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Effects of magnesium on matrix metalloproteinase-2 production in cultured rat cardiac fibroblasts

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Abstract The precise correlation between magnesium and cardiac disease remains to be established. Matrix metalloproteinases (MMPs) are important in cardiac disease such as heart failure. Cardiac fibroblasts are the most abundant cell type in the heart and play an important role in the regulation of collagen degradation by MMPs. To assess the association between magnesium and MMPs, we examined the effects of different extracellular magnesium concentrations (0 – 3.0 mmol/L) on MMP-2 production in cultured rat cardiac fibroblasts. Using gelatin zymography and western blotting, we found that magnesium reduced MMP-2 production dose-dependently, and this effect was inhibited by the tyrosine kinase inhibitors, genistein or herbimycin A. The results of this study indicated that the beneficial effect of magnesium supplementation on the cardiac disease may be due, at least in part, to the inhibitory effect of magnesium on production of MMPs in cardiac fibroblasts, which appears to be mediated by a protein tyrosine phosphorylation related signal transduction pathway.

Key words Magnesium – matrix metalloproteinase – cardiac fibroblasts – protein tyrosine kinase – heart failure

Introduction

Magnesium is the second most common intracellular electrolyte, after potassium, and the fourth most abundant cation in the body. Approximately 40 % of the magnesium contained in the adult human body resides in the muscles and soft tissues, about 1 % in the extracellular fluid, and the remainder in the skeleton [20]. Magnesium has been implicated in the negative correlation between cardiovascular disease and hardness of drinking water [7]. A reduced level of cardiac total magnesium has been observed in patients with congestive heart failure, acute myocardial infarction, and in patients undergoing cardiac surgery [11, 25]. Animal model studies of severe magnesium restriction have demonstrated progressive cardiovascular lesion formation, heightened inflammatory cell infiltration, decreased levels of endogeneous antioxidants (glutathione, vitamine E, and ascorbate), and higher plasma levels of pro-oxidant metals and lipid peroxidation products [15]. Magnesium therapy may has a direct depressive effect on the cardiac conduction system, an arterial dilatory effect, an ion stabilizing effect, an energy generation improving effect, and a platelet aggregation inhibiting effect [28]. However, the most recent clinical trial on magnesium in acute myocardial infarction showed no benefit of magnesium [19]. In the heart, the magnesium levels are relatively high but the myocardium is highly vulnerable to magnesium deficiency [4]. Magnesium may be protective by a direct action on the myocardium [35]. Thus, the relation between magnesium and cardiac disease remains not fully understand

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and may should be strengthen at the point of myocardium.

Important changes occur in the extracellular matrix components of the myocardium as the left ventricle begins to fail. The extracellular matrix of the heart includes a fibrillar collagen network, a basement membrane, and proteoglycans [21]. The matrix metalloproteinases (MMPs) are a family of zinc-dependent enzymes demonstrated to contribute to tissue remodeling in a number of disease states [22]. MMP-2 is one of the MMPs that may have particular relevance to myocardial remodeling [21]. The potent gelatinase, MMP-2, can also cleave fibrillar collagen [1] in addition to basement membrane [9]. Increased MMP-2 activity in heart failure or left ventricular remodeling has been identified in humans and animals [27, 30]. MMP activity contributes to left ventricular dilation and progression to left ventricular dysfunction in a rodent heart failure model, and direct MMP inhibition can attenuate this process [23].

Fibroblasts are the most abundant cell type in the heart and play a major role in synthesizing components of the cardiac extracellular matrix [8]. Cardiac fibroblasts have also been shown to play an important role in the regulation of collagen degradation by MMPs [6]. Therefore, the present study was designed to study whether magnesium affects MMP-2 production in cardiac fibroblasts and further elucidate the mechanisms.

Methods

Materials

The chemicals used in this study were obtained from the following sources: Dulbecco's modified Eagle's medium (DMEM) without magnesium was obtained from GIBCO (Grand Island, NY), methyl-[^{3}H] thymidine was obtained from Daiichi Radioisotopes (Tokyo, Japan). Mouse monoclonal Anti-MMP-2 antibody was obtained from Fuji Chemical Industries (Toyama, Japan). Genistein and herbimycin A were purchased from Wako (Osaka, Japan). All other chemicals were of reagent grade or were of the highest grade commercially available.

Cell culture

Primary cultures of neonatal rat cardiac fibroblasts were prepared as previously described with some modifications [10]. Briefly, hearts were removed from 1- to 2-day-old Wistar rats anesthetized by ether under aseptic conditions and placed in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS). The hearts were washed with PBS, and the atria and aorta were discarded. The ventricles were minced with scissors into 1- to 3-mm^3 fragments, and they were then enzymatically digested 4 times for 10 to 15 min each with 10 ml of PBS containing 0.07% trypsin. The liberated cells were collected by centrifugation at 1000 g for 5 min and incubated in 100-mm glass culture dishes for 60 min at 37 °C in a humidified incubator with 5% CO_2-95% air. During this time, the majority of myocardiocytes remained in suspension, whereas the nonmyocardiocytes attached more readily to the dish. Subsequently, the medium containing the myocardiocytes was aspirated and discarded. Highly enriched cultures of cardiac nonmyocytes were prepared by 2 passages of the cells adherent to the culture dish during the replating procedure [26]. Until the second passage, cells were maintained in DMEM supplemented with 10% fetal calf serum. The nature of cells was determined by immunofluorescence staining with anti-vimentin, and more than 95% of cells were stained positively. All cardiac fibroblast cultures used in this study were between passages 3 and 4. At the subconfluent stage, the culture medium was replaced with serum-free medium for 16 h and then cells were exposed to various treatments for 24 h.

Preparation of culture medium

Magnesium sulfate or magnesium chloride was added to DMEM without magnesium to the final magnesium concentrations of 0 to 3.0 mmol/L before use. The level of 0 to 0.5 mmol/L magnesium corresponds to the lowest physiological level of this ion that can be measured in human serum, 1.0 mmol/L to the physiological level, and 3.0 mmol/L to the highest level reached by therapeutic supplementation of magnesium in patients with arrhythmia or preeclampsia.

DNA synthesis

Fibroblasts were plated in 6-well plates (1×10^5 cells/well), incubated at 37 °C in a humidified atmosphere of 95% air–5% CO_2, and maintained in DMEM with 10% fetal calf serum until subconfluent (50 to 70% confluent). After incubation with magnesium (0 to 3.0 mmol/L), [^{3}H]thymidine (1 μCi/ml) was added and followed by incubation for 24 h. DNA synthesis was determined by measuring the trichloroacetic acid-insoluble radioactivity [3]. After incubation, the trypsinized fibroblasts were suspended in ice-cold 10% trichloroacetic acid. The lysates were placed on ice for 10 min, followed by a passage through paper filters (Whatman, Maidstone, UK). The filters were washed with cold 10% trichloroacetic acid and dried. Incorporated [^{3}H]thymidine was measured in an Aloka LSC-3500 scintillation counter (Aloka, Tokyo, Japan).

Gelatin zymography

After various treatments for 24 h, medium samples were harvested, centrifuged at 2000 × g for 10 min and normalized for cell protein content using Bio-Rad assay [32]. Samples of 20 μg protein were applied without reduction to a 7.5% polyacrylamide slab gel impregnated with 1 mg/ml gelatin [31]. After electrophoresis, the gel was washed at room temperature for 30 min in washing buffer (50 mmol/L Tris-Cl, pH 7.5, 15 mmol/L $CaCl_2$, 1 μmol/L $ZnCl_2$, and 2.5% Triton X-100), then incubated overnight at 37 °C with shaking in the same buffer but containing 1% rather than 2.5% Triton X-100. The gel was stained with a solution of 0.1% Coomassie brilliant blue R-250. Clear zones against the blue background indicated the presence of gelatinase. To quantify the amount of gelatinase production, the stained zymograms were scanned on a densitograph (ATTO, Tokyo, Japan). A protein marker and control MMPs (Sigma-Aldrich, ST. Louis, MO) were electrophoresed in parallel for molecular weight identification.

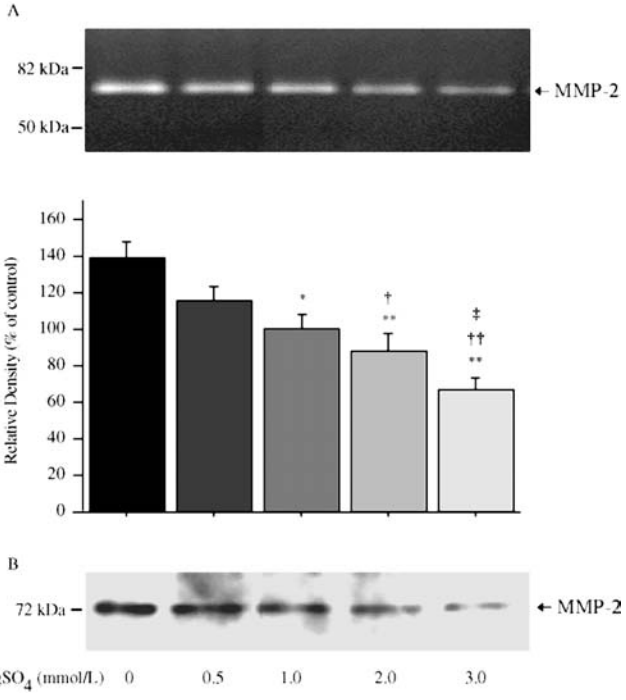

Fig. 1 Influence of magnesium on production of MMP-2 as determined by zymography (**A**) and western blotting (**B**). Clear zones against the blue background indicated the presence of MMP-2. Columns indicate data of gelatinolytic activity as percentages of the amount of control (1.0 mmol/l $MgSO_4$), and represent the means ± standard errors (S.E.) for four separate experiments performed in duplicate. * $p < 0.05$ vs. $MgSO_4$ 0 mmol/l, ** $p < 0.01$ vs. $MgSO_4$ 0 mmol/l, † $p < 0.05$ vs. $MgSO_4$ 0.5 mmol/l, †† $p < 0.01$ vs. $MgSO_4$ 0.5 mmol/l, ‡ $p < 0.05$ vs. $MgSO_4$ 1.0 mmol/l

Western blotting

After various treatments for 24 h, medium samples were harvested with the protease inhibitors phenylmethane sulfonyl fluoride (0.1 mmol/L) and leupeptin (10 μg/ml) from cells, centrifuged at 2000 × g for 10 min, measured of protein concentration and separated by electrophoresis on 7.5% sodium dodecyl sulfate polyacrylamide gels followed by transfer onto polyvinylidene difluoride membranes (Immobilon P, Millipore, 0.22 μm pore size). The membranes were blocked in 5% skim milk in PBS containing 0.1% Tween 20 at room temperature for 1 h, and probed with anti-MMP-2 monoclonal antibody overnight. After washing 3 times with PBS containing 0.1% Tween 20, the membranes were incubated with secondary antibody conjugated with horseradish peroxidase for 1 h as described previously [24], and finally developed by ECL detection system (Amersham Pharmacia Biotech, Buckinghamshire, England).

Statistical analysis

Results were presented as percentages of the control and represent the means ± standard errors (S.E.) for four separate experiments performed in duplicate. Differences among all data were analyzed for statistical significance by one-way analysis of variance (ANOVA) followed by unpaired Student's t-test. Differences of $p < 0.05$ were considered statistically significant.

Results

Effects of magnesium on [^{3}H]thymidine incorporation in cardiac fibroblasts

There have no significant difference between the different concentrations of magnesium on thymidine incorporation, indicating that magnesium did not influence the DNA synthesis of cardiac fibroblasts (data not shown).

Effects of magnesium on the production of MMP-2 in cardiac fibroblasts

Gelatin zymograms of cardiac fibroblasts-conditioned media showed that the major MMP expressed was MMP-2. Magnesium decreased the gelatinolytic activity of MMP-2 significantly in a dose-dependent manner (Fig. 1A). These effects were not toxic as determined by trypan blue exclusion.

Corresponding with the data of gelatin zymography, western-blotting analysis demonstrated that magnesium induced a decrease in MMP-2 expression dose-dependently (Fig. 1B).

Enzymatic activity of MMP-2 was inhibited after addition of 10 mM EDTA, an inhibitor of MMPs, into incubating buffer. In contrast, gelatinolytic bands displayed after zymography were unaffected by incubation in phenylmethylsulfonyl fluoride, an inhibitor of serine proteases (data not shown).

Effects of anions and ions on production of MMPs

To verify that the altered MMP production by magnesium sulfate was due to magnesium ions and not a non-specific effect of sulfate anions, the effects of magnesium chloride on the production of MMP-2 were compared with those of magnesium sulfate. No significant differences were seen in the degree of decrease in MMP-2 production between magnesium sulfate and magnesium chloride (Fig. 2A), indicating that the effect on MMP-2 production was due to neither sulfate nor chloride anions but to magnesium ions. To examine whether other divalent cations, such as zinc and copper, have any effects on MMP-2 production, cardiac fibroblasts were treated with zinc sulfate or copper sulfate culture medium, which were made in the same way as the magnesium sulfate treatments, to the final concentrations of 0 to 100 μmol/L for 24 h. No influences were found in MMP-2 production by cardiac fibroblasts treated with either zinc sulfate or copper sulfate (Fig. 2B), indicating that the effect of magnesium on MMP-2 production in cardiac fibroblasts was specific but not a common character of divalent cations.

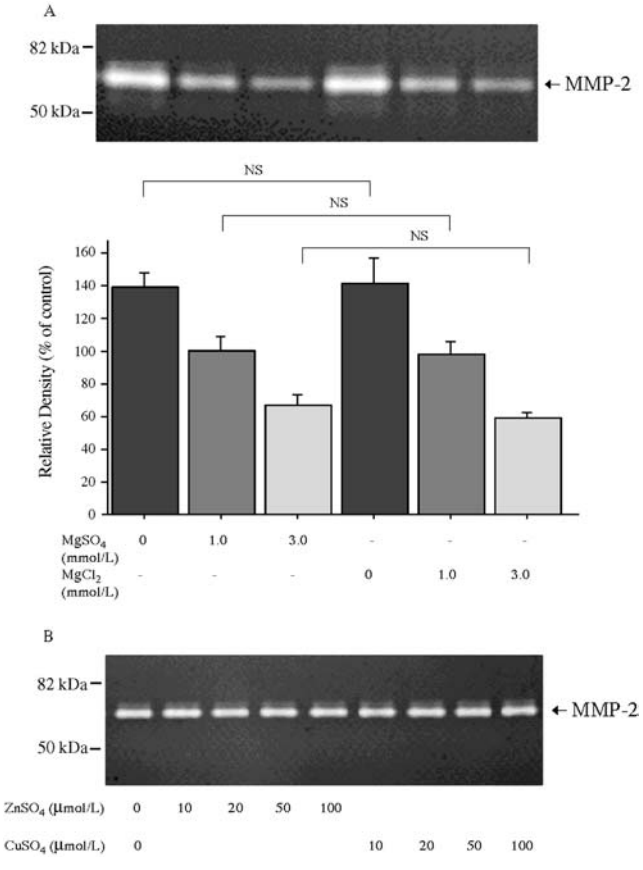

Fig. 2 Effects of anions and ions on production of MMPs as described in **A** and **B**. Clear zones against the blue background indicated the presence of MMP-2. Columns are as described in Fig. 1. 1.0 mmol/L $MgSO_4$ was regarded as control in **A**. The group treated with neither $ZnSO_4$ nor $CuSO_4$ was regarded as control in **B**

Effects of magnesium on the zymography system

To detect the effects of magnesium on the zymography system, subconfluent cardiac fibroblasts were treated with 0 mmol/L magnesium for 24 h, then the 0 mmol/L culture medium was adjusted to different magnesium concentrations (0 to 3.0 mmol/L) before zymography. Magnesium did not influence MMP detection under these conditions. The lack of an inhibitory effect of magnesium on MMP production after removal of cells demonstrated the feasibility of studying the effects of magnesium on MMP detection by the zymography system (Fig. 3).

Effects of protein tyrosine kinase inhibitors on magnesium-induced MMP-2 production

To evaluate whether the effects of magnesium on MMP-2 were related to protein tyrosine kinase, subconfluent

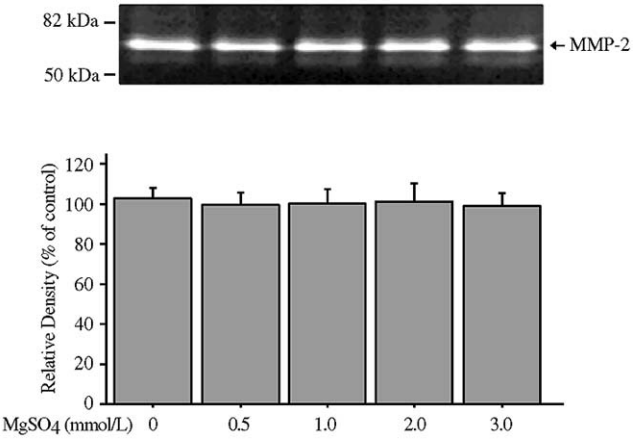

Fig. 3 Effects of magnesium on the zymography system. The conditioned media of 0 mmol/L magnesium sulfate ($MgSO_4$) which had been cultured for 24 h were collected, adjusted to different magnesium concentrations and normalized for cell protein content, then analyzed for the presence of MMP-2 by zymography. Clear zones against the blue background indicated the presence of MMP-2. Data shown are representative of four separate experiments performed in duplicate

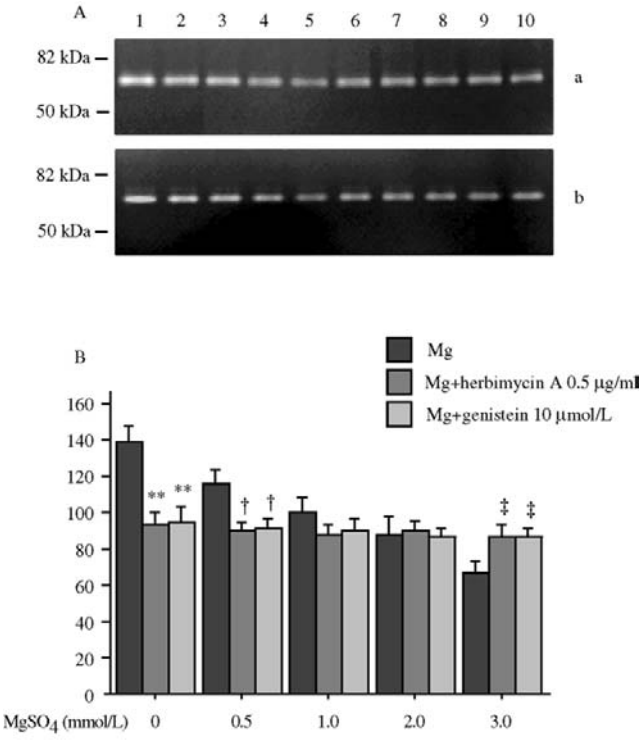

Fig. 4 Effects of protein tyrosine kinase inhibitors on magnesium-induced MMP-2 alteration. Magnesium sulfate ($MgSO_4$)-induced alterations of MMP-2 production (at concentrations of 0 mmol/L, 0.5 mmol/L and 3.0 mmol/L) were significantly inhibited by two protein tyrosine kinase inhibitors, genistein and herbimycin A. Clear zones against the blue background indicate the presence of MMP-2. Lane 1 to lane 5 represent $MgSO_4$ groups with concentrations of 0 mmol/L to 3.0 mmol/L, lane 6 to lane 10 represent combined treatment groups of herbimycin A (a) and genistein (b) with $MgSO_4$ of 0 mmol/L to 3.0 mmol/L, separately shown by zymography (**A**). The statistical data are shown in **B**. Columns are as described in Fig. 1. ** $p < 0.01$ vs. $MgSO_4$ 0 mmol/L, † $p < 0.05$ vs. $MgSO_4$ 0.5 mmol/L, ‡ $p < 0.05$ vs. $MgSO_4$ 3.0 mmol/L

cardiac fibroblasts were treated with two protein tyrosine kinase inhibitors, genistein and herbimycin A for 10 min before treatment with magnesium in culture media at different concentrations (0 to 3.0 mmol/L). A pretreatment with either genistein (10 μmol/L) or herbimycin A (0.5 μg/ml) with magnesium resulted in an inhibition of the alterations on MMP-2 production induced by magnesium (Fig. 4).

Discussion

The major finding of this study was that extracellular magnesium supplementation reduced the production of MMP-2 in rat cardiac fibroblasts. Our previous report of the effects of magnesium on the production of MMP-2 in cultured rat vascular smooth muscle cells [37] and the clinical report [29] that intravenous administration of magnesium decreased serum levels of MMP-1 in patients with acute myocardial infarction provided some supports on the present study.

It had been reported that magnesium deficiency enhances circulating levels of many pro-oxidant, proinflammatory, and mitogenic factors like substance P, which are postulated to be involved in the pathogenesis of the cardiopathic lesions of magnesium deficiency [33]. A study treating rat ventricular fibroblasts with serum of magnesium deficient rat suggested that in hypomagnesemia, serum factors may stimulate cardiac fibroblast proliferation via a superoxide anion-mediated mechanism and contribute to the fibrogenic response in the heart [16]. However, the direct effects of magnesium on cardiac fibroblasts especially at the point of MMPs have not been examined to our knowledge. In a review which preceded the publication of LIMIT-2 and ISIS-4, Woods concluded that following coronary occlusion, intravenous $MgSO_4$ protects by a direct action on the myocardium [35]. The present study that in rat cardiac fibroblasts, hypomagnesium (magnesium concentrations of less than 1.0 mmol/L) increased MMP-2 production, while hypermagnesium (magnesium concentrations of over than 1.0 mmol/L) decreased MMP-2 production might provide some evidence for the understanding of the effects of magnesium deficiency and administration in cardiac disease such as heart failure after myocardial infarction.

Although magnesium is called a natural calcium antagonist, the L-type calcium antagonist, nifedipine, increased MMP-2 production in the same rat cardiac fibroblasts through a pathway related to nitric oxide in another study (unpublished data), this indicated that magnesium acts on MMP-2 expression through a pathway different from nifedipine. Magnesium can act on potential-operated channels, receptor-operated channels, and leak-operated channels in the cardiovascular system [2]. Using CAI (carboxy amino-triazole), a receptor-operated calcium influx inhibitor, Kohn et al. found a marked reduction in MMP-2 activity and suggested a role for calcium-mediated signal transduction in the expression of metalloproteinases [14]. Therefore, it is possible that magnesium affected MMP-2 expression in cardiac fibroblasts through a calcium-mediated signal transduction pathway like CAI.

Protein phosphorylation on tyrosine residues by protein tyrosine kinases is an important regulator of cell function [12]. Li et al. [18] and our laboratory [32] have demonstrated that MMP-2 production or activation was induced through protein tyrosine phosphorylation. We showed that in rat vascular smooth muscel cells, magnesium affected MMP-2 production through a pathway related with protein tyrosine kinase [37]. Therefore, it is not difficult to assume that protein tyrosine kinase might also play an important role on effects of magnesium in MMP-2 in cardiac fibroblasts. In the present study, we observed that in agreement with our previous study in

vascular smooth muscle cells, a hypomagnesium (magnesium concentrations of 0.5 to 0 mmol/L)-induced increase in MMP-2 production was blocked by either genistein or herbimycin A, and a hypermagnesium (magnesium concentrations of 3.0 mmol/L)-induced decrease in MMP-2 production was also inhibited by either genistein or herbimycin A. Our data suggest that the effects of magnesium on production of MMP-2 in cardiac fibroblasts might also be related, at least in part, to a protein tyrosine kinase pathway, in complete agreement with its effects on vascular smooth muscle cells [37].

Unlike nifedipine, magnesium possesses the ability to antagonize the N-type calcium channel [5] and thapsigargin-sensitive calcium stores [36], in addition to its L-type calcium channel inhibitory effect. N-type calcium channel and thapsigargin-sensitive calcium stores have been suggested to be regulated via a tyrosine kinase pathway [17, 34]. Furthermore, protein tyrosine kinase inhibitors, genistein and herbimycin A, have been shown to elevate intracellular Ca^{2+} through a dihydropyridine-insensitive Ca^{2+} influx pathway in rat osteoclasts [13]. Therefore, although no studies can be found related to CCBs and tyrosine kinases directly in cardiac fibroblasts, we can not exclude the possibility that magnesium influences MMP-2 production in cardiac fibroblasts by tyrosine kinase via inhibition of the upper N-type calcium channel and/or thapsigargin-sensitive calcium stores.

From the present study and our previous data on vascular smooth muscle cells, we concluded that magnesium possesses the ability to decrease MMP-2 production, which might be mediated through a protein tyrosine kinase related pathway.

Conclusions

In cultured rat cardiac fibroblasts, magnesium significantly reduced the production of MMP-2 in a dose-dependent manner, and this effect was inhibited by both genistein and herbimycin A – two protein tyrosine kinase inhibitors. Our data suggest that the beneficial effect of magnesium supplementation on cardiac disease such as heart failure may be due, at least in part, to the inhibitory effect of magnesium on the production of MMP-2 in cardiac fibroblasts, which appears to be associated with a protein tyrosine kinase pathway.

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