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Matrix metalloproteinase-2-induced epidermal growth factor receptor transactivation impairs redox balance in vascular smooth muscle cells and facilitates vascular contraction

Alejandro F. Prado[a,b], Laena Pernomian[c], Aline Azevedo[d], Rute A.P. Costa[e], Elen Rizzi[a], Junia Ramos[a,b,c,d,e,f,g], Adriana F. Paes Leme[e], Lusiane M. Bendhack[f], Jose E. Tanus-Santos[a], Raquel F. Gerlach[g,*]

[a] Department of Pharmacology, Ribeirao Preto Medical School, University of Sao Paulo, Ribeirao Preto, SP, Brazil
[b] Laboratory of Structural Biology, Institute of Biological Sciences, Federal University of Para, Belem, PA, Brazil
[c] Department of Biochemistry and Immunology, Faculty of Medicine at Ribeirao Preto, University of São Paulo, SP, Brazil
[d] Department of Biomechanics, Medicine and Rehabilitation of the Locomotor System, Ribeirao Preto Medical School, University of Sao Paulo, Ribeirao Preto, SP, Brazil
[e] Mass Spectrometry Laboratory, Brazilian Biosciences National Laboratory, LNBio, CNPEM, Campinas, Brazil
[f] Department of Physics and Chemistry, Faculty of Pharmaceutical Sciences from Ribeirao Preto, University of São Paulo, Ribeirao Preto, SP, Brazil
[g] Department of Morphology, Physiology and Basic Pathology, Faculty of Dentistry of Ribeirao Preto, University of Sao Paulo, Av. Café, S/N - Ribeirao Preto, SP 14040-904, Brazil

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ABSTRACT

Increased reactive oxygen species (ROS) formation may enhance matrix metalloproteinase (MMP)-2 activity and promote cardiovascular dysfunction. We show for the first time that MMP-2 is upstream of increased ROS formation and activates signaling mechanisms impairing redox balance. Incubation of vascular smooth muscle cells (VSMC) with recombinant MMP-2 increased ROS formation assessed with dihydroethidium (DHE) by flow cytometry. This effect was blocked by the antioxidant apocynin or by polyethylene glycol-catalase (PEG-catalase), and by MMP inhibitors (doxycycline or GM6001). Next, we showed in HEK293 cells that MMP-2 transactivates heparin-binding epidermal growth factor (HB-EGF) leading to EGF receptor (EGFR) activation and increased ROS concentrations. This effect was prevented by the EGFR kinase inhibitor Ag1478, and by phospholipase C (PLC) or protein kinase C (PKC) inhibitors (A778 or chelerythrine, respectively), confirming the involvement of EGFR pathway in MMP-2-induce responses. Next, we showed that intraluminal exposure of aortas to MMP-2 increased vascular MMP-2 levels detected by immunofluorescence and gelatinolytic activity (by *in situ* zimography) in association with increased ROS formation. This effect was inhibited by MMP inhibitors (phenanthroline or doxycycline) and by apocynin or PEG-catalase. MMP-2 also increased aortic contractility to phenylephrine and this effect was prevented by MMP inhibitor GM6001 and by apocynin or PEG-catalase, showing again that increased ROS formation mediates functional effects of MMP-2. These results show that MMP-2 activates the EGFR and triggers downstream signaling pathways increasing ROS formation and promoting vasoconstriction. These findings may have various implications for cardiovascular diseases.

1. Introduction

Imbalanced matrix metalloproteinase (MMP) activity has long been acknowledged as a complex and critical alteration involved in a variety of diseases [1]. Among other MMPs, mounting evidence implicates MMP-2 as a major player in disease processes, particularly in cardiovascular diseases [2,3]. In fact, increased circulating levels of MMP-2 have been associated with worse prognosis in patients with cardiovascular disease [4] and were suggested to predict mortality [5]. Moreover, acutely increasing circulating MMP-2 levels significantly impaired both cardiac and vascular function [6], although the precise mechanisms explaining these findings have not been fully elucidated.

Current knowledge supports the notion that enhanced tissue levels of reactive oxygen species (ROS) contribute to MMP-2 activation [7,8] or induce its expression [9] promoting cardiovascular dysfunction [10]. This notion is supported by studies showing that antioxidant drugs

* Corresponding author.
E-mail address: rfgerlach@forp.usp.br (R.F. Gerlach).

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attenuate MMP-2 activity and MMP-2-mediated pathophysiological cardiovascular alterations [11,12] and these findings are very similar to those found with the use of MMP inhibitors [13–15]. Therefore, both antioxidant drugs and MMP inhibitors have been shown to prevent many of the deleterious effects associated with imbalanced MMP-2 activity. However, the possibility that MMP-2 is upstream of increased ROS formation and activates cell signaling mechanisms that impair redox balance by enhancing ROS formation has not been explored yet. This hypothesis is supported by few studies showing that other MMPs can activate pathways associated with increased ROS production [9,16–18]. For example, MMP-7 induced ROS production by activating epidermal growth factor receptor (EGFR) after stimulation of alpha-1 adrenergic receptor in mesenteric arteries from rats [16,17,19]. Moreover, increased expression of intracellular cardiac MMP-2 in transgenic mice promoted lipid peroxidation after ischemia reperfusion injury [18].

There is strong evidence that MMP-2 proteolytic activity cleaves the transmembrane protein pro-heparin-binding epidermal growth factor (HB-EGF) into soluble HB-EGF leading to EGF receptor (EGFR) transactivation [16,20,21]. In addition, previous studies showed that EGFR stimulation promotes ROS formation in the cardiovascular system [17,19,22] and contributes to vasoconstriction [16]. However, there is no evidence linking increased MMP-2 activity and enhanced vascular ROS formation as a result of increased MMP-2 mediated gelatinolytic activity. In this study, we tested the hypothesis that MMP-2-mediated proteolytic activity leads to transactivation of EGFR and triggers prooxidant alterations of the vascular biology that contribute to increased vascular contractility. We now provide evidence that MMP-2 is upstream of ROS formation and we show mechanisms directly linking increased MMP-2 activity with impaired redox balance resulting in vascular dysfunction.

2. Material and methods

2.1. Animals

This study complied with the guidelines of the Ribeirao Preto Medical School, University of Sao Paulo (Protocol no. #120/2010), and the animals were handled according to the guiding principles published by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male *New Zealand* rabbits (2.5–3.0 kg) and male rats (200 ± 10 g) from the colony at University of São Paulo were maintained at room temperature (22–25 °C) on light/dark cycle (12 h) and had free access to standard rat chow and water.

2.2. Materials

Tyrphostin AG 1478, Phenylephrine, Apocynin, Peg-Catalase (PG-Cat), Dihydroethidium (DHE), phenanthroline, Phenylmethylsulfonyl fluoride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). GM6001 was purchased from Merck-Millipore (Tokyo, Japan). MMP-2 polyclonal antibody was purchased from NovusBio (Littleton, CO, USA). DQ Gelatin fluorogenic substrate and Alexa 647-conjugated anti-rabbit secondary antibody was purchased from Molecular Probes (Eugene, OR, USA). The MMP-2 recombinant protein was produced in our laboratory and specific details on its production as well as enzymatic activity data on various lots are described in a previous manuscript [23].

2.3. Cell culture

The *Rattus norvegicus* vascular smooth muscle cell (VSMC) line A7r5 obtained from American Type Culture Collection (ATCC CRL-1444) (Rockville, MD, USA) was maintained at 37 °C under an atmosphere of 5% CO_2 in culture flasks with Dulbecco´s modified Eagle´s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (Life technologies, Cat# 15240112). The cells were used from third to fifth passages after unfreezing.

2.4. Assessment of the effects of MMP-2 and inhibitors on vascular smooth muscle cells ROS production by cell flow cytometry

To assess the effects of MMP-2 on ROS concentrations in vascular smooth muscle cells (VSMC), we incubated VSMC with MMP-2 and assessed ROS concentrations with dihydroethidium (DHE) probe by flow cytometry. Cytofluorographic analysis was performed using a Becton–Dickinson FACS Canto (San Jose, CA, USA) with an argon ion laser tuned to 488 nm. Acquisition was set at 10.000 events. Changes in fluorescence intensity (FI) emitted by DHE were measured in isolated VSMC cells initially analyzed without DHE (Blank) as a control to ensure that there was no interference of DHE emitted fluorescence. After that, the cells were incubated with DHE (10 μM) for 30 min, as previously detailed [24], either in the presence of MMP-2 (16 nmol/l) or vehicle (PBS), which was added immediately after DHE (30 min MMP-2 incubation) or during the last 10 min (10 min MMP-2 incubation).

To confirm that cell incubation with MMP-2 affects ROS concentrations, we carried out control experiments to examine the effects of antioxidant agents including apocynin (a ROS scavenger; 100 μmol/l) [25], diphenyl iodonium (DPI; a flavoprotein inhibitor) 10 μmol/l, or polyethylene glycol-catalase (PEG-catalase, which catalyzes the breakdown of intracellular H_2O_2 into H_2O and O_2; 3000 U/ml). These experiments were carried out as described above, either in the presence of MMP-2 (30 min MMP-2 incubation) or vehicle.

To confirm that MMP-2 proteolytic activity affects ROS concentrations in VSMC, we examined the effects of MMP inhibitors (doxycycline 100 μmol/l or GM6001 1 μmol/l) on MMP-2-induced changes in ROS concentrations using the same conditions as described above, either in the presence of MMP-2 (30 min MMP-2 incubation) or vehicle.

2.5. Effects of MMP-2-induced EGFR transactivation on cellular ROS concentrations

MMP-2 proteolytic activity is known to promote EGFR transactivation [16,20,21,26], which activates cell signaling. To examine whether MMP-2-induced cleavage of HB-EGF results in increased ROS concentrations and the mechanism involved in this effect, we designed a series of cell experiments.

Firstly, we examined the cleavage of HB-EGF by MMP-2 using a reporter protein in cell culture conditions. A plasmid encoding HB-EGF-AP, a chimeric protein used for alkaline phosphatase (AP) reporter assay, was kindly provided by Dr. Michael R. Freeman (Department of Surgery, Harvard Medical School, Boston) [27–30]. HEK293 cells were stably transfected with the HB-EGF-alkaline phosphatase (AP) plasmid and seeded (1×10^4) into 96-well plates (Corning) for 24 h. The following day, the cells were starved for 4 h in a DMEM FBS-free medium. After 4 h, the cells were treated in a phenol-free medium with MMP-2 (16 nM) for 10 and 30 min. Briefly, 100 μl of conditioned media were collected from each well and added for 30 min to other individual well of a 96-well plate containing 100 μL of AP buffer (0.5 mol/l Tris-HCl, pH 9.5, containing 5 mmol/l *p*-nitrophenyl phosphate disodium, 1 mmol/l diethanolamine, 50 μmol/l $MgCl_2$, 150 mmol/l NaCl, 5 mmol/l EDTA) and the absorbance was measured at 405 nm. Three independent experiments were performed in duplicates.

Secondly, to further validate the idea that MMP-2 proteolytic activity promotes EGFR transactivation, we assessed the effects of MMP-2 on ROS concentrations in VSMC by incubating VSMC with MMP-2 in the presence of AG1478 (an EGFR kinase inhibitor) 3 or 10 μmol/l (or vehicle) as described above. ROS concentrations were assessed with DHE probe by flow cytometry, as described above.

Thirdly, given that EGF binding to its receptor activates downstream cell signaling mediated by phospholipase C (PLC) [31,32] and protein kinase C (PKC) [31,32], we assessed the effects of MMP-2 on ROS