with BCP crystals. Since amorphous calcium phosphate clusters/aggregates/precipitates are precursors of BCP crystals and coexist with calcium-containing crystals in calcified tissues (2-6), these amorphous clusters/aggregates/precipitates, similar to BCP crystals, may have played a significant role in pathological calcifications and in the development of crystals associated diseases such as crystals associated OA. The superior activity of CaNaPC over its precursor NaPC in the inhibition of amorphous calcium phosphate-DNA coprecipitates-induced cell death may, at least in part, explain its powerful anti-calcification activity *in vivo*. The findings suggest that CaNaPC through a dual action of inhibiting both the detrimental biological effects of formed BCP crystals and preforming amorphous calcium phosphate clusters/aggregates/precipitates, could present as a better disease-modifying drug for crystals associated OA than its parent NaPC.

2. INTRODUCTION

Basic calcium phosphate [BCP] and calcium pyrophosphate dihydrate [CPPD] crystals are the two most common forms of pathologic articular minerals. They are found inside the shoulder joints of Milwaukee shoulder syndrome patients and frequently in the knee joints of OA patients (7-9). The presence of BCP crystals correlates strongly with radiographic evidence of cartilaginous degeneration and synovial thickening and it is associated with larger joint effusions (10-12). There is compelling evidence that these crystals engender multiple biological effects that may promote joint degeneration (13-16). Studies have demonstrated that BCP crystals stimulate the proliferation of fibroblast-like synoviocytes [FLSs] and chondrocytes and induce the production of matrix metalloproteinase [MMP] (16-19). Both of the growth

stimulating- and MMP production inducing-effects of BCP crystals can partially be attributed to an abnormal calcium signaling caused by the deposition of BCP crystals on cells and the dissolution of endocytosed crystals (20-22). In addition to crystals associated OA, calcium-containing crystals and minerals are also associated with many other diseases including arteriosclerosis, diabetes, kidney stones, renal disease and certain human malignancies such as breast cancer (2, 23-26). Therefore, the development of calcification inhibitors to treat or to manage soft tissue calcification has recently drawn considerable interest.

PC is a naturally occurring compound and has been identified in mammalian mitochondria (27, 28). Its role in mitochondria is thought to be in a protective capacity. Mitochondria are often subjected to high concentrations of phosphate and calcium ions, so by inhibiting the interactions between minerals and mitochondrial membranes and by controlling nucleation and crystal growth, PC could offer protection to the vulnerable mitochondrial membrane. PC is strongly adsorbed to hydroxylapatite crystals and inhibits crystal growth or is adsorbed to amorphous calcium phosphate precipitates and inhibits the conversion of amorphous precipitates to crystals (27, 29). Since its original identification, PC has been shown to be a very powerful calcification inhibitor. It is considered to be one of the most powerful of the calcification inhibitors known to date, particularly when compared to the natural endogenous pyrophosphate molecule. PC inhibits BCP crystals-induced MMP synthesis and mitogenesis (30-32), nitric oxide-induced calcification of articular cartilage and the formation of chondrocyte-derived apoptotic bodies (33). In addition, PC inhibits BCP crystal deposition and disease progression in murine progressive ankylosis (34) and more recently, we demonstrated that NaPC inhibited the endocytotic activity of cells induced by BCP crystals (16). These data together suggest that PC is a potential disease-modifying drug for crystals associated diseases, such as OA. One aspect to consider which is unknown at this time is the concentration of PC needed to completely avert the dangers of BCP damage. Cell-based studies indicate that at least a concentration of 0.1 mM NaPC is needed to completely block BCP crystals-stimulated induction of MMPs. Since such a concentration would be difficult to achieve *in vivo* unless specific targeting were employed, the use of NaPC might be limited, implying that a more potent calcification inhibitor still needs to be developed.

Recently, a new PC salt, CaNaPC, has been reported. The structure of CaNaPC can be described as polymeric in nature with $Ca(PC)_2(H_2O)$ "monomers" linked through Na^+ bridges (1, 35). It was shown that CaNaPC was more powerful than its precursor NaPC as a calcification inhibitor. CaNaPC inhibited the formation of chemically-induced calcified plaque in rat at least 3-6 times more effectively than NaPC (1). The mechanisms underlying such improved anti-calcification activity are unclear. In this study, we examined the new PC salt CaNaPC together with its precursor NaPC using several cell-based assays including BCP crystals-simulated induction of MMP-1, thymidine uptake, endocytosis and amorphous calcium phosphate-DNA coprecipitates-induced cell death assays. We found that CaNaPC was much more powerful than its precursor NaPC in the inhibition of amorphous calcium phosphate-DNA coprecipitates-induced cell death.

3. MATERIALS AND METHODS

Telomerase immortalized human foreskin fibroblast cell line BJ1 was obtained from BD Bioscience (San Diego, CA) and cultured in Dulbecco's minimum essential medium [DMEM] supplemented with 10% fetal bovine serum [FBS], penicillin, streptomycin, and fungizone. Cervical carcinoma Hela cell line was obtained from the ATCC and cultured in McCoy' 5A medium supplemented with 15% fetal bovine serum, penicillin, streptomycin, and fungizone. DMEM, FBS, and stock antibiotic/antimycotic mixture were products of Invitrogen (Carlsbad, CA). BCP crystals were synthesized as described (36). The synthesis of NaPC and CaNaPC has previously been reported (1, 37).

3.1. RT-PCR

BJ1 cells were plated in 60 mm plates and grown to confluence. The cells were rendered quiescent by incubation in 0.5% FBS for 24 hours. Fresh medium containing increasing concentrations of NaPC or CaNaPC was added and followed immediately by 200 μg of BCP crystals (50 μg/ml). Twenty-four hours later, total RNA was extracted using Trizol Reagent according to the manufacturer's instructions (Invitrogen) and the RNA samples were subjected to RT-PCR using the ThermoScript RT-PCR System (Invitrogen). Briefly, 1 μg of each RNA sample was reverse transcribed at 60 ^{0}C for 60 minutes, followed by enzyme inactivation at 85 ^{0}C for 5 minutes. PCR was then performed using specific primers for human MMP1 (5'-GATCATCGGGACAACTCTCCT-3' and 5'-TCCGGGTAGAAGGGATTTGTG-3'). As an internal control, the housekeeping gene, β-actin, was amplified using specific primers for human β-actin (5'-GCTCGTCGTCGACAACGGCTC-3' and 5'-CAAACATGATCTGGGTCATCTTCTC-3').
Amplifications of the MMP1 gene were carried out for 30 cycles by denaturing at 95 ^{0}C for 30 seconds, annealing at 55 ^{0}C for 30 seconds and extending at 72 ^{0}C for 45 seconds, with a final extension at 72 ^{0}C for 10 minutes. The products were electrophoresed on 2 % agarose gels, stained with ethidium bromide and photographed using a light image system (ChemiImager 4000, Alpha Innotech Corporation, San Leandro, CA).

3.2. [^{3}H]Thymidine uptake

BJ1 cells were plated in 24-well cluster plate and grown until confluent. The cells were rendered quiescent by incubation in 0.5% FBS for 24 hours. Fresh medium containing different concentrations of NaPC or CaNaPC were added and followed immediately by 25 μg of BCP crystals/per well (100 μg/ml). Twenty three hours later, [^{3}H]thymidine (1 μCi/ml) was added to the wells and pulse-labeled for 1 hour (triplicates). The cells were washed three times with phosphate-buffered saline [PBS] and macromolecules were precipitated with 5% trichloroacetic

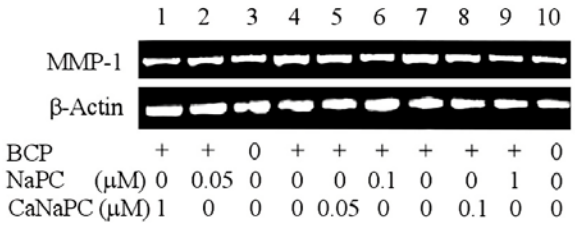

Figure 1. Inhibition of BCP crystals stimulated induction of MMP1 by NaPC and CaNaPC. Human fibroblast BJ1 cells were treated with BCP crystals in the presence of increasing concentrations of NaPC or CaNaPC. Twenty-four hours later, RNAs were extracted and given a number code. The RNA samples were then examined by a "blind" assessment by a second person using RT-PCR for MMP1 message. Lanes 3 and 10, control; Lanes 4 and 7, BCP crystals treatment; Lanes 2, 6 and 9, BCP crystals treatment in the presence of increasing concentrations of NaPC; Lanes 5, 8 and 1, BCP crystals treatment in the presence of increasing concentrations of CaNaPC. Both NaPC and CaNaPC inhibited the BCP crystals stimulated induction of MMP1 in a dose-dependent manner.

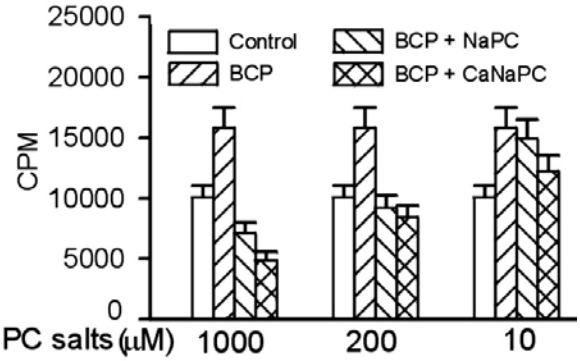

Figure 2. Inhibition of BCP crystals stimulated mitogenesis by NaPC and CaNaPC. Human fibroblast BJ1 cells were treated with BCP crystals in the presence of different concentrations of NaPC or CaNaPC. Twenty three hours later, [^{3}H]thymidine (1 μCi/ml) was added to the wells and pulse-labeled for 1 hour. Bar 1 in all three bar groups, control; Bar 2 in all three bar groups, BCP crystals treatment; Bar 3 in all three bar groups, BCP crystals treatment in the presence of decreasing concentrations of NaPC; Bar 4 in all three bar groups, BCP crystals treatment in the presence of decreasing concentrations of CaNaPC. Both NaPC and CaNaPC inhibited the BCP crystals stimulated thymidine uptake in a dose-dependent manner. Two independent experiments, each in triplicate, were performed. Data were expressed as means ± SEM.

acid solution. The precipitate was washed with PBS and dissolved in 1 ml 0.1 N NaOH, 1% SDS. Levels of trichloroacetic acid-precipitable ^{3}H were determined in triplicate, using a liquid scintillation counter (Packard Instruments, Downers Grove, IL).

3.3. BCP crystals stimulated endocytotic assay

Hela cells were plated at a density of 6 × 10^4 cells/well in 24-well cluster plates and grown until about 75% confluent. Fresh medium containing increasing concentrations of NaPC or CaNaPC and 1 μg pCMV-luciferase reporter plasmid were added into each well and followed immediately by 80 μg of BCP crystals (160 μg/ml). Eighteen hours later, cells were lysed and luciferase activity was determined using an EG&G Berthold Autolumat LB953 Rack Illuminometer. The luciferase activities were normalized relative to the amount of protein in the lysate as determined by using a total protein assay kit from PIERCE (Rockford, IL). Three independent treatments, each run in duplicate, were performed. Data were expressed as means ± SEM.

3.4. Calcium phosphate–DNA coprecipitates-induced cell death assay

Hela cells were plated at a density of 6 × 10^4 cells per well in 24-well cluster plates and grown until about 75% confluent. The cells were washed once with medium without serum and fresh medium containing increasing concentrations of NaPC and CaNaPC were added and followed immediately by 100 μl calcium phosphate–pUC18 DNA plasmid co-precipitates. Calcium phosphate-DNA plasmid coprecipitates were prepared following the procedure described previously (16). Eighteen hours later, cells were gently washed with medium once and subsequently incubated with medium containing 10% serum for another 24 hours. Cells were then photographed using phase contrast microscopy. Similar experiments were also performed using BJ1 cells.

4. RESULTS

4.1. NaPC and CaNaPC inhibited the BCP crystals-stimulated induction of MMP1 and mitogenesis

To determine whether CaNaPC had more potential as a disease modifying drug than NaPC for crystals associated OA, the activity of the two PC salts in the inhibition of BCP crystals-stimulated induction of MMP1 was compared. BJ1 cells were exposed to BCP crystals in the presence of increasing concentrations of PC salts. Using a "blind" trial, RNA samples were prepared, numbered and examined by a second person using RT-PCR. A typical result is shown in Figure 1. MMP1 was induced by BCP crystals and the induction was inhibited by both NaPC and CaNaPC in a dose dependent manner. However, no significant difference between the inhibitory activities of the two PC salts was found.

The activity of the two PC salts was compared for their ability to inhibit BCP crystals stimulated mitogenesis using [^{3}H]thymidine uptake assay. As shown in Figure 2, [^{3}H]thymidine uptake was stimulated by BCP crystals and the BCP crystals stimulated [^{3}H]thymidine uptake was inhibited by both NaPC and CaNaPC in a dose dependent manner. Although a small difference between CaNaPC and NaPC was observed at all concentrations, the difference was too small to explain the 3-6 fold enhanced potency of CaNaPC over NaPC in the inhibition of chemically-induced calcified plaques in rat (1). It is known that BCP crystals-stimulated mitogenesis is dependent on crystals dissolution. Therefore, it is possible that the small

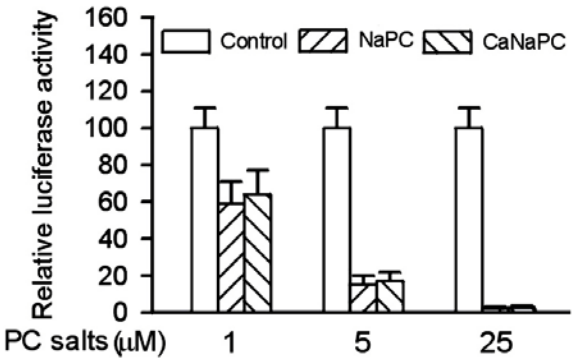

Figure 3. Inhibition of BCP crystals stimulated endocytotic activity by NaPC and CaNaPC. Hela cells were plated in 24-well cluster plates and treated with pCMV-luciferase reporter plasmid and BCP crystals in the presence of increasing concentrations of NaPC or CaNaPC. Eighteen hours later, cells were lysed and luciferase activity was determined. The luciferase activities were normalized relative to the amount of protein in the lysate. Bar 1 in all three bar groups, BCP crystals treatment; Bar 2 in all three bar groups, BCP crystals treatment in the presence of increasing concentrations of NaPC; Bar 3 in all three bar groups, BCP crystals treatment in the presence of increasing concentrations of CaNaPC. Both NaPC and CaNaPC inhibited the BCP crystals stimulated endocytotic activity in a dose dependent manner. Three independent treatments, each in duplicate, were performed. Data were expressed as means ± SEM.

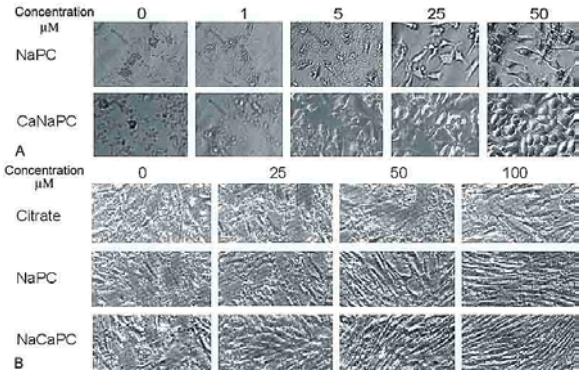

Figure 4. Inhibition of the amorphous calcium phosphate-DNA co-precipitates induced cell death by NaPC and CaNaPC. (A) Hela cells were plated in-24 well cluster plates and treated with 120 μl per well of amorphous calcium phosphate-pUC18 plasmid co-precipitates in the presence of increasing concentrations of NaPC or CaNaPC. Forty-two hours later, cells were photographed. The experiments have been repeated a minimum of five times using two different batches of PC preparations and similar results were obtained. (B) BJ1 cells were plated in 6 well cluster plates and treated with 200 μl per well of amorphous calcium phosphate-pUC18 plasmid co-precipitates in the presence of increasing concentrations of NaPC or CaNaPC. Forty-two hours later, cells were photographed.

difference between the two PC salts observed here can be attributed to a small difference between the ability of the two PC salts to enter the cells and inhibit the dissolution of the endocytosed crystals.

4.2. NaPC and CaNaPC inhibited the BCP crystals-stimulated endocytotic activity

Recently, we have shown that BCP crystals stimulate the endocytotic activity of cells and that several calcification inhibitors inhibit the BCP crystals-stimulated endocytotic activity (16). Since no significant differences between the inhibitory activities of the two PC salts were found which might explain the observed 3-6 fold enhanced potency of CaNaPC over NaPC (1), the two salts were further examined using the BCP crystals-stimulated endocytotic assay. As shown in Figure 3, the endocytosis of the pCMV-luciferase plasmid was stimulated by BCP crystals and the BCP crystals-stimulated endocytosis was inhibited by both NaPC and CaNaPC in a dose dependent manner. However, no significant difference between the inhibitory activities of the two PC salts was found.

4.3. CaNaPC was more powerful than NaPC in the inhibition of calcium phosphate–DNA coprecipitates-induced cell death

When cells are treated with a large amount of BCP crystals (more than 150 μg/ml), instead of proliferating, many cells will die. Also, if cells are treated with a large amount of calcium phosphate-DNA coprecipitates, even more cells will die. Since it is known that pathological soft tissue calcifications are, in many cases, associated with cell death, and that amorphous calcium phosphate precipitates coexist with crystals in calcified tissues, a study was initiated to examine whether PC could inhibit the calcium phosphate-DNA coprecipitates-induced cell death and whether CaNaPC and NaPC had different inhibitory activity in the calcium phosphate-DNA coprecipitates-induced cell death. To this end, we treated Hela cells with a large amount of calcium phosphate-DNA coprecipitates in the presence of increasing concentrations of NaPC or CaNaPC. Forty-two hours post treatment, the cells were photographed. A typical result is shown in Figure 4A. Larger amount of amorphous calcium phosphate-DNA coprecipitates-induced massive cell death, but both NaPC and CaNaPC inhibited the amorphous calcium phosphate-DNA coprecipitates-induced cell death in a dose dependent manner. Significantly, CaNaPC was much more powerful than NaPC in the inhibition of the amorphous calcium phosphate-DNA coprecipitates-induced cell death. More cells (2-4 fold) survived in the presence of CaNaPC than in the presence of NaPC. Similar results were obtained when human foreskin fibroblast BJ1 cells were used (Figure 4B).

5. DISCUSSION

The observations from the present study indicate that CaNaPC is much more powerful than its precursor NaPC in the inhibition of amorphous calcium phosphate-DNA coprecipitates- induced cell death, whereas, both PC salts had a similar inhibitory activity in the inhibition of BCP crystals stimulated induction of MMP1, mitogenesis

and endocytosis. Since it is known that amorphous calcium phosphate precipitates coexist with BCP crystals in calcified tissues and that pathological calcifications are in many cases associated with cell injury, apoptosis and necrosis, the superior activity of CaNaPC over its precursor NaPC in the inhibition of amorphous calcium phosphate-DNA coprecipitates-induced cell death suggests that CaNaPC is more powerful than NaPC in the inhibition of amorphous calcium phosphate associated effects and may, at least in part, explain its powerful anti-calcification activity *in vivo*. PC is known to inhibit the formation and growth of BCP crystals through its strong binding to amorphous calcium phosphate precipitates and crystal surfaces (27, 29). Another important effect of such binding is that it blocks the injurious contact between minerals and cells. Electron microscopic, thermodynamic, and kinetic approaches have revealed that there are two major stages in the conversion of soluble Ca^{+2} and phosphate into crystalline hydroxylapatite, namely, precipitation of soluble ions to amorphous precipitation, and then hydration and crystallization. The amorphous calcium phosphate is an obligatory precursor for the formation of BCP crystals. Numerous studies have found that amorphous calcium phosphate precipitates coexist with BCP crystals in calcified tissues (2, 4, 5, 38, 39). Therefore, besides the crystals, the amorphous calcium phosphate precipitates must have also played an important role in pathological calcification processes and in the induction of detrimental biological effects of calcium containing minerals, and, in doing so, have sped up the progression of crystals associated diseases. An agent that can effectively block both the detrimental biological effects of crystals and the precursor, amorphous calcium phosphate precipitates, should be a better calcification inhibitor and potentially a better disease modifiying drug for crystals associated diseases than an agent that can only effectively block one of the two detrimental biological effects.

The molecular mechanisms underlying the superior activity of CaNaPC over NaPC in the inhibition of the amorphous calcium phosphate-DNA coprecipitates-induced cell death are currently unclear. Possible explanations could be that CaNaPC has the capacity to bind to more amorphous calcium phosphate than NaPC so that it can block the interaction between amorphous calcium phosphate and cells more effectively or that CaNaPC, which might have less negative charge than NaPC, is able to enter the cells more easily than NaPC and acts as an intracellular factor, such as a calcium buffer molecule. These potential mechanisms are currently under investigation in our laboratory.

In summary, CaNaPC is much more powerful than NaPC in the inhibition of the amorphous calcium phosphate-DNA coprecipitates-induced cell death. The result suggests that CaNaPC may be much more powerful than NaPC in the inhibition of amorphous calcium phosphate associated effects. Since amorphous calcium phosphate precipitates coexist with BCP crystals in calcified tissues, the death and apoptosis of chondrocytes, especially those chondrocytes in the superfacial and calcified zones of articular cartilage, are important issues with crystals associated OA. Hence, the present data suggest that CaNaPC (a new salt of PC), has potential to be a better disease-modifying drug for crystals associated diseases such as crystals associated OA than its parent molecule.

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