MMP-2 is localized to the mitochondria-associated membrane of the heart

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1Department of Pediatrics, University of Alberta, Edmonton, Alberta, Canada; 2Department of Pharmacology, University of Alberta, Edmonton, Alberta, Canada; 3Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, Alberta, Canada; and 4Mazankowski Alberta Heart Institute/Cardiovascular Research Centre, University of Alberta, Edmonton, Alberta, Canada

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Hughes BG, Fan X, Cho WJ, Schulz R. MMP-2 is localized to the mitochondria-associated membrane of the heart. Am J Physiol Heart Circ Physiol 306: H764–H770, 2014. First published December 27, 2013; doi:10.1152/ajpheart.00909.2013.—Matrix metalloproteinase-2 (MMP-2) has been extensively studied in the context of extracellular matrix remodeling but is also localized within cells and can be activated by prooxidants to proteolyze specific intercellular targets. Although there are reports of MMP-2 in mitochondria, a critical source of cellular oxidative stress, these studies did not take into account the presence within their preparations of the mitochondria-associated membrane (MAM), a subdomain of the endoplasmic reticulum (ER). We hypothesized that MMP-2 is situated in the MAM and therefore investigated its subcellular distribution between mitochondria and the MAM. Immunogold electron microscopy revealed MMP-2 localized in mitochondria of heart sections from mice. In contrast, immunofluorescence analysis of an MMP-2:HaloTag fusion protein expressed in HL-1 cardiomyocytes showed an ER-like distribution, with greater colocalization with an ER marker (protein disulfide isomerase) relative to the mitochondrial marker, MitoTracker red. Although MMP-2 protein and enzymatic activity were present in crude mitochondrial fractions, once these were separated into purified mitochondria and MAM, MMP-2 was principally associated with the latter. Thus, although mitochondria may contain minimal levels of MMP-2, the majority of MMP-2 previously identified as “mitochondrial” is in fact associated with the MAM. We also found that calreticulin, an ER- and MAM-resident Ca2+ handling protein and chaperone, could be proteolyzed by MMP-2 in vitro. MAM-localized MMP-2 could therefore potentially impact mitochondrial function by affecting ER-mitochondrial Ca2+ signaling via its proteolysis of calreticulin.

matrix metalloproteinase 2; mitochondria-associated membrane; mitochondria; calreticulin

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The most ubiquitous and best understood of the intracellular MMPs, 72 kDa MMP-2, is a bona fide intracellular protease (7, 40) with ~40% of newly synthesized protein retained within the cytosol because of an inefficient signal peptide (2). In fact, the intracellular activity of MMP-2 contributes to acute myocardial ischemia and reperfusion injury (45). Two further NH2-terminal-truncated isoforms also exist that lack the signal peptide entirely (2, 29). While MMP-2 can be activated by the proteolytic removal of its NH2-terminal inhibitory pro-peptide domain, 72 kDa MMP-2 is also directly activated by peroxynitrite (22, 44), a major contributor of oxidative stress (36). One key source of intracellular peroxynitrite are the mitochondria. Recent reports suggest that MMPs might be localized to the mitochondria, including MMP-2 (29, 33, 38, 45), MMP-9 (25, 34, 43), and MMP-1 (27). Of particular note, a constitutively-active MMP-2 transgene, transient oxidative stress, or hypoxia increased mitochondrial MMP-2 levels in the heart (29). Hypoxia-reoxygenation and ischemia-reperfusion injury involve increased peroxynitrite biosynthesis that could activate MMPs localized in or near mitochondria and thus contribute to the mitochondrial dysfunction which characterizes the pathology of this disorder.

The mitochondrial localization of MMP-2, a ubiquitous and abundant myocardial MMP, is, however, uncertain. The methods that were used to isolate mitochondria (25, 29, 33, 38, 43) result in preparations that copurify with a portion of the endoplasmic reticulum (ER) that is proteinaceously bound to mitochondrial Ca2+. The MAM, although contiguous with the ER, has specialized functions in lipid metabolism and oxidative protein folding and plays an important role as the ER/mitochondrial interface which, for example, controls ER/mitochondrial calcium (Ca2+) flux (37). MMP-2 is known to be localized to the ER (2). Thus the MMPs identified in so-called “crude” mitochondrial fractions obtained via differential centrifugation may actually be of ER origin. Immunofluorescence microscopy, also used to demonstrate the mitochondrial localization of MMPs (27, 34), may not have the resolution necessary to differentiate between proteins within mitochondria or the MAM (3) that are in close juxtaposition (as little as 10 nm apart) (12).

We therefore set out to determine the precise localization of MMP-2 at or near the mitochondria within the heart. We found that under normal physiological conditions, MMP-2 is preferentially localized to the MAM and much less so to the mitochondria. We also show that MMP-2 proteolyses the ER and MAM Ca2+ handling protein calreticulin, illustrating a potential mechanism for the biological action of MMP-2 in the MAM.
MATERIALS AND METHODS

Isolation of mitochondria and MAM. All experiments involving animals were approved by the Institutional Animal Care and Use Committee and were performed according to the recommendations given by the Guide to the Care and Use of Experimental Animals, published by the Canadian Council on Animal Care, and institutional guidelines for the care and use of animals. Male Sprague-Dawley rats (250–300 g) were anesthetized with pentobarbital sodium (240 mg/kg). Hearts were rapidly removed and perfused with oxygenated Krebs-Henseleit buffer at 37°C for 10 min at a constant perfusion pressure of 60 mmHg and 37°C to clear them of blood.

Crude mitochondrial and mitochondrial-free (“cytosolic”) fractions were obtained by differential centrifugation as previously described (19), with all procedures carried out on ice or at 4°C. It should be noted that although we follow the prevailing custom of referring to the mitochondria-free supernatant from differential centrifugation as “cytosol” for simplicity, it does retain the ER and other components of the light membrane t-PAGn. Crude mitochondrial fractions were either resuspended in hypotonic buffer consisting of 10 mM HEPES (pH 7.4), supplemented with protease inhibitor, and flash frozen in liquid N2 or subjected to density gradient centrifugation to obtain purified mitochondria (32). In brief, crude mitochondria were overlaid on a 30% Percoll solution in homogenization buffer supplemented with mitochrondia (32). In brief, crude mitochondria were overlaid on a 30% Percoll solution in homogenization buffer supplemented with protease inhibitor cocktail and centrifuged at 10,000 g for 15 min. The MAM was isolated by collecting the upper band, diluting with homogenization buffer, and then centrifugation at 100,000 g for 15 min. The supernatant was then centrifuged at 100,000 g for 1.5 h. The MAM pellet was collected using a pipette. Crude and purified mitochondrial fractions were subjected to three freeze-thaw cycles (liquid nitrogen/37°C) to rupture mitochondrial membranes before protein determination.

Western blot analysis. For protein analysis, equal amounts of total protein measured using the bicinchoninic acid assay were loaded onto SDS-PAGE gels, electrophoresed, transferred to polyvinylidene difluoride membranes, and incubated with either rabbit monoclonal anti-MMP-2 (Abcam ab92536), polyclonal anti-protein disulfide isomerase (PDI) (PAI-16920; Pierce), or polyclonal anti-voltage-dependent anion channel (VDAC; ab15895, Abcam), followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (CLCC42007, Cedarlane). Protein bands were detected using ECL Prime reagent (Amersham). Membranes were either exposed to autoradiography film or scanned using a charge-coupled device-based imager (Carestream 4000 MM Pro Image Station).

Gelatin zymography. Gelatin zymography was used to detect MMP-2 activity as previously described (42). In brief, tissue or cell fractions were either diluted with 10 mM HEPES (pH 7.4) and mixed with nonreducing loading buffer. Samples were run on 8% polyacrylamide gels copolymerized with 2 mg/ml gelatin. Gels were rinsed in 2.5% Triton X-100 (3 x 20 min), and incubated 18 h at 37°C in zymography activity buffer, consisting of 50 mM Tris, 150 mM NaCl, 5 mM CaCl2, and 0.05% Na3EDTA (pH 7.5). For some gels, the MMP inhibitor EDTA (10 mM) was added to the activity buffer to confirm that the observed gelatinolytic activity was due to MMP activity. Gels were stained with 0.05% Coomassie Brilliant blue G-250 for 3 h and then destained in 4% methanol:8% acetic acid solution.

Immunogold electron microscopy. Immunogold electron microscopy was essentially performed as previously described (20), with the following exceptions: heart samples from MMP-2 WT and KO mice were fixed in 0.1% glutaraldehyde, 4% paraformaldehyde, and 4% sucrose in 0.1 M sodium phosphate buffer (pH 7.4) overnight at 4°C and rinsed in 0.1 M sodium phosphate buffer before dehydration with a graded alcohol series. Primary antibodies against MMP-2 (monoclonal mouse anti-MMP-2 IgG, Millipore MAB3308 or monoclonal rabbit anti-MMP-2 IgG, ab92536, Abcam) were used at a dilution of 1:10,000 overnight at 4°C. Colloidal gold (18 nm)-conjugated, donkey anti-mouse IgG secondary antibodies were used at a 1:10 dilution for 90 min at room temperature.

Transduction of cardiomyocytes with HaloTagged MMP-2. The HL-1 cardiomyocyte cell line (10) was kindly provided by William Claycomb (Louisiana State University School of Medicine, New Orleans, LA). Cells were maintained with Claycomb Media (Sigma-Aldrich). cDNA encoding full-length MMP-2 lacking a stop codon was subcloned into a lentiviral construct containing a COOH-terminal HaloTag fragment fused to the insert (“HaloTagged” MMP-2). Lentiviral vectors were produced by transient transfection of 293T cells with the four packaging plasmids system. For transduction of HL-1 cardiomyocytes, 1 x 106 cells were incubated for 8 h with 5 x 108 transduction units of lentiviral vector in the presence of 6 μg/ml polybrene (Invitrogen). Transduced cells were treated with 2 μg/ml puromycin as a selection agent.

To visualize the HaloTagged protein, cells were incubated with the R110Direct HaloTag ligand (Promega) as per the manufacturer’s instructions. Mitochondria were visualized by incubating cells with 150 nM Mitotracker red C7MRos (Molecular Probes) for 30 min. Cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.2% Triton X-100 in PBS for 10 min. Some cells were incubated with primary antibodies against protein disulfide isomerase (Pierce PAI-16920) and then secondary antibody conjugated to Alexa Fluor 594. Cells were mounted with ProLong Gold Antifade reagent with 4,6-diamidino-2-phenylindole (DAPI; Life Technologies) and imaged using a spinning disc confocal microscope running Velocity 6.1.1 (PerkinElmer). For colocalization analysis, Velocity was used to generate Costes Pearson’s correlations for individual cells.

In vitro MMP-2 proteinase assay. Recombinant human calreticulin (1 μg, ab91577, Abcam) was incubated with 0.1, 0.7, or 2 μM of recombinant human MMP-2 for 4 h at 37°C. One sample was incubated with the MMP inhibitor EDTA (10 mM) to confirm that the observed degradation was due to MMP activity. Immediately after incubation, MMP-2 was inactivated by addition of 6x SDS-PAGE loading buffer and heating at 95°C for 5 min. Samples were electrophoresed on a 10% SDS-PAGE gel. Proteins and their fragments were visualized with Coomassie blue.

Statistics. Immunogold binding was compared between WT and KO heart sections using unpaired t-tests (Prism 6, GraphPad Software). Colocalization of MMP-2 with ER or mitochondrial markers was quantified using Costes Pearson’s correlation as calculated by Velocity. The statistical significance of the differences was determined using unpaired t-tests.

RESULTS AND DISCUSSION

To determine if MMP-2 is found within heart mitochondria, we first performed immunogold electron microscopy using ventricular heart sections from WT and MMP-2 KO mice. Initial attempts with a primary antibody dilution of 1:1,000 yielded nonspecific binding in KO sections (results not shown), whereas 1:10,000 dilution resulted in immunogold-bound MMP-2 localized to mitochondria within WT sections (Fig. 1A) and negligible binding in mitochondria within KO sections (Fig. 1B). MMP-2 was also localized to myofibrils as we have previously shown (39, 45). The degree of MMP-2 immunogold binding in mitochondria from WT and KO hearts was quantified as the number of gold particles (per μm2) of mitochondrial surface area (0.73 vs. 0.13, P = 0.006; Fig. 1C). We obtained similar results for MMP-2 located within the mitochondrial-associated membrane of the heart.
using a different anti-MMP-2 antibody (results not shown, ab92536, Abcam). Thus, as determined by immunogold electron microscopy, MMP-2 is unambiguously present within mitochondria of ventricular cardiac myocytes in WT but not MMP-2 KO mice.

To better understand the subcellular distribution of MMP-2, we expressed HaloTagged MMP-2 in HL-1 cardiomyocytes and used fluorescence confocal microscopy to compare their distribution with markers for ER (PDI) and mitochondria (MitoTracker red). HaloTagged MMP-2 showed some possible colocalization with mitochondria (Fig. 2, A–C). However, MMP-2 did not exhibit the filamentous staining pattern that is typical of mitochondria. Instead, the overall distribution of MMP-2 more closely resembled that of the ER marker PDI (Fig. 2, D–F), with a perinuclear distribution typical of ER. Consistent with these observations, analysis of the staining distribution of MMP-2 versus PDI yielded a stronger correlation coefficient than that of MMP-2 versus MitoTracker red (0.63 vs. 0.46, $P = 0.016$; Fig. 2G).

This preferential association of MMP-2 with the ER suggested to us that at least some of what was identified as mitochondrial MMP-2 may in fact be of MAM origin. To investigate this, we isolated cytosolic and crude and purified mitochondrial fractions from rat hearts, as per the schema in Fig. 3A. As previously reported, 72 kDa MMP-2 protein was present in both cytosolic (8) and crude mitochondrial fractions (29) (Fig. 3B). As expected, the crude mitochondrial fraction, despite being enriched with the mitochondrial protein VDAC, also contained substantial amounts of the ER protein PDI (Fig. 3B). To determine whether MMP-2 protein was proteolytically active, we performed gelatin zymography on the same fractions, showing significant 72-kDa gelatinolytic activity in the cytosol and, to a lesser extent, in the crude mitochondrial fraction (Fig. 3C). However, when we further separated the crude mitochondria into purified mitochondria and MAM fractions, we found that 72 kDa MMP-2 protein (Fig. 3D), and gelatinolytic activity (Fig. 3E) was chiefly associated with the MAM fraction, characterized by the retention of PDI and minimal levels of VDAC (Fig. 3D). The gelatinolytic activity found in the MAM was inhibited by EDTA, confirming that this was due to MMP proteolytic activity (Fig. 3E). No gelatinolytic activities in the molecular weight range of dimeric MMP-2 (24), MMP-9, or lower molecular weight MMP-2 cleavage products were observed for any samples (data not shown).

The minimal presence of MMP-2 in mitochondria is consistent with in silico analysis. A collection of six programs designed to predict, via differing algorithms, if a given protein is likely to be localized to the mitochondria [Predotar (41), MitoProt II (9), TargetP (15), CELLO (47), PSORT II (18), and MITOPRED (26)] predicted a nonmitochondrial localization for human MMP-2, as well as for MMP-1 and MMP-9 (results not shown).

Zhou et al. (48) showed that transgenic mice expressing a mutant, constitutively activated MMP-2 and subjected to ex vivo ischemia-reperfusion injury exhibited cardiac mitochondrial dysfunction. These mice also exhibited an NH$_2$-terminally truncated isofrom of MMP-2 within crude mitochondrial fractions (29); this MMP-2 isoform exhibited an immunohistochemical distribution consistent with mitochondria (30). Our results suggest that this MMP-2 may have been instead localized to the MAM, which cannot be distinguished from mitochondria via conventional light microscopy (3). Indeed, the MAM plays an important role in the maintenance of normal mitochondrial function, notably through the regulation of Ca$^{2+}$ release into the cytosol in close proximity to mitochondrial outer membrane anion channels. One important ER and MAM regulator of Ca$^{2+}$ homeostasis, calreticulin (31), has been previously identified as a putative MMP-2 target by in vitro degradomic analyses (7, 13). To test whether MMP-2 is capable of proteolyzing calreticulin, we incubated these proteins to-
together at 37°C and then analyzed the products using SDS-PAGE. MMP-2 was capable of proteolyzing calreticulin in a concentration-dependent fashion, and this was blocked by the metalloproteinase inhibitor EDTA, confirming that calreticulin proteolysis was due to MMP proteolytic activity (Fig. 4).

The proteolysis of calreticulin by MMP-2 therefore provides a potential biological mechanism through which MAM-localized MMP-2 could modulate mitochondrial and cellular function. Calreticulin has been identified as a protective factor against hypoxic injury (23, 28, 46), possibly through its ability to limit mitochondrial Ca\(^{2+}\) accumulation (4). Decreased calreticulin due to increased MAM MMP-2 activity in the setting of ischemia-reperfusion injury could therefore lead to a further increase of mitochondrial Ca\(^{2+}\), which is known to induce mitochondrial dysfunction (35) and which is consistent with

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Fig. 2. Fluorescent images of HL-1 cardiomyocytes expressing HaloTagged MMP-2. Scale bar = 17 μm. MMP-2 was visualized with HaloTag R110Direct ligand (green). An antibody against protein disulfide isomerase (PDI), recognized by an Alexa Fluor 594-conjugated secondary antibody (red) was used as a marker for ER. MitoTracker red CMXRos was used to visualize mitochondria. DAPI (blue) was used to stain DNA. A–C: HaloTagged MMP-2 (green) and MitoTracker (red). D–F: HaloTagged MMP-2 (green) and PDI (red). G: Quantification of MMP-2 colocalization with mitochondria (MitoTracker) and ER (PDI). *P = 0.016. Note that while the contrast of the representative images shown above was adjusted for ease of visualization (using the “auto-contrast” feature of Volocity), the statistical analysis was performed on the original, unadjusted images.
Fig. 3. Subcellular distribution of MMP-2 in rat heart tissue. Homogenates were fractionated according to the scheme shown in A, in which crude mitochondria (mito-cr) were first obtained and then further subfractionated into purified mitochondria (mito-p) and mitochondria-associated membrane (MAM) fractions. B: representative immunoblot (of n = 3) for MMP-2 showing that it is present in the cytosol (cyto) and in crude mitochondrial fractions. Antibodies against markers for endoplasmic reticulum (PDI) and mitochondria (VDAC, voltage-dependent anion channel) were used to confirm fraction purity. HT, conditioned media from HT-1080 cells used as a positive control. Homogenates from the hearts of MMP-2 KO and WT mice were used as further controls for antibody specificity. C: gelatin zymograms showing MMP-2 activity in the cytosol and crude mitochondria. D: representative immunoblot (of n = 2) for MMP-2 showing that MMP-2 is present in the MAM fraction. E: gelatin zymograms showing that MMP-2 activity is present in the MAM fraction, but negligibly in purified mitochondria, and that this gelatinolytic activity is blocked by the MMP inhibitor EDTA (10 mM).

In conclusion, our results demonstrate for the first time that proteolytically competent MMP-2 is predominantly localized to the MAM rather than the mitochondria under normal physiological conditions. There is, however, a distinct localization of MMP-2 within the mitochondria as evidenced by immunogold EM, but in an amount that is below the detection threshold of conventional biochemical methods within purified mitochondria. This does not rule out the possibility that cell stress could induce a translocation of MMPs to mitochondria as proposed (29, 34); however, this needs confirmation with methodologies such as those used here. The close association of MAM with mitochondria could also explain why a transgenic, NH2-terminal-truncated isoform of MMP-2 showed a staining pattern consistent with a mitochondrial distribution (30). We speculate that MMP-2 may modulate ER-mitochondrial Ca2+ signaling via control of Ca2+ homeostasis by control of calreticulin levels. To our knowledge this is the first evidence of an ER-resident target of MMP proteolysis. Since all MMPs must pass through the ER as part of the secretory pathway, it is possible that the ER represents a previously unidentified cellular compartment in which MMPs play an important role.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

B.G.H. and R.S. conception and design of research; B.G.H., X.F., and W.J.C. performed experiments; B.G.H. analyzed data; B.G.H., X.F., and R.S. interpreted results of experiments; B.G.H. prepared figures; B.G.H. drafted manuscript; B.G.H., X.F., and R.S. edited and revised manuscript; B.G.H., X.F., W.J.C., and R.S. approved final version of manuscript.
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