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Calcium influx inhibits MT1-MMP processing and blocks MMP-2 activation

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Abstract We have previously reported that concanavalin A (ConA)-induced MMP-2 activation involves both transcriptional and non-transcriptional mechanisms. Here we examined the effects of calcium influx on MT1-MMP expression and MMP-2 activation in MDA-MB-231 cells. The calcium ionophore ionomycin caused a dose-dependent inhibition of ConA-induced MMP-2 activation, but had no effect on MT1-MMP mRNA levels. However, Western analysis revealed an accumulation of pro-MT1-MMP (63 kDa), indicating that ionomycin blocked the conversion of pro-MT1-MMP protein to the active 60 kDa form. This suggests that increased calcium levels inhibit the processing of MT1-MMP. This finding may help to elucidate the mechanism(s) which regulates MT1-MMP activation.

Key words: Gelatinase A; Activation; Concanavalin A; MT-MMP; Calcium; Human breast cancer

1. Introduction

Members of the matrix metalloproteinase (MMP) enzyme family have been broadly implicated in both physiological and pathophysiological tissue remodeling [1], and are generally secreted as latent zymogens requiring specific activation for catalytic activity [2]. MMP-2, also called 72 kDa type IV collagenase or gelatinase A, shows specificity for the helical regions of collagen type IV, and has been implicated in basement membrane invasion and metastasis of carcinomas [3]. Activation of the MMP-2 proenzyme is mediated at the cell surface by a subfamily of membrane-associated MMPs (MT-MMP), identified recently by PCR homology screening (reviewed in [4]). MT1-MMP is co-expressed with MMP-2 in embryonic mesenchyme [5] and is dramatically upregulated in the stroma surrounding various carcinomas [6–11]. It is also up-regulated in gliomas [12], and in the tumor cells of cervical [13] and lung [8,14] carcinomas. Furthermore, human breast [9,15] and cervical [16] carcinoma cell lines expressing MT1-MMP show enhanced invasive and metastatic tendencies. Collectively, these data suggest that MT1-MMP, and its activation of MMP-2, are important in both the local expansion and distant metastasis of carcinomatous tumors. Further understanding of the regulation and mechanism of MT1-MMP-mediated MMP-2 activation may ultimately provide new avenues for cancer diagnosis and therapy.

Despite constitutive expression of MT1-MMP mRNA and protein, MMP-2 activation by MDA-MB-231 human breast cancer cells requires additional stimulation, and is subject to complex regulation [17]. MMP-2 activation by these cells can be potently induced by concanavalin A (ConA), which stimulates the steady state levels of MT1-MMP mRNA and protein, and also mediates some non-transcriptional events which appear crucial for MT1-MMP functionality [17]. Calcium is a well-known intracellular second messenger which mediates a wide range of cellular responses [18,19]. It is required for metalloproteinases activity [20] and calcium influx has been implicated in TGF-β-induced MMP-2 expression [21]. However, increased calcium inhibits ConA-, TNFα- and PMA-induced MMP-2 activation [22]. In this paper, we examined the effect of calcium influx on ConA-induced MT1-MMP functionality in MDA-MB-231 cells. Our results suggest that calcium influx blocks the constitutive processing of MT1-MMP without affecting the steady-state mRNA levels. It is known that MT1-MMP is constitutively processed into the active form by all cells so far studied [12,14,15,23], however the mechanism for such activation is not yet clear. Our observation here that MT1-MMP processing is sensitive to calcium influx may help in the elucidation of this mechanism.

2. Materials and methods

2.1. Cells and chemicals

The MDA-MB-231 cell line was originally obtained from ATCC (Rockville, MD) and provided by the Lombardi Cancer Center (LCC) Cell Culture Shared Resource. Cells were cultured in IMEM/FCS [Richter's IMEM (Biofluids, Rockville, MD) supplemented with 10% FCS (Gibco, Gaithersburg, MD)] in a 95% air, 5% CO_2 atmosphere at 37°C. Cells were confirmed as mycoplasma-free by the LCC Cell Culture Shared Resource using the Genprobe kit (Gen-Probe, San Diego, CA) and maintained by weekly passage. Concanavalin A (tissue culture grade) and ionomycin were purchased from Sigma (St. Louis, MO). Anti-MT1-MMP monoclonal antibody 114-1F2 was kindly provided by Dr. K. Iwata, Fuji Chemical Industries, Ltd., Takaoka, Japan). Lysates from MT1-MMP-transfected COS-1 cells were prepared as previously described [14] and employed as controls for Western analysis.

2.2. Treatment of cells with ConA, and ionomycin

MDA-MB-231 cells were plated (1×10^5 cells/0.5 ml IMEM/FCS) in Costar 24-well clusters, incubated overnight, and then washed in unsupplemented IMEM twice before treatment and further incubation in MMP-2 supplemented serum free medium (MMP-2/SFM) as previously described [17]. SFM was prepared by supplementing phenol red-

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Abbreviations: Gel A, gelatinase A; ConA, concanavalin A; MMP-2, matrix metalloproteinase-2; MT1-MMP, membrane type 1 matrix metalloproteinase

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free Richter's IMEM (Biofluids, Rockville, MD) with glutamine, sodium pyruvate, HEPES, vitamins, trace elements and buffered non-essential amino acids (100×stock, Biofluids), human plasma fibronectin (1 mg/500 ml) and ITS supplement (2 ml/500 ml, Becton Dickinson, Bedford, MA). SFM was then mixed in a ratio of 3:1 with SFM conditioned by MMP-2-transfected MCF-7 cells for 24 h. This MMP-2/SFM formulation provides a reproducible source of latent MMP-2 for activation analysis [9,15,17]. Activation was induced with ConA (20 μg/ml) in MMP-2/SFM, in the presence or absence of various concentrations of ionomycin. After incubation, conditioned media was frozen at −20°C until testing for the activation of the exogenous MMP-2 by gelatin zymography.

2.3. Gelatin zymography

10% Sodium dodecyl sulfate-polyacrylamide gels copolymerized with 2 mg/ml gelatin were used for electrophoresis to resolve latent and activated species of MMP-2 as previously described [17]. Bands were quantified by densitometry analysis using a Model DNA 35/ Sparc Station 2 (pdi NY, NY).

2.4. Slot-blot analysis of MT1-MMP RNA levels

Slot-blot analysis was used because we showed previously that our MT1-MMP probe recognized only one band on Northern analysis of these cells [17]. Cells were plated in 10 cm dishes and treated as described above except for the omission of exogenous MMP-2 from the incubation media. Total RNA was prepared from cells using either 4 M guanidinium thiocyanate and phenol-chloroform or an RNA purification kit (Qiagen). For slot-blot analysis, 10 μg total RNA was loaded onto nitrocellulose filters in a Minifold II slot-blot apparatus (Schleicher and Schuell Inc., Keene, NH). The filter was then prehybridized for 1 h in 50% formamide at 42°C and then hybridized overnight at 42°C with a random prime-labeled ^{32}P-MT-MMP cDNA PCR fragment probe encompassing bases 98 to 541 [17]. Filters were washed with 0.1×SSC and 1% SDS at 65°C and the bands visualized and quantified by phosphorimaging as well as autoradiography. MT1-MMP mRNA levels were normalized by densitometry of the methylene blue-stained blot before probing.

2.5. Western analysis of MT1-MMP protein levels

Replicate cultures in 10 cm dishes were plated and treated as described above for slot-blot analysis. Monolayers were rinsed with Tris-buffered saline (TBS: 10 mM Tris-HCl, pH 7.5, 150 mM NaCl), scraped in ice-cold TBS (1 ml/plate), and sonicated on ice (20 s, maximal setting, Microson Ultrasonic Cell Disrupter, Heat Systems Ultrasonics Inc., Farmingdale, NY) in the presence of protease inhibitors (1 μM PMSF; 0.1 μM aprotinin, 1 μM leupeptin, 1 mM EDTA, 1 μM pepstatin (Boehringer Mannheim Co., Indianapolis, IN). Extracts were dissolved in 200 μl 6×reducing SDS-PAGE sample buffer, electrophoresed (20 μl/lane) on 12% SDS-PAGE mini-gels, proteins transblotted to a nitrocellulose filter (Amersham) in a Hoeffer Transfer Cell (Hoeffer, Scientific Instruments, San Francisco, CA), and blotted with anti-MT-MMP monoclonal antibody 114-1F2 as previously described [14,17]. Bands were visualized by enhanced chemiluminescence (ECL kit, Amersham).

3. Results and discussion

Like most invasive human breast cancer cell lines, MDA-MB-231 cells do not express MMP-2, but can be induced to activate exogenous MMP-2 when treated with certain stimuli, including 3-dimensional fibrillar collagen gels, the phorbol ester TPA, and the plant lectin ConA [9,15,24,25]. Among these agents, ConA provides the most rapid induction [26], which is mediated by a rapid non-transcriptional effect requiring the continued presence of ConA, as well as a slower increase in MT1-MMP mRNA levels [17]. In the course of screening inhibitors of various signal transduction pathways to characterize mechanisms of MT1-MMP regulation, we found that ionomycin (100 nM), a calcium ionophore, completely blocked MMP-2 activation induced by ConA treatment of MDA-MB-231 cells. Fig. 1 shows the activation of MMP-2 induced by ConA treatment (lanes 1, 2), and the dose-dependent inhibition of this activation by ionomycin (lanes 2, 4, 6, 8). ConA-induced MMP-2 activation was completely inhibited by 100 nM ionomycin (lane 4), while 10 nM (lane 6) and 1 nM (lane 8) ionomycin showed no inhibition. Ionomycin did not influence the status of the latent MMP-2 seen in the absence of ConA (lanes 1, 3, 5, 7).

Because we have found that MT1-MMP expression correlates best with both collagen-induced [9] and ConA-induced [15] MMP-2 activation in human breast cancer cells, we further examined the effect of ionomycin on MT1-MMP expression. MT1-MMP mRNA levels were examined by slot blot analysis (Fig. 2A) and normalized against methylene blue staining of the filter (not shown). Fig. 2B shows the normalized MT1-MMP mRNA levels from each of these treatments as indicated in the figure. The MT1-MMP mRNA level from control and ConA treated MDA-MB-231 cells, shown in Fig. 2B, are consistent with the pattern of the MT1-MMP mRNA expression levels observed in our previous studies [15,17]. When the cells were treated in the combination of ConA and ionomycin, the MT1-MMP RNA level remains the same as that from ConA treated cells. Ionomycin showed no effect on ConA-induced MT1-MMP mRNA expression. These data suggested that ionomycin may inhibit the non-

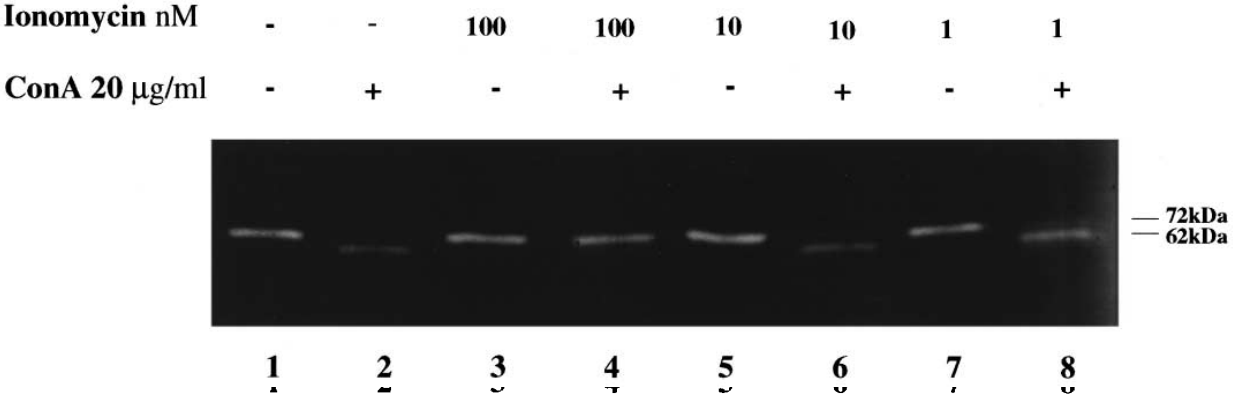

Fig. 1. Zymographic analysis of the effect of the calcium ionophore, ionomycin, on ConA-induced MMP-2 activation. Cells were plated overnight in 24 well plastic dishes in FCS/IMEM SFM, and treated with MMP-2/SFM only (lane 1), 20 μg/ml ConA (lane 2), 100 ng ionomycin (lane 3), 100 ng/ml ionomycin plus 20 μg/ml ConA (lane 4), 10 nM ionomycin (lane 5), 10 nM ionomycin plus 20 μg/ml ConA (lane 6), 1 nM ionomycin (lane 7), or 1 nM ionomycin plus 20 μg/ml ConA (lane 8) for 24 h.

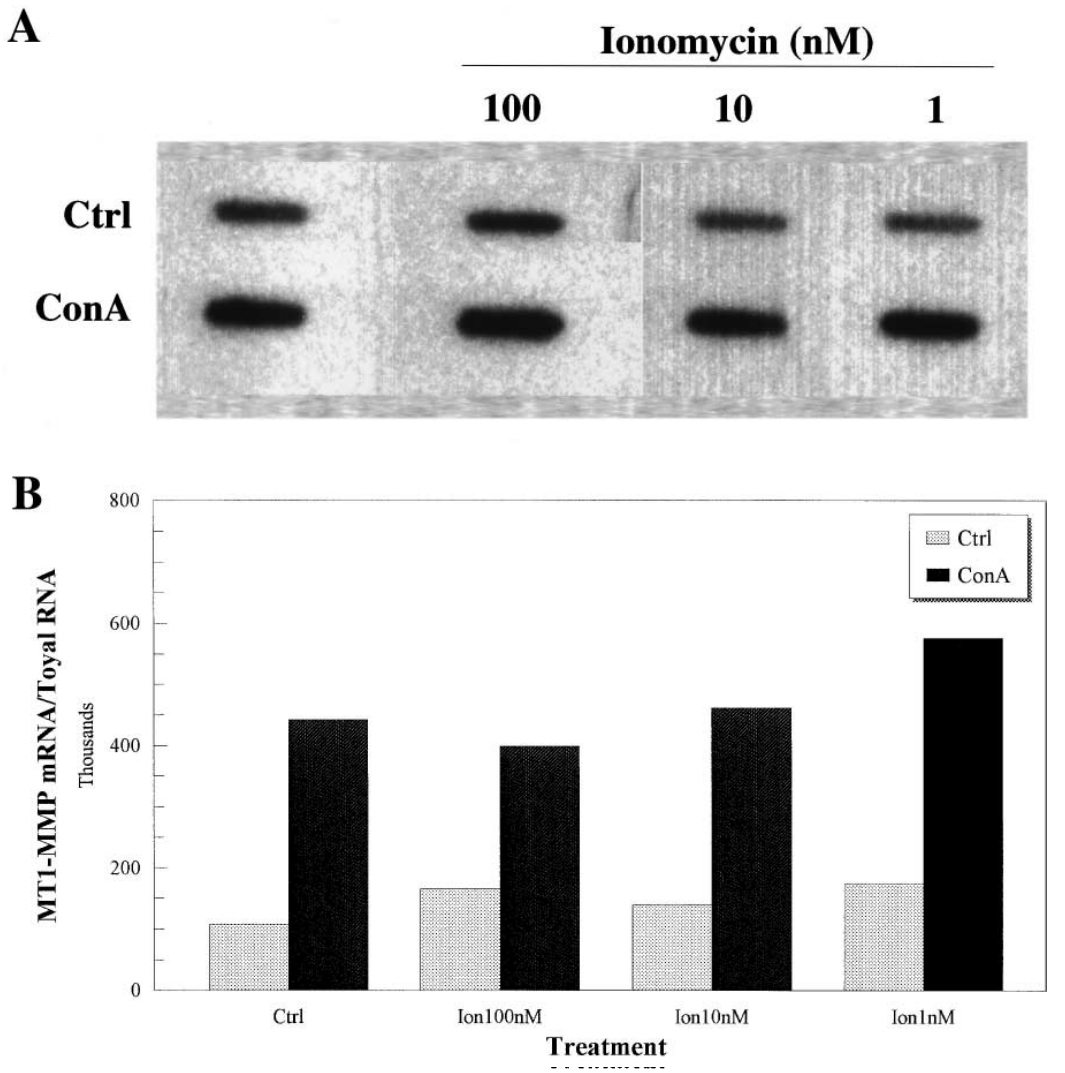

Fig. 2. Lack of effect of ionomycin ConA-induced increase in MT1-MMP mRNA levels. Cells were plated overnight on 10% FCS/IMEM, into 10 cm diameter plastic dishes and then incubated in SFM with the indicated concentrations of ionomycin in the presence (gray bar) or absence (dark bar) of 20 μg/ml ConA. Total RNA was isolated 24 h after addition of the indicated agents. Ten μg of total RNA from each sample were loaded onto nylon membranes in the slot blot apparatus, and probed as described in Section 2. MT1-MMP mRNA levels in slot-blots shown in (A). The MT1-MMP signals were quantified by phosphorimaging and normalized by the density of methylene blue staining of the total RNA on the membrane. The results are expressed as the ratio of MT1-MMP to total RNA loaded on the membrane as shown in (B).

transcriptional component of ConA regulation that we have described previously [17].

We further examined MT1-MMP protein profiles by Western blotting (Fig. 3). As described previously [15], there were two bands specifically recognized by anti-MT1-MMP monoclonal antibodies from ConA-treated cells lysates (lane 3). The slower migrating band of approximately 63 kDa co-migrated with the major species present in lysates from MT1-MMP-transfected Cos-1 cells (lane 1). This band has been previously identified as pro-MT1-MMP by N-terminal sequencing [14]. The faster migrating band is about 10 kDa smaller (about 50-55 kDa), and its appearance correlated with MMP-2 activation potential and thus it appears to represent mature MT1-MMP. This observation is consistent with the previously isolated and N-terminal sequenced native mature MT1-MMP protein from TPA-stimulated HT-1080 cells [27]. MT1-MMP from ConA treated MDA-MB-231 cells occurs predominantly as the faster migrating band. This pattern has also been reported for ConA- or TPA-treated CCL-137 human lung fibroblasts [28], and is also consistent with other reports [23,29]. When MDA-MB-231 cells were treated with 100 nM ionomycin together with ConA, a dramatic increase in the slower-migrating pro-MT1-MMP protein band was detected together with a loss of the faster migrating band (lane 5), indicating that cellular processing of MT1-MMP is blocked. This inhibitory effect was also ionomycin dose-dependent and corresponded to the concentration of ionomycin required for inhibition of MMP-2 activation (Fig. 1). Interestingly, ionomycin also appeared to block processing of MT1-MMP in unstimulated cells. Although sometimes low levels of MT1-MMP protein are detected in MDA-MB-231 cell lysates under the basal conditions [15], they are close to the level of sensitivity limit of the Western analysis. In this experiment, MT1-MMP protein was undetectable in control cells (lane 2). However, treatment with ionomycin alone resulted in a pronounced band corresponding to pro-MT1-MMP (lane 4), again indicating a block in the cellular processing of MT1-MMP. This data further suggests that ionomycin prevents

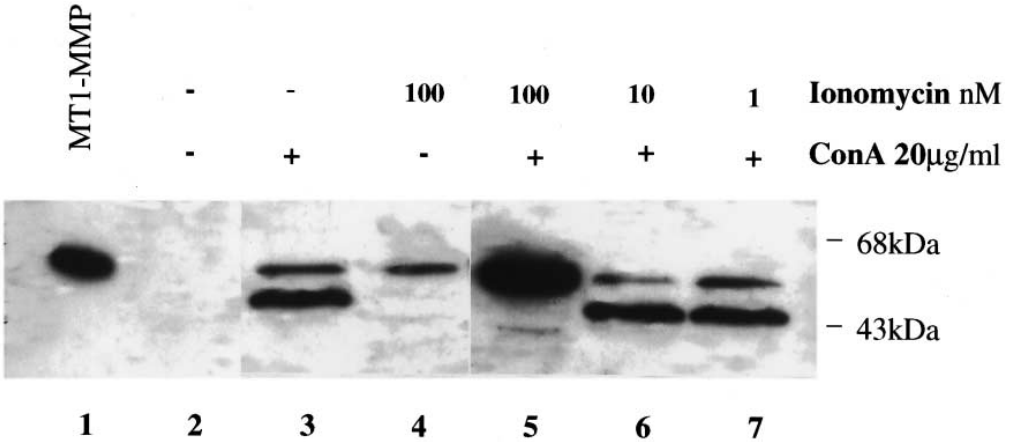

Fig. 3. Western analysis of the effect of ionomycin on ConA-induced MT1-MMP protein levels. Cells cultured in duplicate dishes were treated as described in the legend to Fig. 1. Cell lysates were electrophoresed, transferred to nitrocellulose membranes, and blotted with 114-1F2 anti-MT1-MMP monoclonal antibody. Lanes: 1, MT1-MMP cDNA transfected Cos1 cell lysate; 2, untreated MDA-MB-231 cells; 3, cells treated with 20 μg/ml ConA; 4, cells treated with 100 nM ionomycin; 5, cells treated with 100 nM ionomycin plus 20 μg/ml ConA; 6, cells treated with 10 nM Ionomycin plus 20 μg/ml ConA; 7, cells treated with 1 ng/ml Ionomycin plus 20 μg/ml ConA.

constitutive cellular processing of MT1-MMP, rather than interfering with ConA-induced regulation.

It is also possible, however, that the increased cellular calcium blocks the intracellular transport of the MT1-MMP, and thus prevent it from reaching its activator molecule. Initial reports consistent with the MT1-MMP activation being mediated by furin enzymes in the Golgi apparatus [23] have been challenged recently by observations with wild-type pro-MT1-MMP transfection studies [30], and the precise mechanism and localization of the MT1-MMP activator is not yet known. However, recombinant forms of MT1-MMP appear to remain predominantly latent in the intracellular environment compared to the predominantly active secreted form [23,30], leading to speculation that activation may be mediated by a plasma membrane-associated entity.

Lohi et al. have reported that 500 nM ionomycin inhibited ConA-, PMA- and TNFα-induced MMP-2 activation in HT1080 fibrosarcoma cells [22], broadening the scope of this observation to diverse cell types. Ionomycin treatment inhibits ConA-treated MDA-MB-231 cells from processing MT1-MMP protein and resulting MMP-2 activation without affecting ConA-induced increases in MT1-MMP mRNA levels. These data confirm that MT1-MMP expression and MMP-2 activation are sequentially regulated and that post translational modification of MT1-MMP is required for its function. Increased calcium levels may be involved in the control of MT1-MMP protein processing and hence the activity of MT1-MMP. This may be a direct effect, since furin enzymes which have been implicated in MT-MMP activation are calcium dependent (reviewed in [31]), as are other MMP's which are involved in cross-activation of other family members, and could also participate in MT1-MMP activation (reviewed in [1]). Plasmin, which is also capable of pro-MT1-MMP processing [32], is not calcium-dependent. The calcium effects may also be indirect, since altered intracellular calcium levels profoundly effect a plethora of cellular pathways. For example, calcium/calmodulin-dependent kinases and phosphatases could influence factors which directly mediate or regulate MT1-MMP trafficking and/or processing. In this light, it is interesting to note that the cycling of furin between the plasma membrane and Golgi apparatus is dependent on serine and tyrosine residues in the furin cytoplasmic tail [33]. Our data, indicating that the localization and/or activation of MT1-MMP is sensitive to increased calcium levels, may provide some clues for the elucidation of this pivotal process.

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