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ROLE OF THE HUMAN CHYMASE (CMA1) IN THE CONVERSION OF BIG-ENDOTHELIN-1 TO ENDOTHELIN-1 (1-31)

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RÉSUMÉ

Rôle de la chymase humaine (CMA 1) dans la conversion de la big-endothéline-1 en endothéline-1 (1-31)

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Mémoire présenté à la Faculté de médecine et des sciences de la santé en vue de l'obtention du diplôme de maître ès sciences (M.Sc.) en pharmacologie, Faculté de médecine et des sciences de la santé, Université de Sherbrooke, Sherbrooke, Québec, Canada, J1H 5N4

La voie de conversion de Big ET-1 en ET-1, chymase dépendante a été établie *in vitro*. Ce n'est que récemment, en 2009 que notre groupe a démontré que la conversion de Big ET-1 en ET-1 (1-31) peut avoir lieu *in vivo* chez la souris (Simard et al., 2009), sachant que ET-1 (1-31) est convertie en ET-1 via NEP *in vivo* (Fecteau et al., 2005). En plus, en 2013, notre laboratoire a démontré que la mMCP-4, l'analogue murin de la chymase humaine, produit l'ET-1 (1-31) à partir du précurseur Big ET-1 (Houde *et al*., 2013). Jusqu'a présent, dans la littérature, on ne trouve pas de caractérisations spécifiques de chymases (humaine ou murine) recombinantes. En fait, le groupe de Murakami, en 1995, a publié une étude caractérisant, d'une façon chymostatin dépendante, la CMA1 (chymase humaine) en utilisant l'Angiotensine I comme substrat (Murakami et al., 1995). Cependant, le chymostatin est un inhibiteur non-spécifique de la chymase. Il a été démontré que le chymostatin peut inhiber l'élastase, une enzyme pouvant convertir l'Angiotensine I en Angiotensine II (Becari et al., 2005). Basé sur ces observations, l'hypothèse formulée dans la présente étude est que la CMA1 recombinante ou extraite des cellules LUVA (lignée humaine de mastocytes) ou des fractions solubles des aortes humaines convertit la Big ET-1 en ET-1 (1-31) d'une façon TY-51469 (un inhibiteur spécifique de la chymase) sensible. Dans un deuxième volet, on a étudié la cinétique enzymatique de CMA1 en vers le substrat Big ET-1 et Ang I. L'affinité de CMA1 contre la Big ET-1 était plus grande comparé à l'Ang I (K_M Big ET-1 : 12.55 μM et Ang I : 37.53 μM). Cependant CMA1 était plus efficace dans le clivage de l'Ang I comparé à la Big ET-1 (K_{cat}/K_M Big ET-1 : 6.57 x 10^{-5} $\mu M^{-1}.s^{-1}$ et Ang I : 1.8 x 10^{-4} $\mu M^{-1}.s^{-1}$). Dans un troisième volet impliquant des expériences *in vivo*, l'effet presseur de la Big ET-1, l'ET-1 et l'Ang I a été testé chez des souris conscientes mMCP-4 KO comparé à des souris de type sauvage. L'augmentation de la pression artérielle moyenne a été plus importante chez les souris de type sauvage après l'administration de Big ET-1 que chez les souris mMCP-4 KO. Cet effet n'a pas été observé après l'administration d'ET-1 et/ou d'Ang I ce qui explique le rôle de la chymase dans l'effet de la conversion de Big ET-1 en ET-1 (1-31).

Mots clés : Chymase, Enzymes recombinantes, Spectrométrie de masse, radio-télémétrie, analyse *in silico*

ABSTRACT

Role of human chymase (CMA 1) in the conversion of big-endothelin-1 to endothelin-1 (1-31)

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Thesis submitted to the Faculty of Medicine and Health Sciences for Masters of Science (M.Sc.) in Pharmacology, Faculty of Medicine and Health Sciences, Université de Sherbrooke, Sherbrooke, Quebec, Canada, J1H 5N4

The chymase-dependant pathway responsible for converting Big ET-1 to ET-1 was established in vitro. It has only been recently, in 2009, that our group demonstrated that the conversion of Big ET-1 to ET-1 (1-31) can occur in vivo in mice (Simard et al., 2009), knowing that ET-1 (1-31) is converted to ET-1 via NEP in vivo (Fecteau et al., 2005). In addition, our laboratory demonstrated in 2013 that mMCP-4, the murine analogue of human chymase, produces ET-1 (1-31) from the Big ET-1 precursor (Houde et al. 2013).

Thus far, in the literature, there are no specific characterizations of recombinant chymases (human or murine). In fact, the group of Murakami published in 1995 a study characterizing the CMA1 (human chymase) in a chymostatin-dependent fashion, using Angiotensin I as a substrate (Murakami et al., 1995). However, chymostatin is a non-specific inhibitor of chymase. It has been shown that chymostatin can inhibit elastase, an enzyme that can convert Angiotensin I to Angiotensin II (Becari et al., 2005).

Based on these observations, the proposed hypothesis in the present study suggests that recombinant as well as extracted CMA1 from LUVA (human mast cell line), in addition to soluble fractions of human aortas, convert Big ET-1 into ET-1 (1-31) in a TY-51469 (a chymase-specific inhibitor) sensitive manner.

In a second component, we studied the enzyme kinetics of CMA1 with regard to the Big ET-1 and Ang I substrate. The affinity of CMA1 against Big ET-1 was greater compared to Ang I (KM Big ET- 1: 12.55 μM and Ang I: 37.53 μM). However, CMA1 was more effective in cleaving Ang I compared to Big ET-1 (Kcat / KM Big ET-1: 6.57 x 10-5 μM-1.s-1 and Ang I: 1.8 x 10-4 MM-1.s-1).

In a third component involving in vivo experiments, the pressor effects of Big ET-1, ET-1 and Ang I were tested in conscious mMCP-4 KO mice compared to wild-type mice. The increase in mean arterial pressure after administration of Big ET-1 was greater in wild-type mice compared to mMCP-4 KO mice. This effect was not observed after administration of ET-1 and / or Ang I.

Key words: Chymase, Recombinant enzymes, Mass spectrometry, radio-telemetry, in silico analysis

TABLE OF CONTENT

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AAA	Abdominal aortic aneurysms
ACE	Angiotensin converting enzyme
ADH	antidiuretic hormone
Ala	Alanine
AMC	7-amino-4-methylcoumarin
Ang I	Angiotensin I
Ang II	Angiotensin II
Apo A I	Apolipoprotein A I
Apo B	Apolipoprotein B
Apo E	Apolipoprotein E
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid (Aspartate)
AT1R	Angiotensin type 1 receptor
bFGF	Basic fibroblast growth factor
Big ET-1	Big endothelin-1
BMCP	Bipotent Basophil/Mast cell progenitors
C1	Complement 1
C3a	Complement 3 a
Ca^{2+}	Calcium
CaM	Calmodulin
CLP	Common lymphoid progenitor

CMA1	Human chymase
CMP	Common myeloid progenitor
CPA	Carboxypeptidase A
CTAP-III	Connective tissue activating peptide- III
CTMC	Connective tissue mast cells
DAG	Diacylglycerol
DM	Diabetes Mellitus
DMI	Diabetes Mellitus type I
DMII	Diabetes Mellitus type II
DPPI	Dypeptidyl Peptidase I
ECE	Endothelin Converting Enzyme
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelial Nitric Oxide synthase
ET-1	Endothelin-1
ET-2	Endothelin-2
ET-3	Endothelin-3
ET_A	Endothelin A receptor
ET_B	Endothelin B receptor
Gly	Glycine
GMP	Granulocyte/Monocyte progenitor
GPCR	G protein coupled receptors
HDL3	High Density Lipoprotein 3
His	Histidine
HPLC	High Performance Liquid Chromatography

HTN	Hypertension
IBS	Irritable bowel syndrome
IgE	Immunoglobulin E
IL	Interleukin
Ile	Isoleucine
IP_3	inositol-1,4,5-triphosphate
K_{cat}	Productivity constant of Michaelis
K_M	Michaelis constant
KO	Knockout
LC-MS	Liquid chromatography–mass spectrometry
LDL	Low Density Lipoprotein
Leu	Leucine
MC	Mast cells
MCT	Mast cells with tryptase only granules
MCTC	Mast cells with tryptase and chymase granules
MEP	Megakaryocyte/Erythrocyte progenitor
MLC	Myosin light chain
MLCK	Myosin light chain kinase
mMCP-4	Mouse mast cell protease- 4
MMPs	Matrix metalloproteases
MPP	Multipotent hematopoietic progenitor
NEP	Neutral endopeptidase
NO	Nitric Oxide
PAR-1	Protease activated receptor
PGD2	Prostaglandin D2

PGE2	Prostaglandin E2
Phe	Phenylalanine
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl fluoride
Pro	Proline
RAS	Renin-Angiotensin system
SBTI	Sterol Biosynthesis inhibitor
SCF	Stem cell factor
Ser	Serine
SMC	Smooth muscle cells
SR	Sarcoplasmic Reticulum
Suc	Succynate
TGF-β1	Transforming growth factor beta-1
TIMP-1	Tissue inhibitor of metalloproteinase-1
TLR	Toll-like receptors
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine
VIP	Vasoactive intestinal peptide
VSMC	Vascular smooth muscle cells
WAT	White adipose tissue

I- INTRODUCTION

1.1 Mast cells development

Mast Cells (MC) are immune cells maturing from Mast cell-committed progenitor (MCcP) and originating from hematopoietic progenitors in the bone marrow .The multipotent hematopoietic progenitor stem cells (MPP) develop into common myeloid progenitor (CMP) and common lymphoid progenitor (CLP) (Kondo *et al.*, 1997; Akashi *et al.*, 2000).CMP will further divide into Megakaryocyte/Erythrocyte progenitor (MEP) and Granulocyte/Monocyte progenitor (GMP).The lineage origin of Mast cell-committed progenitor was a debate since the literature shows conflicting data. It was known for a certain time that they belong to the CMP/GMP lineage (Suda *et al.*, 1983). However in 2005, Chen and collaborators concluded that it could be directly derived from MPP. On the other hand, Franco and collaborators found that MCcP are closer to the MEP lineage relying on gene expression profiling of cells (Franco *et al.*, 2010). In contrast, Arinobu and colleagues verified that MCcP belonged to GMP lineage (Arinobu *et al.*, 2005). Moreover, Bipotent Basophil/Mast cell progenitors (BMCP) were identified in the spleen of C57BL mice (Arinobu, 2005; Iwasaki, 2006; Qi *et al.*, 2013); these progenitors were isolated and were capable of developing basophils and MC within the GMP fraction in the bone marrow (Qi *et al.*, 2013). When put together, the data suggests that within the GMP pathway, bipotent progenitors give rise to the MCcP. These MCcP circulate in the blood and are home to tissues where they will mature into MC. MCcP express several markers and receptor such as CD34, CD13, c-kit, stem cell factor (SCF) and the IgE receptor FcεRI just like the mature MC; however they are less granulated than MC. Homing of the MCcP is triggered by several cytokines and mediator such as IL-3 and SCF (Kawakami and Galli, 2002).

The maturation of MCcP give rise to two types of MC, the MCT and MCTC depending on the type of tissue they are maturing in (Irani *et al.*, 1986; Kitamura *et al.*, 1989; Metcalfe *et al.*, 1997; Kawakami and Galli, 2002). The MCT are present essentially in the mucosa of the gastrointestinal tract and the bronchi. Their granules contain mostly Tryptase. On the other hand, the MCTC are present in the skin, lymph nodes and submucosa of the gastrointestinal tract. Their granules contain Tryptase and Chymase (Irani *et al.*, 1986; Irani *et al.*, 1989; Kitamura *et al.*,

1989; Metcalfe *et al.*, 1997). It has been shown that MCTC are similar to murine connective tissue MC while MCT resemble mucosal MC. (Miyazaki *et al.*, 2006; Pejler *et al.*, 2010).

1.2 Mast Cell dependent diseases

MC have been known for their role in allergy (Williams and Galli,2000). Once activated, by Immunoglobulin E (IgE) binding on their Fcε receptor, they will degranulate and thus release hormonal mediators and proteases, triggering an allergic reaction and resulting in sustained inflammation (Galli,1993; He and Shi, 2013).

In addition to their pivotal role in allergy, mast cells are key players in mediating the immune response. Due to their Fcγ receptors I and III and toll-like receptors (TLR) types 2 and 4, they sense the microenvironment thus guiding the innate and acquired immunity (Frossi *et al.*, 2004; Mekori *et al.*, 2000; Okumura *et al.*, 2003). For example, MC-deficient mice are not protected against sepsis secondary to bacterial peritoneal infection (Schneider *et al.*, 2007). Given this role, their implication in inflammatory, immune and autoimmune diseases becomes consequential. It has, for example been shown that MC have an involvement in Rheumatoid arthritis, inflammatory bowel disease, metabolic diseases, atopic dermatitis, idiopathic pulmonary fibrosis, liver fibrosis and psoriasis.

In addition, their function is becoming clearer in neoplasms such as gynecological neoplasms and prostate cancer. MC are associated with tumor progression and cancer cells invasion in prostate cancer (Li *et al.*, 2015; Johansson *et al.*, 2010). Moreover, new studies are presenting MC as a target for immunotherapy in prostate cancer (Olford and Marshall, 2014). Furthermore, their implication in vascular and cardiovascular diseases is of significant importance especially regarding atherosclerosis, plaque erosion and abdominal aortic aneurysms (AAA) (Bot *et al.,* 2008; Bot and Biessen, 2011).

1.3 Mast Cells in Cardiovascular diseases

In the 1950's, MC were thought to have a protective role in atherosclerosis (Constantinides, 1953; Cairns and Constantinides, 1954) since their number was found to be inversely correlated with the disease. Myocardial tissue from atherosclerotic patients showed a reduced number of mast cells compared to healthy subjects (Constantinides, 1953; Cairns and Constantinides, 1954). However in the following years the tendency shifted more towards the proatherogenic function of Mast cells. Later studies showed that the number of MC increased in the intima and adventitia of atherosclerotic plaques with the progression of the disease. (Laine *et al.*, 1999; Kaartinen *et al.*, 1994a; 1994b; Kovanen *et al.*, 1995). These results were further confirmed to be true in human aorta, coronary arteries and carotid arteries (Atkinson *et al.*, 1994; Jeziorska *et al.*, 1997). Both types of Mast cells were found to be present in the plaque the MCTand MCTC (Kaartinen *et al.*, 1994b). In addition, Mast cell granules were found to be ingested by foam cells and smooth muscle cells suggesting a role for MC in plaque expansion (Kaartinen *et al.*, 1995). Furthermore, some studies showed that MC expressing bFGF an angiogenic factor, were co-localized along with intraplaque neovessels. Indeed, the group of Kaartinen hypothesized in 1995 that with the release of histamine, the neovessels could bleed causing intraplaque hemorrhage and therefore plaque destabilization (Kamat *et al.*, 1987Kaartinen *et al.*, 1995; Lappalainen *et al.*, 2004).

1.4 Mast Cell Proteases

The numerous physiological and pathological roles of MC are suggested to be dependent on their granule content. The MC degranulation can be initiated by various stimuli such as Fc receptor mediated activation- in allergic reactions- (Genovese *et al.*, 2000), complement receptor mediated activation (Nilsson *et al.*, 1996) or Toll-like receptor activation. MC activation enhances de novo synthesis of cytokines, prostaglandins (PGE2, PGD2) and Leukotrienes (Galli *et al.*, 2005). Once degranulated, MC release several mediators such as histamine (known role in allergies), proteases and cytokines.

MC granules contain several proteases such as matrix metalloproteases (MMPs) (Baram *et al.*, 2001), cathepsin D, C, and E (Dragonetti *et al.*, 2000;Henningsson *et al.*, 2005;Wolters *et al.*, 2000), Chymase, Tryptase and Carboxypeptidase A (MC-CPA); of which the last three are known to be MC-specific. Tryptases and chymases belong to theserine protease class, while MC-CPA is a zinc-dependent metalloprotease. These proteases have different substrate specificities. Tryptases have a Trypsin-like activity; Chymases have a Chymotrypsin like activity; whereas MC-CPA cleave at the C-terminal of peptides. It is believed that these MC proteases, especially the chymases and tryptases are at the basis of the role of MC in cardiovascular and metabolic diseases (Sun *et al.*, 2011; Yang *et al.*, 2008; Lutgens *et al.*, 2006).

1.5 Chymase Synthesis

The chymase, a mast cell protease found in the granules, is synthesized as a preproenzyme. The preproenzyme has a signal on its N terminal responsible for the guidance of the peptide to the endoplasmic reticulum lumen (for review see Nakano *et al.*, 1997; Watts *et al.*, 2007; Pejler *et al.*, 2010 and Takai *et al.*, 2010). Although there is no consensus regarding the biosynthesis of the active chymase from prepro-chymase (Nakano *et al.*, 1997; Watts *et al.*, 2007; Pejler *et al.*, 2010 and Takai *et al.*, 2010), it is postulated that this signal peptide is 2 amino acids (aa) long. Once cleaved, the preproenzyme will give rise to a proenzyme that will also be cleaved on the N-terminal site leading to the active enzyme. It is noteworthy that the active enzyme itself is stored in the granules unlike other known zymogens. The prochymase is constituted of 226 aa. An additional 2 aa on the N-terminal will be cleaved (from the prochymase) to lead to the active chymase (Caughey *et al.*, 1991; Huang *et al.*, 1991; Serafin *et al.*, 1991; Urata *et al.*, 1991). Dypeptidyl Peptidase I (DPPI) or cathepsin C is believed to be the enzyme responsible for this cleavage. A paramount role for heparin has been demonstrated in this cleavage (Murakami *et al.*, 1995; McEuen *et al.*, 1998).The N-terminal of the proenzyme is attached to a region on the proenzyme which makes it inaccessible for cleavage by DPPI. Heparin will cause a conformational change in the prochymase, by binding to a heparin binding site on the proenzyme, exposing the N-terminal to DPPI. The cleavage can thus occur and activation of the enzyme takes place (Murakami *et al.*, 1995; McEuen *et al.*, 1998). In a DPPI Knockout (KO) mouse model, there was a failure in generating active chymase in connective tissue MC,

demonstrating further the importance of DPPI in the synthesis of active chymase (Wolters *et al.*, 2001).

Once liberated from the granules, the active chymase will be bound to the extracellular matrix (ECM). In the ECM, endogenous chymase inhibitors (such as α1-antitrypsin, α2 antichymotrypsin, α 2-macroglobulin, and eglin C) block the activity of the chymase. However, the fact that it will be bound to heparin renders it resistant to the endogenous inhibitors and preserves its activity for several weeks (Lindstedt *et al.*, 2001).

In Humans, a single chymase has been identified to date, the CMA1. Despite the fact that it is an α-chymase, it shows similarities in proteolytic activities to the mouse mast cell protease 4 (mMCP-4) which is a β-chymase present in murine connective tissue MC (Wu *et al.*, 2005; Andersson *et al.*, 2008; Urata *et al.*, 1990).

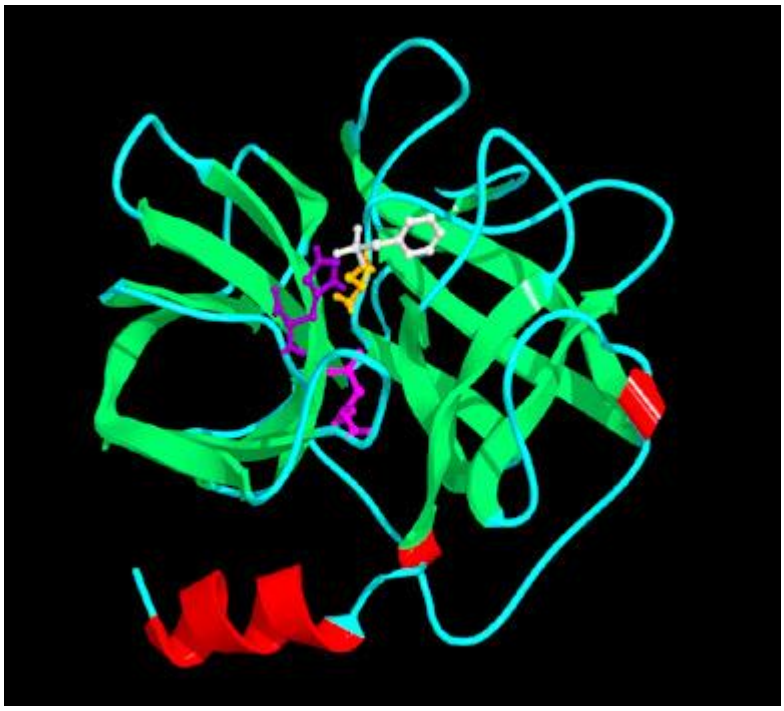

Figure1. Structure of CMA1: CMA1 and PMSF-bound inhibitor (in grey)- complex. Catalytic residues are shown in ball-and-stick representation: His66 in purple, Asp110 in pink and Ser203 in orange. α-helices are shown in red and β-pleated sheets are shown in green. (MEROPS database)

1.6 Chymase substrates

Chymase plays an important role in physiology and pathophysiology. This role is attained due to its broad cleavage specificity which explains its ability to process a large number of proteins/peptides. In table I, some chymase substrates are listed. It is important to note that some of these substrates were identified *in vitro* either by direct incubation or by the use of chymase inhibitors; other substrates were identified *in vivo* in mice models with chymase Knock-out (KO) genes.

Table I. Chymase substrates modified from Pejler et al. 2007

Substrates	Chymase activity	References
Procollagen	Activation	Kofford *et al.*, 1997
Procollagenase	Activation	Saarinen *et al.*, 1994
Pro-MMP-9	Activation	Chen *et al*., 2002; Tchougounova *et al.*, 2005
Fibronectin	Degradation	Lazaar *et al.*, 2002 ; Tchougounova *et al.*,2003
Vitronectin	Degradation	Banovac *et al.*, 1993
TIMP-1	Inactivation	Frank *et al.*, 2001
Substance P	Degradation	Caughey *et al.*, 1988
VIP	Degradation	Caughey *et al.*, 1988
Bradykinin	Inactivation	Reilly *et al.*, 1985
Kallidin	Inactivation	Reilly *et al.*, 1985
Big-endothelin 1/2	Cleavage	Kido *et al.*, 1998; Nakano *et al.*, 1997;Takai *et al.*, 1998
Neurotensin	Hydrolysation	Goldstein *et al.*, 1991
Hepatocyte growth factor	Inactivation	Raymond *et al.*, 2006
CTAP-III	Activation	Schiemann *et al.*, 2006
Pro-IL-18	Activation	Omoto *et al.*, 2006
IL-6, IL-13	Degradation	Zhao *et al.*, 2005
TGF-β1	Activation	Taipale *et al.*, 1995
SCF	Liberation	de Paulis *et al.*, 1999; Longley *et al.*,1997
apoE, apoA-I, apoA-II	Degradation	Lee *et al.*, 2002b, 2003a; Lindstedt *et al.*, 1996
apoB	Degradation	Kokkonen *et al.*, 1986
Phospholipid transfer protein	Degradation	Lee et al., 2003b
PAR-1	Activation	Schechter et al., 1998

Table I. Chymase substrates modified from Pejler et al. 2007 continued

Substrates	Chymase activity	References
C3a	Degradation	Gervasoni *et al.*, 1986; Kajita and Hugli, 1991
Albumin	Degradation	Raymond *et al.*, 2003
Occludin	Degradation	Scudamore *et al.*, 1998
C1 inhibitor	Inactivation	Schoenberger *et al.*, 1989
Ang I	Processing and cleavage	Urata *et al*., 1990
Thrombin	Inactivation	Pejler and Karlstrom, 1993

1.7 The Role of Chymase in Physiology and Pathophysiology

1.7.1 Chymase in wound healing

Wound healing comprises three major stages: the inflammatory phase, the proliferative phase and the remodeling phase (Schilling 1976). Chymase has a role in all stages. It has a paramount role in ECM regulation in direct and indirect ways which is a key step in the inflammatory phase of wound healing (Nishikori *et al.*, 1998; Noli and Miolo 2001, 2010; Younan *et al.*, 2010). This is expected given the abundance of CTMC in the connective tissue (Irani *et al.*, 1986; Kitamura *et al.*, 1989; Metcalfe *et al.*, 1997; Kawakami *et al.*, 2002). Chymase is responsible for the degradation of fibronectin and Vitronectin, both of which are components of the ECM (Lazaar *et al.*, 2002; Tchougounova *et al.*, 2003; Banovac *et al.*, 1993).

On the other hand, in the proliferative and remodeling phases, chymase stimulates fibroblasts by releasing TGF-β1 causing ECM deposition (Lindstedt *et al.*, 2001). In addition, chymase was shown to contribute to angiogenesis and vascular growth, in granulation tissue (Norrby *et al.*, 1986). Furthermore, chymase was demonstrated to cleave precollagen leading to fibril formation (Kofford *et al.*, 1997).

1.7.2 Chymase in cardiovascular diseases and Atherosclerosis

Atherosclerosis is an inflammatory disease of the arteries. As we have mentioned earlier, MC have been shown to be involved in this disease. More evidence has demonstrated a role for chymase in atherosclerosis (Bot *et al.*, 2015).

Chymase can affect SMC directly and indirectly. It indirectly regulates SMC differentiation, migration and proliferation by the activation of TGF-β1 (Otsuka *et al.*, 2006). It can also directly induce their apoptosis (Leskinen *et al.*, 2001) which explains the thinning of the aortic wall media in atherosclerosis. Chymase can also inhibit collagen synthesis and induce the apoptosis of endothelial cells by degrading Vitronectin and Fibronectin of the ECM (Heikkilä *et al.*, 2008) and/or via TGF-β1 which will further cause endothelial dysfunction.

Chymase has also a role in activating the metalloprotease Pro-MMP-9 which has been implicated in atherosclerosis (Wågsäter *et al.*, 2011).

Within the atheroma, chymase is involved in the proteolysis of LDL, the step preceding foam cell formation (Lee *et al.*, 1992). In addition, by degrading ApoE, ApoAI and HDL3, it inhibits the efflux of cholesterol from foam cells, the process that will maintain the presence of foam cells in atherosclerotic plaques (Lee *et al.*, 1992, 1999, 2002 a, 2002 b; Lindstedt L *et al.*, 1996).
Finally, higher chymase levels were found in the serum of patients who suffered from a myocardial infarction or unstable angina compared to individuals without coronary artery disease (Xiang *et al.*, 2011).

1.7.3 Chymase in Metabolic diseases and Diabetes Mellitus

Mast cells have been shown to be involved in the pathogenesis of metabolic diseases such as Obesity, Diabetes Mellitus (DM), type I (DM I) and II (DM II) and in complications of DM. Studies have shown that MC are present in a larger amount in white adipose tissue (WAT) of obese patients compared to lean subjects (Tanaka *et al.*, 2011; Liu *et al.*, 2009).
Mast cell deficient mice gained less body weight, had less adipose tissue inflammation and had improved glucose intolerance (Liu *et al*., 2009).
In a more specific way, chymase has been linked to metabolic diseases and DM. In fact, in a hamster model of DM I there was an increase in blood glucose levels, pancreatic chymase and Ang II formation; all of which were decreased with the inhibition of chymase by TY-51469 (Maeda *et al.*, 2010; Takai *et al.*, 2009).
On the other hand, chymase levels were measured in the blood of patients suffering from pre-diabetes and DM II and were shown to be higher than chymase levels in the blood of controls (with normal blood glucose levels) (Wang *et al.*, 2011).

DM complications, such as nephropathies and retinopathies can be detrimental. Although it has not been directly linked to these complications, chymase has been reported to have an unfavorable role in these processes. Chymase levels were elevated in diabetic nephropathy. These levels were associated with glomerulosclerosis and tubulointerstitial fibrosis (Ritz, 2003) and diabetic vascular diseases secondary to Ang II formation (Koka *et al.*, 2006). Furthermore, once activated by the chymase, pro-MMP-9 is believed to be active in diabetic nephropathy and retinopathy (Van der Zijl *et al.*, 2010; Kowluru *et al.*, 2012).

1.8 The role of Chymase in the conversion of Angiotensin I to Angiotensin II

The role of chymase in Ang II synthesis is well documented (for review see Takai *et al.*, 2010 and Pejler *et al.*, 2010). As mentioned in table I, Ang I is a substrate for chymase (Urata *et al.*, 1990). Chymase was found to cleave Ang I and form Ang II. Ang I is a peptide constituted of ten aa Asp1-Arg2-Val3-Tyr4-Ile5-His6-Pro7-Phe8-His9-Leu10, chymase is responsible for the cleavage of the Phe8-His9 bond to form Ang II, an eight aa peptide (Reilly *et al.*, 1982; Urata *et al.*, 1990).

It is well known that Ang II is a product of the activation of the Renin-Angiotensin system (RAS). The conventional conversion of Ang I to Ang II is done via Angiotensin converting enzyme (ACE). RAS is activated, in normal physiology, when the body senses a decrease in blood flow to the kidneys (decreased intratrarenal pressure), which implies a decrease in blood pressure. Ang II will be formed and will cause a vasoconstriction leading to restoration of normal blood pressure. This is believed to occur in the blood stream.

However this is not the only pathway, since ACE inhibitors could not inhibit totally the production of Ang II (Padmanabhan *et al.*, 1999; Wolny *et al.*, 1997) and in an ACE KO model of mice, the formation of Ang II was not totally repressed either (Wei *et al.*, 2002). The alternative route, involving the chymase was shown to occur mainly in the tissues (as opposed to bloodstream) form Ang II in the tissues where Ang II plays a major role in Pathophysiology.

1.9 The role of Angiotensin II in physiology and Pathophysiology

As mentioned in the previous paragraph, Ang II plays a key role in physiology and Pathophysiology and works on several systems.

The main system we are interested in here is the cardiovascular system where Ang II can stimulate cardiac remodeling and hypertrophy as well as vascular hypertrophy (Humma and Terra, 2002; Mehta and Griendling, 2007). It also causes constriction of the resistance vessels which will increase the systemic vascular resistance hence increasing the arterial pressure (Humma and Terra, 2002).

Ang II also affects the renal system. It stimulates the release of aldosterone in the adrenal cortex causing an increase in sodium reabsorption and water retention; besides the stimulation of

Vasopressin or antidiuretic hormone (ADH) release will further increase fluid retention in the body (Humma and Terra, 2002).
On another level, Ang II impacts the nervous system by facilitating Norepinephrine release and inhibiting its reuptake on sympathetic synapses (Humma and Terra, 2002).

In more details, Ang II affects all the cells in the cardiovascular system. With excess production of Ang II, growth, hypertrophy and migration of vascular smooth muscle cells (VSMC) will take place. In addition, endothelial dysfunction will occur and there will be an increase in the expression of the adhesion molecules. Moreover, with cardiac remodeling, electrophysiological conduction will be altered (Mehta and Griendling, 2007).

These changes in physiology secondary to excess Ang II production implies that this peptide has a supreme role in myocardial infarction, arrhythmias, strokes, diabetic vascular diseases and congestive heart failure (Schieffer *et al.*, 2000 ; Mehta and Griendling, 2007)

1.10 Angiotensin receptors

Most physiologic and pathophysiologic effects of Ang II are mediated by its receptor angiotensin type 1 receptor (AT_1R). Other receptors also exist, such as angiotensin type 2, 3 and 4, however our focus will be on AT_1R since it is responsible for most of the cardiovascular diseases related to the renin-angiotensin system (RAS). AT_1R is a seven-membrane G protein-coupled receptor. It is composed of 359 aa. The receptor is widely distributed in the body: It is present on tissues of the heart, vessels, kidneys, adrenals, liver, lungs and brain (Griendling, Lassegue and Alexander, 1996).

As shown in Fig. 2, once Ang II binds to AT_1R, the $G\alpha_{q/11}$ and $G\alpha_{12/13}$ protein cascade will be activated (Ushio-Fukai *et al.*, 1998). The $G\alpha_{q/11}$ will activate the phospholipase C (PLC) which will cleave the Phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3). IP_3 will increase calcium levels in the cytoplasm by binding to its receptor on the sarcoplasmic reticulum and opening a channel facilitating calcium efflux. Calcium released from the sarcoplasmic reticulum activates the myosin light chain kinase (MLCK) which will phosphorylate the myosin light chain thereby enhancing the interaction

between actin and myosin leading to SMC contraction (Yan *et al.*, 2003). On the other hand, DAG will activate protein kinase C (PKC) which will phosphorylates the Sodium/Hydrogen (Na^+/H^+) exchange and will act as an effector in the Ras/Raf/MEK/ERK pathway (Yan *et al.*, 2003).

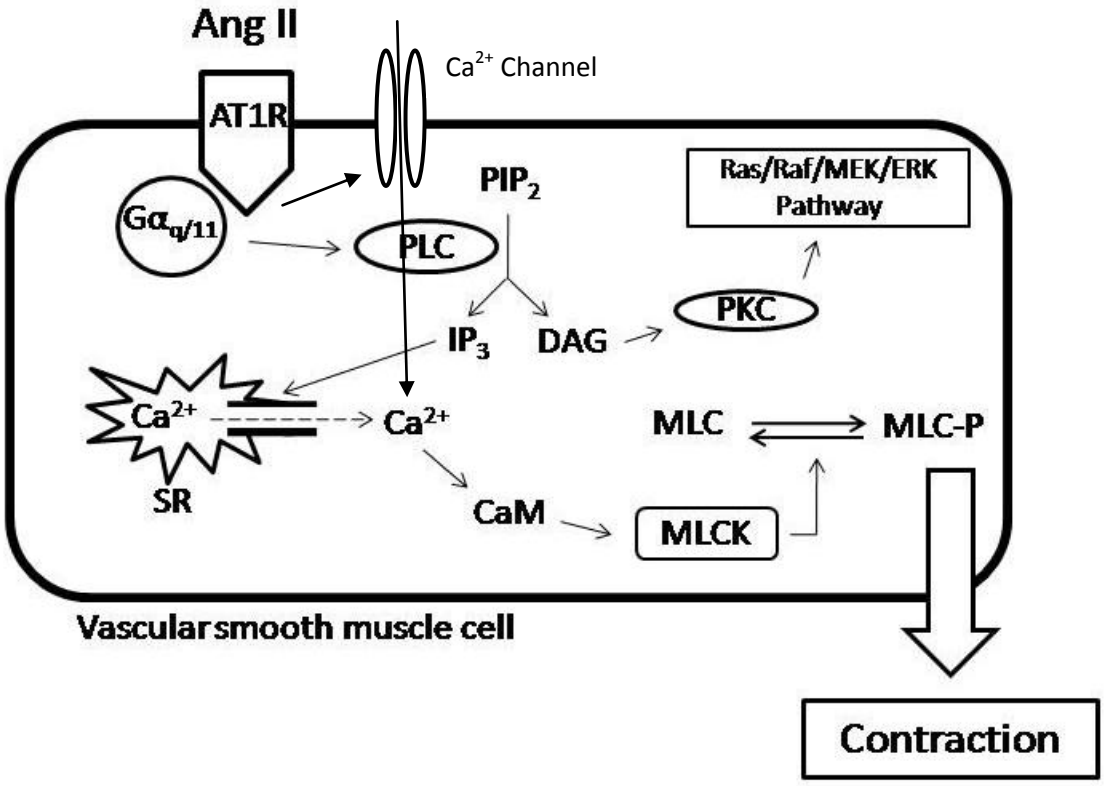

Figure 2 AT_1R signaling pathway after activation by Ang II. PLC: Phospholipase C, PIP_2: Phosphatidylinositol 4,5-bisphosphate, DAG:Diacylglycerol, IP_3: Inositol-1,4,5-triphosphate, SR: Sarcoplasmic Reticulum, Ca^{2+}: Calcium, CaM: Calmodulin, MLC: Myosin light chain, MLCK: Myosin light chain kinase, MLC-P: Phosphorylated myosin light chain, PKC: Protein Kinase C. (Modified from Ushio-Fukai et al., 1998)

1.11 Endothelin-1

As mentioned in table I, Big endothelin-1 (Big ET-1) has been shown *in vitro* to be a substrate for the chymase (Kido *et al.*, 1998; Nakano *et al.*, 1997; Takai *et al.*, 1998). The end product of the processing and cleavage of the Big ET-1 is endothelin-1 (ET-1).

ET-1 is a potent vasoconstrictor constituted of 21 aa. It was first isolated in 1988 from porcine aortic endothelial cells by Yanagisawa (Yanagisawa *et al.*, 1988). Three isomers of endothelin exist ET-1, ET-2 and ET-3 (Inoue *et al.*, 1989). Each peptide is encoded by a different gene. ET-1 is produced by endothelial cells, mainly the vascular endothelium (Masaki, 2000; Simonson and Dunn, 1990). ET-2 is produced by the renal medulla and is implicated mainly in the vascular function in the kidneys; ET-3 was found to be present in nerve endings and implicated in neurotransmission (Waeber *et al.*, 1990; Spyer *et al.*, 1991).

1.12 The role of Endothelin-1 in Physiology and Pathophysiology

ET-1 has several important physiologic actions in the embryologic and adult life. In fact, a repression of the ET-1 gene in mice caused their deaths from cardiovascular and craniofacial anomalies leading to respiratory failure minutes after their delivery (Kurihara *et al.*, 1994; Yanagisawa *et al.*, 1998). In addition, ET-1 has a key role in maintaining the basal vascular tone (Masaki, 2000). It is also involved in sodium excretion from the renal tubules (Hirata *et al.*, 1988, Murray *et al.*, 2008). It is also involved in bronchoconstriction, sputum production and MC degranulation (Rubanyi and Polokoff, 1994; Murray *et al.*, 2008).

On the other hand, ET-1 is involved in several diseases and pathologies. Table II summarizes most of them.

Table II. Diseases and pathologies which ET-1 is involved in

Conditions	References
Atherosclerosis	Attina *et al.*, 2005; Ihling *et al.*, 2001
HTN	Dhaun *et al.*, 2008
Pulmonary HTN	Attina *et al.*, 2005
Metabolic syndrome	Weil *et al.*, 2011
IBS	
Congestive Heart failure	Wei *et al.*, 1994; Kiowski *et al.*, 1995; Pacher *et al.*, 1996
Cancer metastasis	Grant *et al.*, 2003; Said and Theodorescu, 2012
Pain mediation	Hans *et al.*, 2008

1.13 Biosynthesis of Endothelin-1

The first product of the ET-1 gene is Pre-proendothelin, a peptide constituted of 212 aa. This peptide will be processed by a carboxypeptidase to form proendothelin which is the precursor of the Big ET-1. Furin, an enzyme of the subtilisin family will cleave the proendothelin further to generate Big ET-1(Blais *et al*., 2002; D'Orleans-Juste *et al.*, 2003).

1.14 Classical Pathway

Big ET-1 is found in the peripheral circulation. It has some vasoconstrictive capacities however once converted to ET-1 via the Endothelin Converting Enzyme (ECE), the product, ET-1, has a much higher- 140 times higher- vasoconstrictive potency (Rubanyi and Polokoff, 1994).

1.15 Endothelin Converting Enzyme

The ECE cleaves the bond between Trp 21 and Val 22 of the Big ET-1 to generate ET-1(McMahon *et al.*, 1991; D'Orleans-Juste *et al.*, 2003). The ECE is a Zinc-dependent Metalloendopeptidase localized in several cell types such as endothelial cells, SMC, cardiomyocytes and macrophages (Hioki *et al.*, 1991; Hisaki *et al.*, 1993; Takahashi *et al.*, 1995; Barnes *et al*., 1997; Barnes and Turner, 1999; Korth *et al*., 1999). Three isoforms of ECE have been identified, the ECE-1, ECE-2 and ECE-3 (Xu *et al.*, 1994; Shimada *et al.*, 1994 ; Maguire *et al.*, 1997; Schweizer *et al.*, 1997; Fukuchi and Giaid, 1998; Kobayashi *et al.*, 1998; Rossi *et al.*, 1999).

ECE-1 and ECE-2 were shown to generate ET-1 from Big ET-1 (Emoto and Yanagisawa, 1995), however ECE-1 is believed to be more involved physiologically since ECE-2 maximal activity occurs at a more acidic pH (pH= 5.5) (Emoto and Yanagisawa, 1999). Furthermore, ECE-1 binds and process several substrates such as bradykinin, substance P, Ang I and insulin, with different affinities (Hoang *et al.*, 1997; Johnson *et al.*, 1999).

Four isoforms of ECE-1 as well as of ECE-2 have been identified: ECE-1a, ECE-1b, ECE-1c and ECE-1d; ECE-2a-1, ECE-2a-2, ECE-2b-1 and ECE-2b-2 (Shimada *et al.*, 1995; Schweizer *et*

al., 1997; Valdenaire *et al.*, 1999; Ikeda *et al.*, 2002). The isoforms differ in their N terminal sequences which dictate their cellular location.

1.16 Alternative pathway

The ECE dependent pathway does not seem to be the sole pathway leading to the formation of ET-1. It has been shown that in embryos of mice whose ECE-1 and ECE-2 genes were KO, the production of ET-1 was not completely inhibited; but was only decreased by 33% (Yanagisawa *et al.*, 2000). This suggests that other pathways involved in the production of ET-1 exist, independent of ECE. Even though the role of chymase in Ang II biosynthesis is well covered in the literature (for review see Takai *et al*., 2010 and Pejler *et al.*, 2010), less is reported concerning the role of chymase in ET-1 synthesis (for review see Nakano *et al.*, 1997 and Watts *et al.*, 2007). One of the enzymes able to cleave Big ET-1, as mentioned in table I, is the chymase. It has been shown that the latter enzyme can cleave the Big ET-1 at the bond Tyr 31-Gly 32 leading to an intermediate peptide formed of 31 aa, the ET-1 (1-31). This ET-1 (1-31) will be further processed by the neutral endopeptidase (NEP) which cleaves the Trp 21- Val 22 bond to form ET-1 (Hanson *et al.*, 1997; Nakano *et al.*, 1997); as shown in fig. 3. The NEP is a ubiquitous membrane bound metalloendopeptidase responsible not only for the generation of ET-1 from ET-1 (1-31) but has a role in the degradation of ET-1 (Vijavaraghavan *et al.*, 1990; Turner and Tanzawa, 1997).

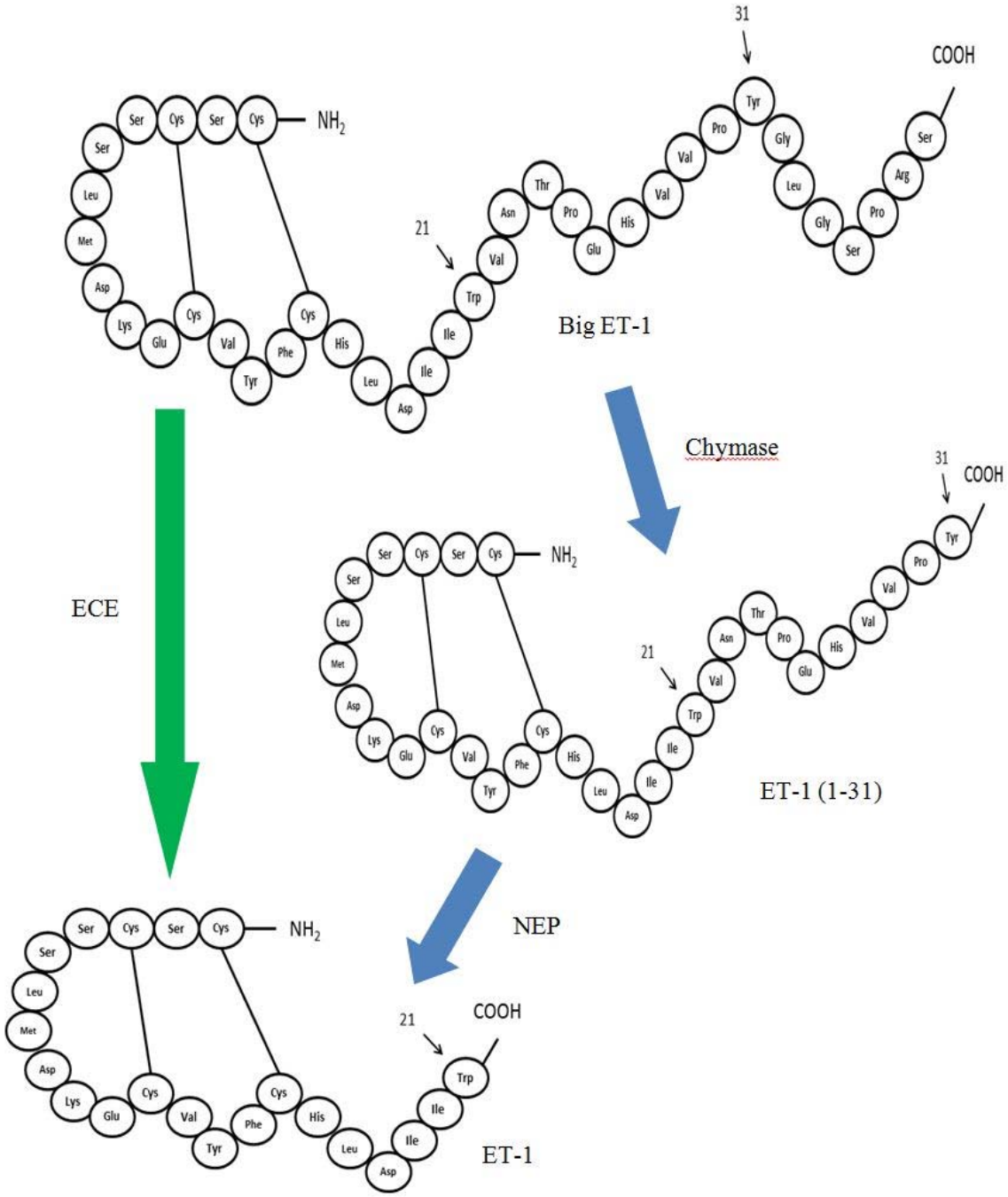

Figure 3: Schematization of the synthesis of ET-1 via the classical pathway (green arrow) and the alternative pathway (blue arrows). (Modified according to Goto et al., 1996, Hanson *et al.*, 1997 and Nakano *et al.*, 1997)

1.17 Endothelin-1 (1-31)

There has not been a clear conclusion in the literature on whether ET-1 (1-31) acts directly on the endothelin receptors or it has to be cleaved into ET-1 to achieve its activity. Many *in vitro* studies have shown that ET-1 (1-31) can act as an agonist on the endothelin receptors. For example, the group of Maguire showed in 2001 that ET-1 (1-31) has vasoconstrictive properties when activating the endothelin receptors in a human mammary artery (Maguire *et al.*, 2001). Some studies have concluded that ET-1 (1-31) is a selective agonist for one of the endothelin receptors, the endothelin A receptor (ET_A) (Mazzochi *et al*., 2000), whereas other studies have shown that ET-1 (1-31) is an agonist of both endothelin receptor, ET_A and endothelin B (ET_B). On the other hand, other studies indicated that ET-1 (1-31) needs to be processed by the NEP to generate ET-1 which will activate the receptors (Hayasaki-Kajiwara *et al.*, 1999).

It is important to note that the studies mentioned in the previous paragraph were conducted *in vitro*. A closer look to the studies performed on animals *in vivo* shows a consensus on the necessity of the conversion of the ET-1 (1-31) to ET-1 by the NEP to get its functionality *in vivo* (Fecteau *et al.*, 2005; Simard *et al.*, 2009).

1.18 Endothelin receptors

There are two known receptors for the endothelin, ET_A and ET_B. They are G protein coupled receptors (GPCR) that have seven transmembrane domains. These receptors are coupled to $G_{q/11}$. They share about 50 % of identical sequence with the main differences existing in the N-terminal (Ogawa *et al.*, 1991; Rubanyi and Polokoff, 1994; Murray *et al.*, 2008). ET_A is localized mainly on the VSMC whereas ET_B is localized on the VSMC along with the endothelial cells (Hosoda *et al.*, 1991; Fan *et al*., 2000; Giannessi *et al.*, 2001).

1.19 ET_A receptor

The binding of ET-1 to ET_A will activate the receptor. Similar to the AT_1 receptor, stimulation of ET_A receptor will activate the protein $G_{q/11}$ and activate phospholipase C (PLC) which will hydrolyze phosphatidylinositol-4,5-bisphosphate (PIP_2) into inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). In addition, activation of both ET_A and AT_1 receptors induces proliferation in non-excitable cells and hypertrophy in excitable cells (Bkaily *et al.*, 2011;

Alvarenga *et al.*, 2016). Both types of receptors induce increase of intracellular calcium via stimulation of L- and R-type calcium channels as well as release of calcium from the endoplasmic reticulum (Bkaily et al., 2005, 2011; Simonson and Dunn, 1990; Giannessi *et al.*, 2001; Becker *et al.*, 2009). Little is known concerning the differences in signaling and biological effects between ET_A and AT_1 receptors activation.

1.20 ET_B receptor

As mentioned earlier and shown in fig. 4, ET_B is present on VSMC and endothelial cells. The receptors present on VSMC will activate G_q and $G_i$will cause an increase in intracellular calcium, in a cascade similar to the activation to ET_A which will cause a contraction of the VSMC and hence a vasoconstriction. However, the activation of ET_B present on the endothelial cells will cause an increase in Nitric Oxide (NO) and prostacyclin causing a relaxation of the VSMC hence a vasodilation (Giannessi *et al*., 2001; Attina *et al.*, 2005; Murray *et al.*, 2008).

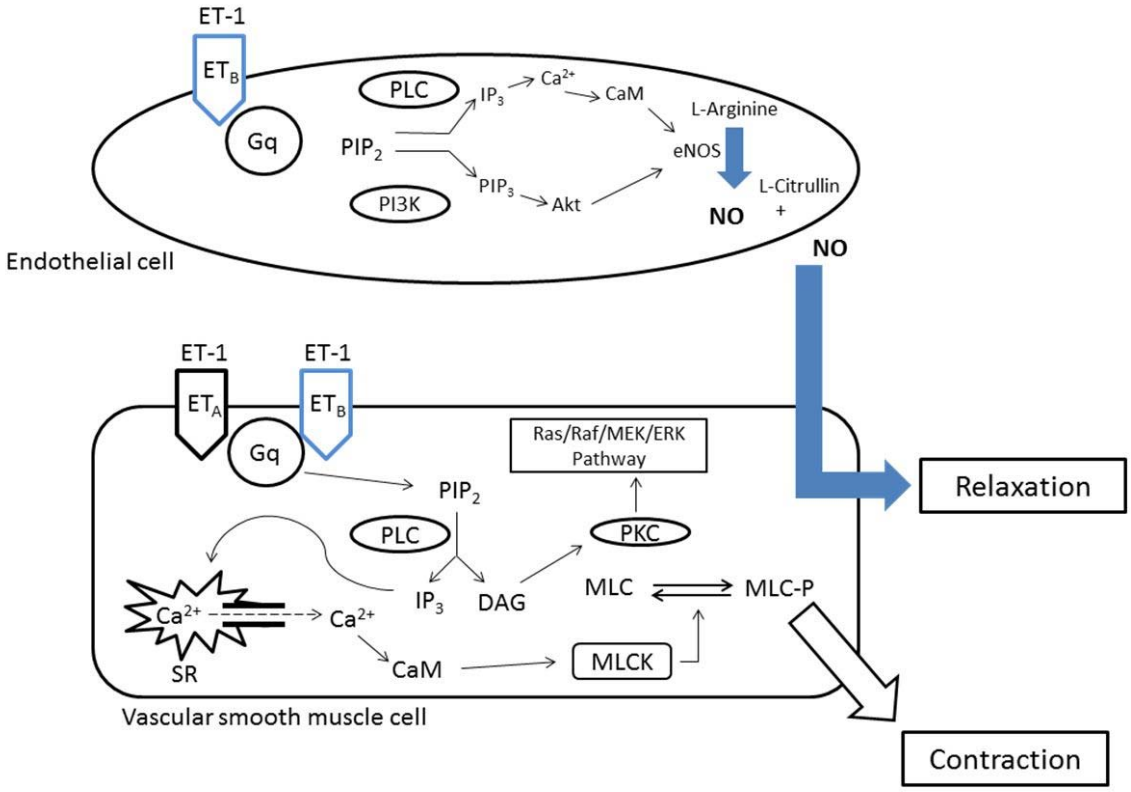

Figure 4: ET_A and ET_B signaling pathway after activation by ET-1. PLC: Phospholipase C, PIP_2: Phosphatidylinositol 4,5-bisphosphate, DAG:Diacylglycerol, IP_3: Inositol-1,4,5-triphosphate, SR: Sarcoplasmic Reticulum, Ca^{2+}: Calcium, CaM: Calmodulin, MLC: Myosin light chain, MLCK: Myosin light chain kinase, MLC-P: Phosphorylated myosin light chain, PKC: Protein Kinase C, NO: Nitric Oxide, PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase, PIP_3: Phosphatidylinositol (3,4,5)-trisphosphate, eNOS: Endothelial Nitric Oxide synthase. (Modified from Ushio-Fukai et al., 1998)

1.21 Chymase inhibitors and clinical relevance

Many chymase inhibitors are currently in clinical trials, some are currently in phase two such as SUN 13834 (Ogata *et al.*, 2011). Table III summarizes some of the chymase inhibitors and their potential clinical relevance.

Table III. Chymase inhibitors and their therapeutic potential

Chymase inhibitors	Therapeutic potential	Reference
SUN-C8257	Atherosclerois, pulmonary fibrosis, skin disease	Doggrell, 2008
SUN13834	Atopic dermatitis	Ogata *et al.*, 2011
BCEAB	Cardiac diseases	Doggrell, 2008
Compound 17	Cardiac diseases, asthma	Doggrell, 2008
NK 3201	Cardiac diseases	Doggrell, 2008
TEI-ES48	Cardiac diseases	Hoshino et al., 2003
RO5066852	Atherosclerosis	Bot *et al.*, 2011
JNJ-10311795	Anti-inflammatory	De Garavilla *et al.*, 2005
Suc-Val-Pro-Phe $(OPh)_2$	Cardiac adhesions	Soga *et al.*, 2004
Y-40613	Atopic dermatitis	Akahoshi *et al.*, 2001; Imada *et al*., 2002
TY-51463	Cardiac diseases, liver fibrosis, gastroentesitinal diseases, diabetes mellitus	Oyamada *et al.*, 2011; Komeda *et al*., 2010 ; Kakimoto *et al.*, 2010 ; Takai *et al.*, 2009
Chymostatin	Glaucoma, chorioretinal, Gastroentestinal diseases	Doggrell, 2008; Groschwitz *et al*., 2009

1.22 Aim of the study and targeted objectives

Since the establishment of the chymase dependent route of conversion of Big ET-1, the production of ET-1 was not demonstrated to occur *in vivo* up until recently in 2009, when our laboratory has shown that the conversion of Big ET-1 into ET-1 (1-31) occurs in mice (Simard *et al.*, 2009); knowing that ET-1 (1-31) is converted to ET-1 via NEP *in vivo* (Fecteau *et al.*, 2005). In addition in 2013, our laboratory has demonstrated that the mMCP-4, which is the murine analog of the human chymase, can generate ET-1 (1-31) from the Big ET-1 *in vitro* and *in vivo* (Houde *et al.*, 2013). However, no information is available concerning the ability of recombinant chymases (murine or human) to cleave Big ET-1. In fact the literature shows a chymostatin dependent characterization of CMA1 in regards to generation of Ang II from Ang I (Murakami *et al.*, 1995). Chymostatin is a general chymotrypsin-like protease inhibitor, not specific to chymase. It was shown to inhibit elastase II as well, an enzyme involved as well in the production of Ang II from its precursor Ang I (Becari *et al*., 2005).

Based on these observations, we hypothesized in this study that the CMA1, whether recombinant, extracted from the LUVA cells (human mast cell line) or in the soluble fractions of human aortas would generate ET-1 (1-31) from Big ET-1 in a chymase inhibitor - sensitive manner.

In a second aim, we also characterized the kinetic enzymatic activity of CMA1 towards its substrate Big ET-1.

In order to achieve these aims, we propose the following objectives:

- To validate that the recombinant human chymase (CMA1) converts Big ET-1 into ET-1 (1-31)
- To verify if CMA1 has a role in the degradation of ET-1 or ET-1 (1-31)
- To determine the Kinetic constants of CMA1 towards a fluorogenic substrate, Big ET-1 and Ang I

- To verify if the chymase extracted from the LUVA cells (human Mast cell line) converts the Big ET-1 into ET-1 (1-31)
- To verify if CMA1 extracted from soluble fractions of healthy human aortas would generate ET-1 (1-31) from the precursor Big ET-1

II- ARTICLE

2.1 TITLE PAGE

Title: Chymase inhibitor-sensitive synthesis of endothelin-1 (1–31) by recombinant mouse mast cell protease 4 and human chymase.

Authors: Walid Semaan, Louisane Desbiens, Martin Houde, Julie Labonté, Hugo Gagnon, Daisuke Yamamoto, Shinji Takai, Tanya Laidlaw, Ghassan Bkaily, Adel Schwertani, Gunnar Pejler, Christine Levesque, Roxane Desjardins, Robert Day, Pedro D'Orléans-Juste

Status of the article: Published in 2015 in Biochemical Pharmacology doi: 10.1016/j.bcp.2015.02.001

Foreword: On the experimental level, I have executed the fluorescence experiments with recombinant chymases, murine Mast cells extracted from peritoneal lavages and LUVA cells. I have also executed the experiments using the HPLC. Moreover I have prepared the LC-MS/MS conversion experiments with recombinant chymases and with human aortas. I have analyzed the results and plotted the graphs using Graphpad. The article was written under the supervision of Drs. Pedro D'Orleans- Juste and Ghassan Bkaily. All the authors have read and revised the article.

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ABSTRACT

Important structural differences imply that human and mouse mast cell chymases may differ with respect to their enzymatic properties. We compared in this study the catalytic efficiencies of recombinant human chymase (rCMA1) and its functional murine homologue recombinant mouse mast cell protease-4 (rmMCP-4) toward a fluorogenic chymase substrate (Suc-Ala-Ala-Pro-Phe-7-amino-4-methylcoumarin (AMC) and by their ability to convert Big-endothelin (ET)-1 into ET-1 (1–31) using a LC/MS/MS system. Activities toward a fluorogenic substrate (Suc-Leu-Leu-Val-Tyr-AMC) and Big ET-1 were also measured in extracts from mouse peritoneal mast cells, LUVA human mast cell-like cells and human aortas. The specificity of these activities was assessed with the chymase inhibitor TY-51469 (2-[4-(5-fluoro-3- methylbenzo[b]thiophen-2-yl) sulfonamido-3-methanesulfonyl-phenyl]thiazole-4-carboxylic acid). For similar affinities, rmMCP-4 showed a higher activity toward the fluorogenic substrate and a higher ability to process Big ET-1 as compared to recombinant CMA1 (chymase activity (k_{cat}/K_M in $\mu M^{-1}s^{-1}$): 2.29×10^{-4} vs. 6.41×10^{-6}; ET-1 (1–31) production: 2.19×10^{-3} vs. 6.57×10^{-5}), and both of these activities of mouse and human chymase were sensitive to TY-51469. Furthermore, extracts from mouse peritoneal mast cells, LUVA cells and human aorta homogenates contained processing activities toward the fluorogenic chymase substrate as well as Big ET-1, all of which were sensitive to TY-51469. Finally, the pressor responses to Big ET-1 but not to ET-1 were significantly reduced in conscious and free moving mMCP-4 KO mice when compared to wild type congeners. Our results suggest that both mouse and human chymases have potent ET-1 (1–31)-producing abilities, with the murine isoform being more efficient.

Keywords: Chymase, Recombinant enzymes, Mass spectrometry, Radiotelemetry, In silico analysis

1. Introduction

Chymases are serine proteases released by activated mast cells, involved in tissue repair and inflammatory processes such as wound-healing/fibrosis [1], cardiac remodelling and angiogenesis [2,3]. In humans and rodents, two types of mast cells have been identified. Human mast cells positive for both chymase and tryptase (MCTC) are similar to murine connective tissue mast cells (CTMC) while those positive only for tryptase (MCT) resemble murine mucosal mast cells (MMC) [4,5]. To date, a single chymase, a-chymase or CMA1 [6] has been identified in humans, expressed in MCTC. In mice, mouse mast cell protease 4 (mMCP-4), predicted as a rodent b-chymase from its deduced amino acid sequence, shows angiotensin II (Ang II)- forming properties, CTMC localization and serglycin storage dependence [7] similar to those afforded by CMA1 [6]. Importantly, like the a-chymase CMA1 [8], mMCP-4 does not share the preferential b-chymase Tyr4-Ile5 cleaving activity on Ang-II that mMCP-1 and rat mast cell protease 1 (rMCP-1, the mMCP-4 rat homolog) possess, making the mouse a more representative model than that of the rat to study human-like Ang-II formation [9]. mMCP-4 plays a protective role in a mouse model of cerebral trauma [10], yet is detrimental in bleomycin-induced lung inflammation and immune complex-induced glomerulonephritis [11,12].

The potent vasopressor peptide endothelin-1 (ET-1) on the other hand, is generated from a larger 38 amino acid precursor Big-endothelin-1 (Big ET-1) via the hydrolytic activity of an endothelin-converting enzyme (ECE) [13]. Besides, other proteases are also involved in the overall production of mature ET-1. Among those, chymase derived from human purified pulmonary tissue cleaves the Tyr31–Gly32 bond of Big ET-1 (1–38) to yield ET- 1 (1–31) [14]. Our group later reported that ET-1 (1–31) requires a further neutral endopeptidase (neprilysin, NEP)-dependent hydrolysis of the Trp21–Val22 bond to produce mature ET-1 in vivo [15]. Whether mMCP-4 is also involved in the in vivo synthesis of endothelins remained unexplored until we recently reported that this particular chymase isoform converts Big ET-1 to ET-1 (1–31) and subsequently to ET-1 in a study using anesthetised mMCP-4$^{-/-}$ mice [16].

Human chymase generated by a recombinant approach produces chymostatin-sensitive Ang II from Ang I, with a K_M of 59 μM [17]. Chymostatin however, a general chymotrypsin-like protease inhibitor, is much less specific than newer generation chymase inhibitors such as TY-

51469 [18]. In addition, the comparative capacities of recombinant mMCP-4 and CMA1 to generate ET-1 (1–31) have not been assessed.

Based on our previous reports on the chymase-dependent conversion of the precursor Big ET-1 to ET-1 (1–31) in the anesthetised mouse model in vivo [16,19], we hypothesized that recombinant or mast cell-extracted mMCP-4 as well as CMA1, would generate the 31-amino acid intermediate in a TY 51469- sensitive fashion.

The first principal aim of this study was therefore to compare, by using recombinant mMCP-4 and its human counterpart CMA1, the capacity of murine and human chymases to generate ET-1 (1– 31). A second aim was to assess the ET-1 (1–31)-producing capacities of chymases derived from mouse (peritoneal mast cells) and human mast cells (LUVA cells, [20]) as well as the role of chymase in the production of ET-1 (1–31) by human aortic biopsies.

Our data show that the murine mMCP-4 and the human CMA1 generate ET-1 (1–31) from the precursor Big ET-1 via recombinant enzymes as well as in cellular or tissue extracts of mouse and human origin.

2. Materials and methods

2.1. Drugs and chemicals

Phosphate buffered saline (PBS) pH 7.4, ammonium hydroxide, bovine serum albumin (BSA), 2-(N-morpholino)ethanesulfonic acid (MES), pluronic F-68, formic acid (FA), trifluoroacetic acid (TFA), and N-ethylmaleimide (NEM) were obtained from Sigma-Aldrich (Oak- ville, ON, Canada). The StemPro-34 SFM culture medium was purchased from Invitrogen (Carlsbad, CA, USA). The RPMI-1640 and I-Max (IPL-41) culture media, fetal bovine serum (FBS) and the antibiotics hygromycin B and penicillin were obtained from Wisent (Montreal, QC, Canada). Dithiotreitol (DTT), dimethyl sulfoxide (DMSO) and HPLC-grade acetonitrile (ACN) were obtained from Fisher Scientific (Ottawa, ON, Canada). Murine active cathepsin C and recombinant CMA1 were obtained from R&D Systems (Minneapolis, MN, USA). Heparin was purchased from LEO Pharma A/S, (Ballerup, Denmark). Triton X-100 was obtained from ICN Biochemical (Aurora, OH, USA). Suc-Ala-Ala-Pro-Phe-7-amino-4-methylcoumarin (AMC), Suc-Leu-Leu-Val-Tyr-AMC and ET-1 (1–31) were obtained from Peptide Institute (Osaka, Japan), Big ET-1, Ang-I, Ang-II and Pro11- DAla12-Ang-I were obtained from American Peptide Company (Sunnyvale, CA, USA), ET-1 was obtained from Tocris Bioscience (Bristol, UK) and ($13C_6$)Leu^6-ET-1 was obtained from Bachem (Bubenford, Switzerland). Suc-Ala-Ala-Pro-Phe-chloromethylketone (CMK) was obtained from MP Biomedicals (Santa Ana, CA, USA). Ketamine was obtained from Bioniche (Belleville, ON, Canada), xylazine from Bimeda (Cambridge, ON, Canada) and buprenorphine from Reckitt Benckiser Healthcare (Slough, United Kingdom). Finally, TY-51469 (2-[4-(5-fluoro-3-methylbenzo[b]thiophen-2-yl)sulfonamido-3-methanesulfonylphenyl]-thiazole-4-carboxylic acid) was graciously provided by Toa Eiyo Ltd. (Osaka, Japan).

2.2. Expression, purification, activation and titration of recombinant mMCP-4 and CMA1

2.2.1. Expression

The vector pAc5.1 (Life Technologies, Burlington, ON, Canada) containing the recombinant DNA of pro-mMCP-4 or pro-CMA1, with poly-histidine and V5 tags, was co-transfected with the selection vector pCoHygro (Life Technologies) into S2 drosophila cells. The S2 cells were grown in I-Max culture medium IPL-41 complemented by 10% of fetal bovine serum (FBS) and hygromycin B (300 mg/ml). The cells were scaled up and finally suspended in serum-free culture medium containing 1% pluronic F-68 at a concentration of 2 x 10^6 cells/ml.

2.2.2. Purification

The crude extract sample containing the secretion of the drosophila cells was concentrated by ultrafiltration on an Ultracell Microcon 10 kDa filter (EMD Millipore, Billerica, MA, USA). This sample was then purified on a nickel affinity column by FPLC with an imidazole gradient (up to 250 mM) for isolation of poly-histidine tag positive samples. Those samples were then put on a Superdex200 26/60 size exclusion column (GE Life Sciences, CA, USA) and the V5-positive fractions (determined by Western blot, data not shown) were pooled and frozen.

2.2.3. Activation

The recombinant enzymes were thawed and diluted to a concentration of 20 mg/ml in maturation buffer (50 mM MES, 0.1% BSA, pH 5.5,). Active murine cathepsin C was diluted to 0.481 ng/ml in cathepsin C buffer (50 mM MES, 50 mM NaCl, 5 mM DTT, pH 5.5). Activation was performed by adding equal volumes of recombinant chymase and cathepsin C, adding 50 µg/ml heparinand incubating 1 h at room temperature. Chymase activation was stopped with NEM (3 mM) and diluted with assay buffer (20 mM Tris, 2 M KCl, 0.02% Triton X-100 (replaced with (0.1%) BSA for Ang- I assays), pH 9.0) to bring the recombinant chymase concentration to 2 mg/ml, and 5 min was afforded to completely stop the cathepsin C-dependent reaction.

2.2.4. Titration

The activated recombinant enzymes (0.025 ng/ml rmMCP-4 or 0.25 ng/ml rCMA1) were incubated for 25 min at 37 ºC with multiple concentrations (1.12 x 10^{-6} to 1.12 x 10^{-9} M) of the

inhibitory substrate Suc-Ala-Ala-Pro-Phe-chloromethylketone (CMK). When cleaved, the CMK compound covalently binds to the active site of recombinant chymase and blocks it. After this incubation, the fluorogenic substrate Suc-Ala-Ala-Pro-Phe-AMC was added and the chymase-like activity was analyzed as described in the main body of the article. The threshold of total inhibition of chymase activity was interpreted as the number of active sites in the recombinant chymases preparations and used as the molar concentration of enzymes for determination of their kinetics.

2.3. Animals

C57Bl/6J mice were purchased from Charles River (Montreal, QC, Canada) and housed in our facilities. Genitor mMCP-4 KO mice [2] were bred in our facilities. All animals were kept at constant room temperature (23ºC) and humidity (78%) under a controlled light/dark cycle (6:00 AM–6:00 PM), with standard chow and tap water available ad libitum. Animal care and experiments were approved by the Ethics Committee on Animal Research of the University of Sherbrooke following the Canadian Council on Animal Care guidelines and the Guide for the Care and Use of Laboratory Animals of the United States National Institutes of Health. All experiments on mice were performed on newly sacrificed animals, except for telemetric hemodynamic recording performed on live animals. The mice underwent general anesthesia, by the intramuscular administration of ketamine/xylazine (87/13 mg/kg). Complete anesthesia was assumed when no withdrawing reflex was found during pressure on any paw of the mouse. Anesthetized mice were killed by cervical dislocation.

2.4. Mast cell preparation

Mouse peritoneal mast cells preparations were used, as they are readily available, fully mature connective tissue resident mast cells containing their full complement of granule proteases without being primed with cytokines [21]. 5 ml of isolation buffer (phosphate buffer solution containing 1 mg/ml of BSA and 37.5 U/ml heparin, pH 7.4) was introduced into the peritoneal cavity of mice after peritoneal skin removal, then collected after 1 min of peritoneal massage and

centrifuged (200 x g, 5 min). In another series of experiments, mast cell-like LUVA cells were maintained in a StemPro1-34 SFM solution at a cell density of 5 x 10^5cells/ml, in the absence of additional growth factors. The suspension was divided into 2 equal volumes of 30 ml and centrifuged (200 x g, 5 min). Treatment of LUVA and mouse mast cell pellets was similar thereafter. The supernatant was discarded and the mast cell rich pellet was suspended in RPMI-1640 medium (enriched with penicillin (100 U/ml), 2 mM L-glutamine and BSA (1 mg/ml)) and incubated for 1 h at 37 ºC. The suspension was centrifuged for 5 min at 200 x g, the supernatant was discarded and the pellet was suspended in isolation buffer. The cells were counted according to the Moore and James method [22] and adjusted to 2 x 10^5mast cells/ml in PBS pH 8. The cells were lysed through sonication and then centrifuged at 200 x g for 5 min. The resulting pellet was washed thrice in 0.1 M PBS pH 8.0 by further centrifugation cycles.

2.5. Human aortas preparation

The human aortas were collected from middle-aged brain-deceased individuals with no histological signs of atherosclerosis. The tissues were weighed and grinded in PBS on ice by a tissue homogenizer (Polytron, ultra-turax T8, IKA, Wilmington, NC, USA) for 30 s. Centrifugation for 20 min at 25,000 x g at 4 ºC took place then the soluble fractions corresponding to the supernatant were collected and frozen at -80 ºC.

2.6. Specific chymase activity in vitro

Activated rmMCP-4 (0.025 ng/ml), activated rCMA-1 (0.25 ng/ml) or mast cell extracts (from 4 x 10^5 peritoneal mouse mast cells or LUVA cells) were incubated in a 96 well plate. Increasing concentrations of the non-fluorescent substrates Suc-Ala-Ala-Pro- Phe-7-amino-4-methylcoumarin (AMC) (for recombinant enzymes) or Suc-Leu-Leu-Val-Tyr-AMC (for mast cell and tissue extracts) were added and the fluorescence AMC-forming activity, as chymase activity, was then measured with a fluorescence spectrophotometer (λex: 370 nm; λem: 460 nm) for 20 min (Molecular Devices, Sunnyvale, CA). Another series of experiments was performed at 10 μM of fluorogenic substrate with pretreatment of the diluted samples with the specific

chymase inhibitor TY-51469 (10–50 μM). To determine enzyme kinetics, the recombinant enzyme active sites were titrated with the inhibitor substrate Suc-Ala-Ala-Pro-Phe-CMK.

2.7. In vitro conversion of Big ET-1 to ET-1 (1–31) and Ang-I to Ang-II

2.7.1. Recombinant enzymes

Big ET-1 and Ang-I dilutions were prepared in 0.1 M PBS pH 8.0. The recombinant enzymes (28.57 ng/ml activated rmMCP-4 or 1428 ng/ml activated rCMA1) were incubated at 37 ºC with Big ET-1 (0.15, 0.75, 1.5, 4.5, 7.5, 15 and 26 μM) or Ang-I (1.5625, 3.125, 6.25, 12.5, 25, 50 and 100 μM) for 20 min, after which the reactions were stopped with an equal volume of water:acetonitrile:dimethylsulfoxide:formic acid (H2O:ACN:DMSO:FA) mix (73:20:6:1) containing ($^{13}C_6$)Leu^6-ET-1 (100 μg/ml) or a H2O:ACN:FA mix (76:20:4) containing Pro11-DAla12-Ang-I (176 ng/ml) (peptides as internal standards). In another series of experiments, 16.7 μM of Big ET-1 was incubated in the presence of TY-51469 (10 μM) and the reaction was stopped as described above. Samples were diluted 1:10 with the same stop solution without the internal standard before LC–MS/MS analysis. Kinetics parameters were calculated using Prism Software (GraphPad, La Jolla, CA, USA).

2.7.2. Mast cell and aortic extracts

In another series of experiments, the soluble fractions of 9 x 10^5 WT peritoneal mouse mast cells, 4 x 10^6 LUVA cells or the soluble fraction of the human aortas (adjusted to 2.2 mg/ml of protein) were pretreated with vehicle or TY-51469 (10 μM) and then incubated with Big ET-1 at 37 ºC at concentrations of 5, 5 and 13 μM, respectively, for 20 min. The reactions were stopped in with the stop solution described above containing ($^{13}C_6$) Leu^6-ET-1 (50 ng/ml) and subsequently processed by solid phase extraction (SPE) before LC-MC/MS analysis, albeit the stop solution for the human aorta assay was ACN:DMSO:FA (88:6:6).

2.8. LC–MS/MS quantification of Big ET-1, ET-1 (1–31) and ET-1

2.8.1. Sample preparation

For in vitro conversion by recombinant enzymes, samples were analyzed directly. For analysis of samples from masts cells or human aortic tissue, SPE was performed consisting of a polymeric mixed mode strong cation exchange 1 ml cartridge containing 30 mg of sorbent (Phenomenex, Strata-X-C, Torrance, CA, USA).Briefly samples were pre-treated with 2% FA and loaded on the SPE column, samples were next washed once with 60% methanol containing 2% FA and eluted with 2 x 700 µl of 75% ACN containing 10% ammonium hydroxide.

2.8.2. System description

Analysis was performed by LC–MS/MS on TripleTOF 5600 mass spectrometer (ABSciex, Foster City, CA, USA) equipped with DuoSpray source. Samples were introduced to the electrospray ionization (ESI) source in a 50 µm ESI probe using a microLC200 system equipped with a 50 mm x 500 µm HALO C18 2.7 µm column.

2.8.3. System conditions

Chromatography was performed with a gradient of water containing 0.2 % formic acid and 3% DMSO (A) and ACN containing 0.2% formic acid 3% DMSO (B) (without DMSO for Ang-II determination). For endothelin peptides analysis, column temperature was set at 50 ºC. A 4 min gradient was run at 40 µl/min. It consisted of the following steps: hold at 10% B from 0 to 0.5 min, 10% B to 75% from 0.5 min to 2.7 min, hold at 100% B from 2.9 to 3.4 min and equilibration from 3.5 to 4 min at 10% B. Source parameters where the following: curtain gaz was set at 28, gaz 1 was set at 17, gaz 2 was set at 28, ion source voltage was set at 5500 and ESI probe temperature was set at 375. For Ang-II analysis, a 2.5 min gradient was run at 40 µl/min. It consisted of the following steps: hold at 15% from 0 to 0.4 min, 15% B to 100% from 0.4 min to 1.4 min, hold at 100% B from 1.4 to 2.9 min and equilibration from 2 to 2.5 min at 15% B. Source parameters where the following: curtain gaz was set at 27, gaz 1 was set at 25, gaz 2 was set at 20, ion source voltage was set at 4900 and ESI probe temperature was set at 400.

2.8.4. Analysis

Compounds were monitored using optimized collision energy parameter in product ion mode at unit resolution with a mass range from 100 to 2000 m/z in high sensitivity MS/MS scan. Data integration and analysis was performed using MultiQuant software V2.0 with the signal finder algorithm (ABSciex) by selecting the appropriate product ion transition with a mass range of 0.05 Da subsequently analyzed.

2.9. Effect of recombinant CMA1 or mMCP-4 on ET-1 (1–31) in vitro

The activated recombinant enzymes were incubated with ET-1 (1–31) (3.2 μM) for 20 min at 37 ºC, after which samples were filtered through a 30 K Amicon centrifugal filter unit (Millipore Corporation, Billireca, MA, USA) (14,000 x g, 20 min) for high performance liquid chromatography (HPLC) analysis. Filtrates were collected and purified by reversed phase HPLC (1100 series) with a Zorbax DSC-18 analytical column (Agilent Technologies, Santa Clara, CA). The flow was 1 ml/min, water was used as solvent, acetonitrile as eluent, 0.1% trifluoroacetic acid as buffer and the acetonitrile fraction going from 28% to 40% in 35 min. Quantification was assessed with absorbance at a wavelength of 214 nm using area under the curve measurements (in arbitrary units).

2.10. Telemetric hemodynamic recording of the pressor activity of Big ET-1 in conscious mice

2.10.1. Probe implantation

Telemetry probe implantation was achieved in accordance to Carlson and Wyss [23] and Butz and Davisson [24]. Briefly, ketamine/xylazine-anesthetised WT and mMCP-4 KO mice were implanted with a catheter-tipped transmitter (TA11PA-C10; Data Sciences International, St. Paul, MN, USA) into the aortic arch via the left common carotid artery. The transmitter was placed subcutaneously along the right flank of the animal. A buprenorphine protocol of 0.1 mg/kg every 8 h for 24 h post-operation was conducted to control surgical pain. The mice had a 10-day recovery period before recording of hemodynamic data.

2.10.2. Hemodynamic recording

The mice were trained to the contention chamber with saline injections through the caudal vein to minimize stress. After two training sessions, the mice received Big ET-1, Ang-I (both at 1 pmol/kg), ET-1 or Ang-II (both at 0.1 pmol/kg) intra-caudally (200 µl injection) and subsequently released in their cage. Hemodynamic recording in the freely moving mouse was performed every 30 s, each data point representing a 10 s average, for 1 h via Acquisition Dataquest 4.33 (Data Sciences International).

2.11. In silico analysis

The X-ray structure of the complex of human chymase (CMA1) and a substrate analog (Protein Data Bank code; 1PJP) [25] was used as the template for homology modeling of mouse chymase (mMCP-4), and the mouse molecular model was optimized by energy minimization using Amber12 force fields [26]. Big ET-1 (28-35) binding structure on the active site of each chymase model was constructed referencing the substrate analog in 1PJP. Each complex model was optimized by 100 ps molecular dynamics simulation using the same force fields and a periodic cube of water molecules as solvent, and the most stable structure during the latest 25 ps iterations was finally optimized by energy minimization. All of the molecular operations, including molecular dynamics simulation, were performed using a package for molecular structural analyses, MOE 2012 (Molecular Operating Environment, Chemical Computing Group Inc., Montréal, QC, CA).

2.12. Data analysis

All data are presented as the means ± the standard error of the means (SEM) unless otherwise stated. Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software Inc, La Jolla, CA, USA). Statistical significance was assessed using Student's t-test and assumed when the p value was lower than 0.05.

3. Results

3.1. Enzyme kinetics of recombinant mMCP-4 and CMA1

In this first series of experiments, the comparative kinetics of recombinant mMCP-4 and CMA1 were established via the hydrolysis of the fluorogenic substrate Suc-Ala-Ala-Pro-Phe-AMC (Fig. 1A and C) or production of ET-1 (1–31) from Big ET-1 quantified by LC–MS/MS (Fig. 2A and C). All measured parameters are summarized in Table 1 which also includes parameters for Ang-I to Ang-II conversion.

Albeit both enzymes possess equivalent affinities against both substrates, mMCP-4 shows higher efficacy (in terms of k_{cat}/K_M) than CMA1 to hydrolyse the fluorogenic peptide or the precursor of ET-1 (1–31) (Table 1). In addition, TY-51469 (10 μM) abolished the activity of both enzymes (Figs. 1B, D and 2B, D) (** $p < 0.01$, $n = 6$). Lower concentrations of TY-51469 (0.5 and 1 μM) partially inhibited the hydrolysis of the fluorogenic peptide (results not shown). In addition, neither recombinant mMCP-4 nor CMA1 degraded ET-1 (1–31) (Fig. 2E). As a last series of controls, the kinetics of both enzymes were experimentally determined against the hydrolysis of angiotensin I to angiotensin II with similar V_{max} and K_M to those observed with both enzymes against the production of ET-1 (1–31) (Table 1). Michaelis–Menten curves for both recombinant enzymes against Ang-I are shown in supplementary figure S1. The activity of in-house produced rCMA1 was similar to the activity of commercially available rCMA1 on Ang-II production from Ang-I (data not shown).

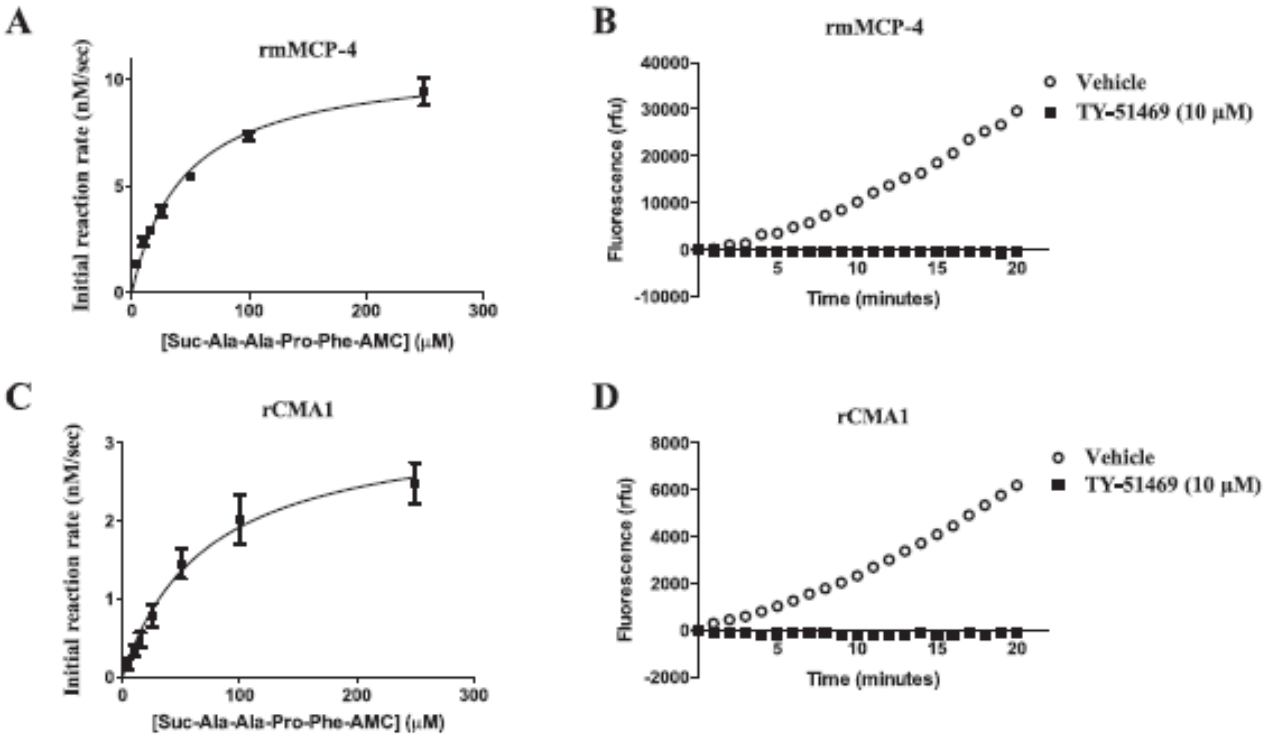

Figure 1: Fluorescence production by recombinant enzymes. The fluorescence-production rate of recombinant mMCP-4 (A; n = 7) and CMA1 (C; n = 6) was measured at different fluorogenic substrate concentrations of 5, 10, 15, 25, 50, 100 or 250 µM. Data points are expressed as means ± SEM. Time-course of the fluorescence production of typical experiments with mMCP-4 (B) and CMA1 (D) with 10 µM of Suc-Ala-Ala-Pro-Phe-AMC which was inhibited in the presence of TY-51469 (10 µM).

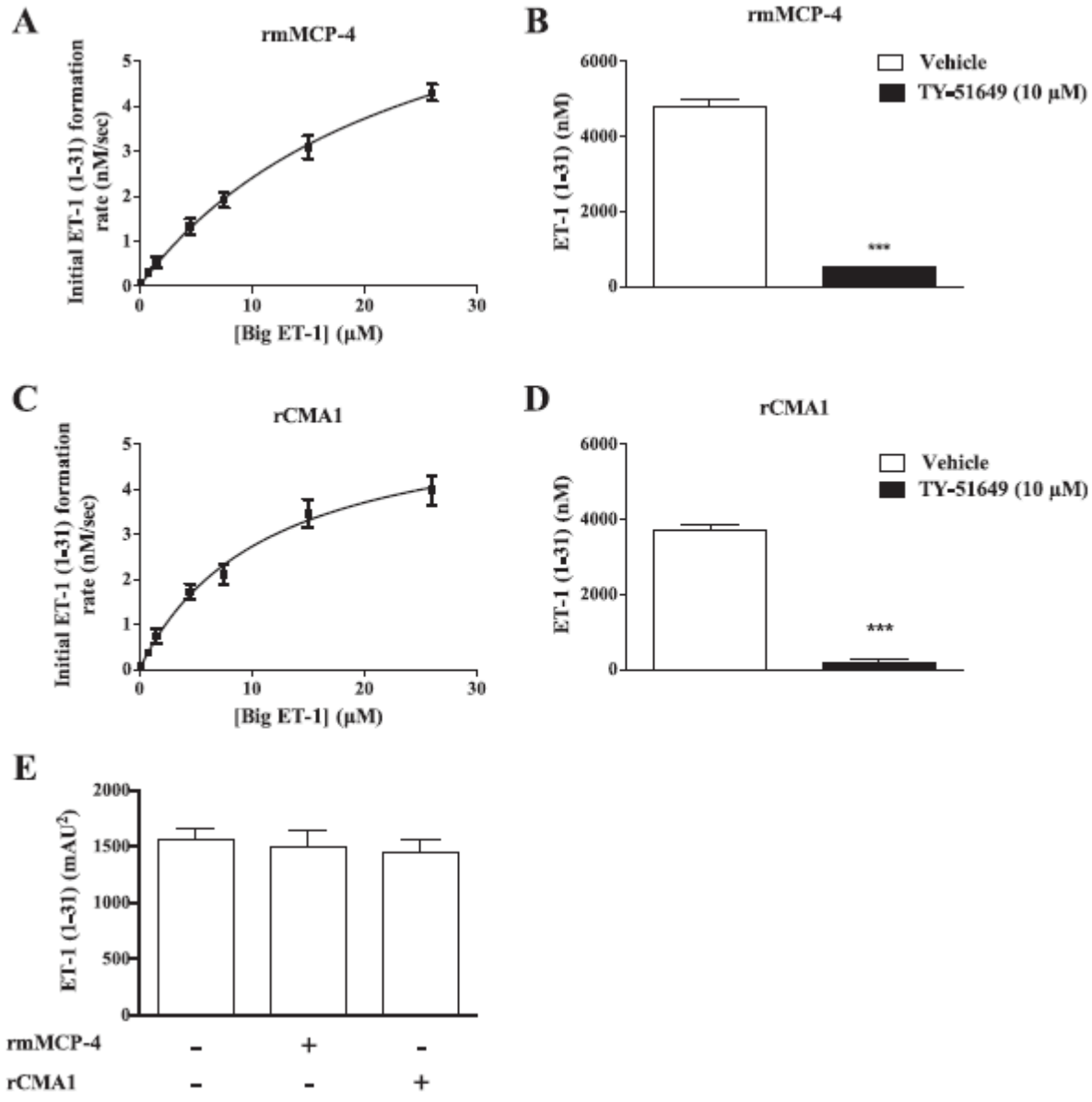

Figure 2: Big ET-1 is converted to ET-1 (1–31) by recombinant enzymes. The rate of reactions at different concentrations of Big ET-1 of 0.15, 0.75, 1.5, 4.5, 7.5, 15 or 26 µM for recombinant mMCP-4 (A; n = 6) and recombinant CMA1 (C; n = 7) is shown. (B and D) Quantification of the in vitro conversion of 16.7 µM of Big ET-1 to ET-1 (1–31) by mMCP- 4 (B) and CMA-1 (D) in the absence (n = 3) or presence (n = 6) of TY-51469 (10 µM) shows that chymase inhibition abolishes the conversion of Big ET-1 to ET-1 (1–31). (E) Reverse-phase HPLC quantification of ET-1 (1–31) (3.2 µM) degradation by mMCP-4 and CMA1 shows no activity of the recombinant enzymes on ET-1 (1–31) (n = 4). Data points are expressed as means ± SEM.

Table 1 Enzyme kinetics of rmMCP-4 and rCMA 1 against a fluorogenic substrate, Big ET-1 (1–38) or Angiotensin I.

	mMCP-4			CMA-1		
Substrates/kinetics	Suc-Ala-Ala-Pro-Phe-AMC	Big ET-1 (1–38)	Angiotensin I	Suc-Ala-Ala-Pro-Phe-AMC	Big ET-1 (1–38)	Angiotensin I
V_{max} ($M \times s^{-1}$)	1.044×10^{-8}	8.21×10^{-9}	2.38×10^{-8}	3.478×10^{-9}	5.58×10^{-9}	4.59×10^{-8}
K_M (μM)	56.96	23.43	19.31	80.31	12.55	37.53
k_{cat} (s^{-1})	1.30×10^{-2}	5.13×10^{-2}	0.149	5.15×10^{-4}	8.25×10^{-4}	6.75×10^{-3}
k_{cat}/K_M ($\mu M^{-} \times s^{-1}$)	2.29×10^{-4}	2.19×10^{-3}	7.70×10^{-3}	6.41×10^{-6}	6.57×10^{-5}	1.80×10^{-4}

3.2. TY-51469-sensitive chymase activity and conversion of Big ET-1 to ET-1 (1–31) in mouse and human-like mast cells

Fig. 3A shows that WT mouse peritoneal mast cell extracts exhibit a TY-51469-sensitive chymase-like activity that is abolished in extracts from mMCP-4$^{-/-}$ mast cells. Soluble extracts from LUVA cells also exhibited chymase-like activity, with higher concentrations of TY-51469 required to significantly inhibit the production of ET-1 (1–31) (Fig. 3C). Conversion of Big ET-1 to ET-1 (1–31) occurred in whole cell extracts of peritoneal mast cells derived from WT, but not in those derived from mMCP-4$^{-/-}$ mice. In addition, TY-51469 (10 μM) reduced by more than 80% the production of ET-1 (1–31) in fractions derived from wild type mice (** $p < 0.01$, $n = 7$, Fig. 3B). Fig. 3D shows the conversion of Big ET-1 to ET-1 (1–31) in whole cell extracts of LUVA cells, which was inhibited by 50% by the specific chymase inhibitor.

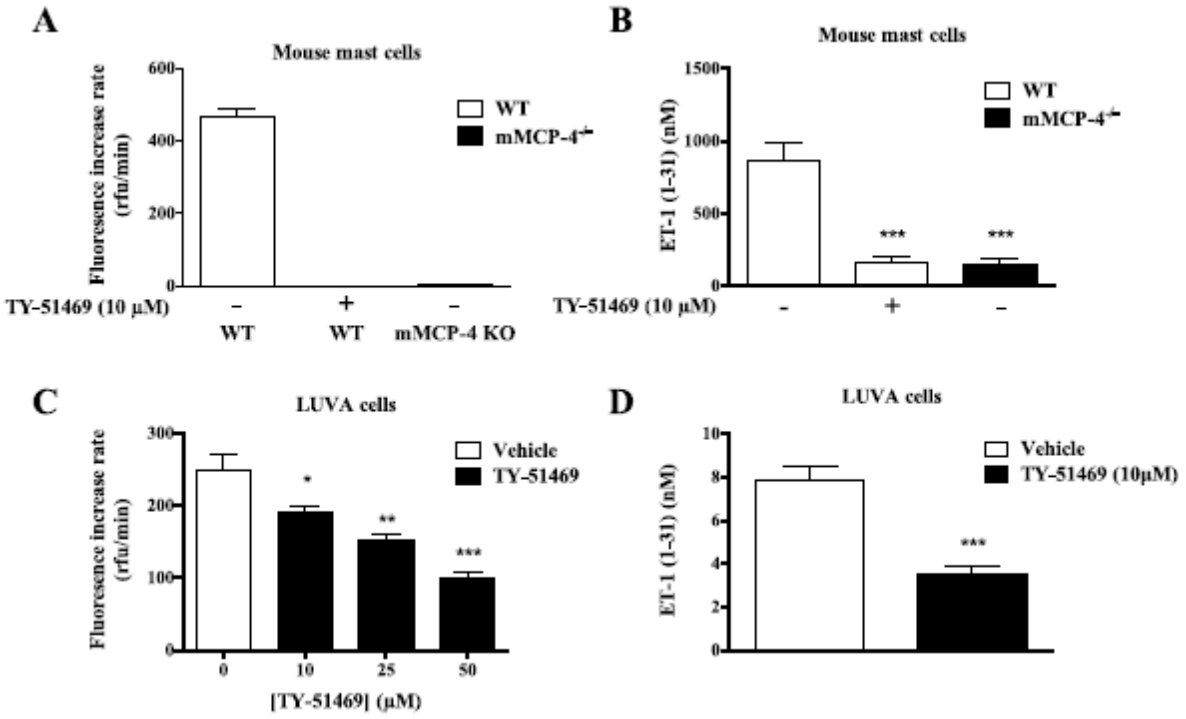

Figure 3: Chymase activity by mast cell extracts. The fluorescence production using Suc-Leu-Leu-Val-Tyr-AMC (10 µM) by mouse peritoneal mast cell extracts from WT and mMCP-4$^{-/-}$ mice (A) and LUVA cells (C) was inhibited by TY-51469 (10 µM). Big ET-1 (15 µM) was converted in vitro to ET-1 (1–31) by homogenates of WT or mMCP-4$^{-/-}$peritoneal mast cells (B) and human LUVA cells (D), and this activity was inhibited by TY-51469 (10 µM) (n = 8). Data points are expressed as means ± SEM.

3.3. Hemodynamics responses to Big ET-1 and Ang-I in conscious WT or mMCP-4_/_ mice

In Fig. 4, panels A–C show the variation in the mean (A), systolic (B) and diastolic (C) arterial pressures, respectively, following caudal vein administration of Big ET-1 (1 pmol/kg). Big ET-1 increased mean arterial pressure by 56.6 ± 7.7 mmHg in WT mice (n = 11) whereas the same response was reduced in mMCP-4$^{-/-}$(34.3 ± 4.2, n = 9, *: $p < 0.05$) by 43%, similarly to systolic and diastolic blood pressure responses in both mouse strains.

In contrast, intravenous (i.v.) administration of ET-1 prompted similar increases of mean arterial blood pressure in WT or mMCP-4$^{-/-}$mice (WT: 42.70 ± 1.89; mMCP-4$^{-/-}$: 43.92 ± 6.39 mmHg, n = 5).

Finally Ang-I (1 pmol/kg, i.v.) (WT: 38.34 ± 1.91;mMCP-4$^{-/-}$: 44.07 ± 3.87 mmHg) or Ang-II (0.1 pmol/kg) (WT: 37.00 ± 3.61; mMCP-4$^{-/-}$: 42.09 ± 3.33 mmHg) also induced similar increases ofMAP in WT and mMCP-4$^{-/-}$mice (n = 5 for each series of experiments).

Basal mean arterial pressure in all telemetry-instrumented mice was averaged at 107.56 ± 5.61 (WT) or 110.70 ±4.17 mmHg (mMCP-4$^{-/-}$) and basal heart rate at 571.65 ± 31.55 bpm (WT) or 615.46 ± 42.00 bpm (mMCP-4$^{-/-}$).

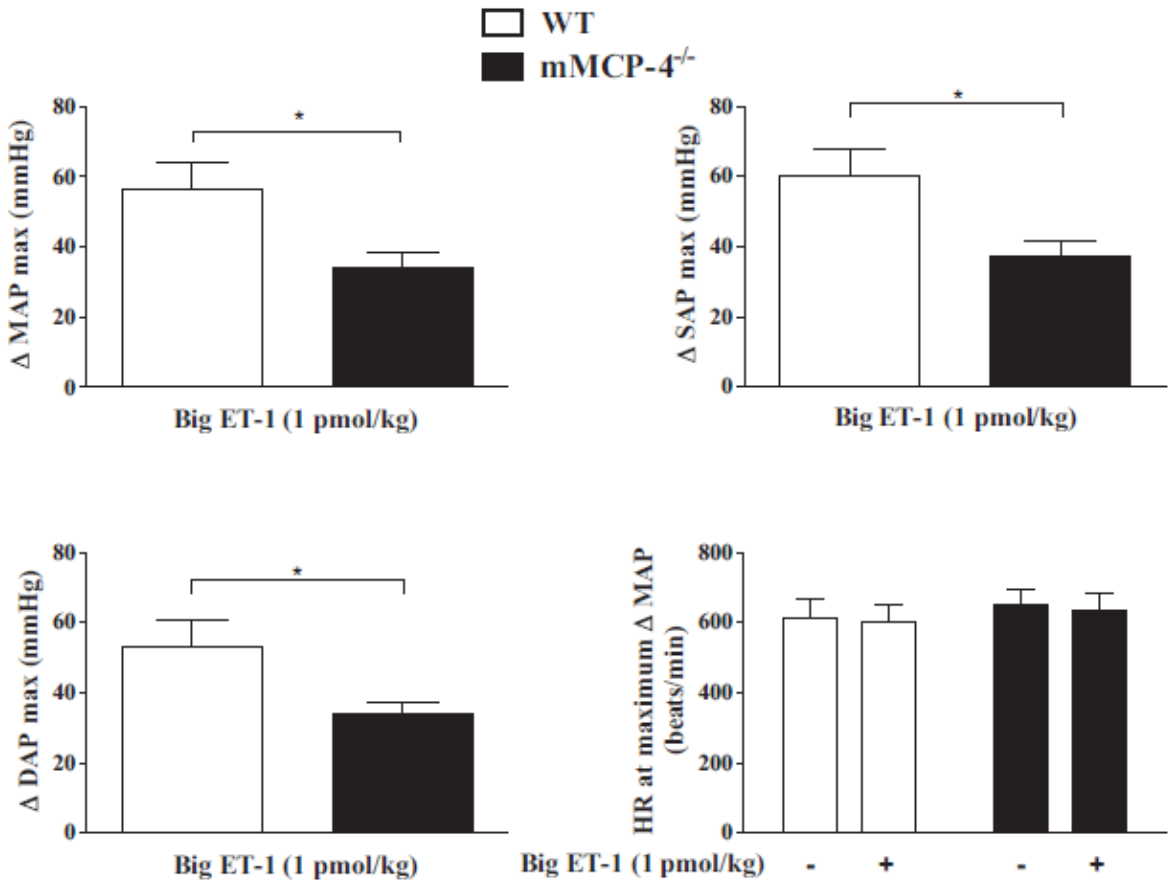

Figure 4: Hemodynamic response to Big ET-1 in instrumented conscious WT or mMCP-4$^{-/-}$ mice. All mice were instrumented with a catheter in the aortic sinus connected with a wireless pressure transducer. Panels A, B and C show the variation in the mean (A), systolic (B) and diastolic (C) arterial pressure measurements, respectively, following caudal vein Big ET-1 administration (1 pmol/kg). Big ET-1 increased mean arterial pressure by 56.6 ± 7.7 mmHg in WT mice (n = 11), and this response was diminished in mMCP-4$^{-/-}$ (34.3 ± 4.2, n = 9, *: $p < 0.05$) by 43%, similarly to systolic and diastolic blood pressures. Big ET-1, on the other hand, did not induce changes in the heart rate (D) in both WT and mMCP-4 KO mice (measures taken at the time of maximal variation of mean arterial blood pressure following saline or Big ET-1 administration). Each data point is expressed as mean ± SEM.

3.4. Human aortic homogenates generate ET-1 (1–31) in a TY-51469- sensitive fashion

Fig. 5 shows the conversion of Big ET-1 to ET-1 (1–31) in soluble fractions of human aorta. Addition of TY-51469 at a concentration of 10 μM resulted in the suppression of ET-1 (1–31) production in aortic extracts. Fig. 5A and B shows representatives spectra of the peptide products detected by LC–MS after incubation of Big ET-1 (13 μM) with aortic extracts in absence (A) or presence of the chymase inhibitor TY-51469 (B). Two major ionization species are detected for Big ET-1 and ET-1 (1–31) ($z = 5$ and $z = 4$ for Big ET-1, $z = 4$ and $z = 3$ for ET-1 (1–31). Co-treatment with the chymase inhibitor TY-51469 abolished the ET-1 (1–31) signal (Fig. 5B). Fig. 5B insert illustrates the quantification of ET-1 (1–31) production over 6 experiments in human aortic homogenates, inhibited by over 90% by TY-51469.

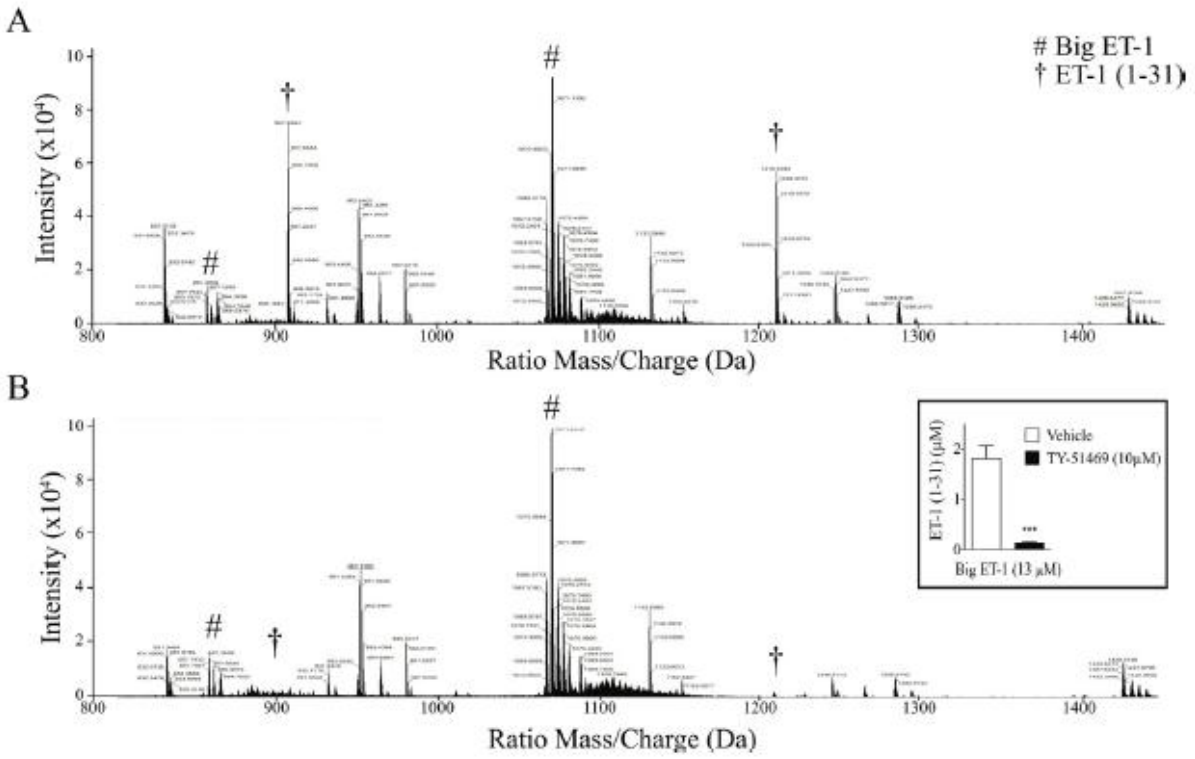

Figure 5: Representative LC/MS/MS mass spectra of the conversion of Big ET-1 to ET-1 (1–31) by human aortic homogenates. Incubation of homogenates of human aortas with Big ET-1 (13 μM) (#) resulted in the production of ET-1 (1–31) (y) specific peaks, in the absence (A) but not in the presence of TY-51469 (10 μM; B). For both peptides, major both ionization species are labeled (from left to right, z = 5 and z = 4 for Big ET-1, z = 4 and z = 3 for ET-1 (1–31)). In the insert is the quantification of the in vitro conversion of Big ET-1 (13 μM) to ET-1 (1–31) in homogenates from human aortas (n = 6). Data points are expressed as means ± SEM.

3.5. In silico analysis

As shown in Fig. 6A, the Tyr^{31} (P1) side-chain of Big ET-1 (28-35) was able to fit the S1 hole of mMCP-4, and the Leu^{33} (P20) side-chain of Big ET-1 interacted with the hydrophobic moiety of Lys^{192} of mMCP-4. By these interactions, the carboxyl group of Tyr^{31} could be fixed by hydrogen bonds to both Gly^{193} and Ser^{195} amine groups in the 'anion hole' of mMCP4. However the part of P30 and P40 (Big ET-1 Gly^{34} and Ser^{35}) being highly flexible, a hydrogen bond was formed between Ser^{35} (of Big ET-1) and Thr^{41} (on mMCP-4) in this structure. On the other hand, Leu^{33} of Big ET-1 (28-35) was stabilized with Phe^{41} in CMA1 by hydrophobic interaction between side chains and hydrogen bonds between main chains (B). Compared with the above model of mMCP-4, these tight interactions of Leu^{33} (of Big ET-1) with Ph^{41} in CMA1 appear to restrict the access of the CMA1 Lys^{192} moiety to the octapeptide,and the carboxyl group of Tyr^{31} of Big ET-1 (28-35) can then only interact with the amine of Gly^{193} in the 'anion hole' of CMA1.

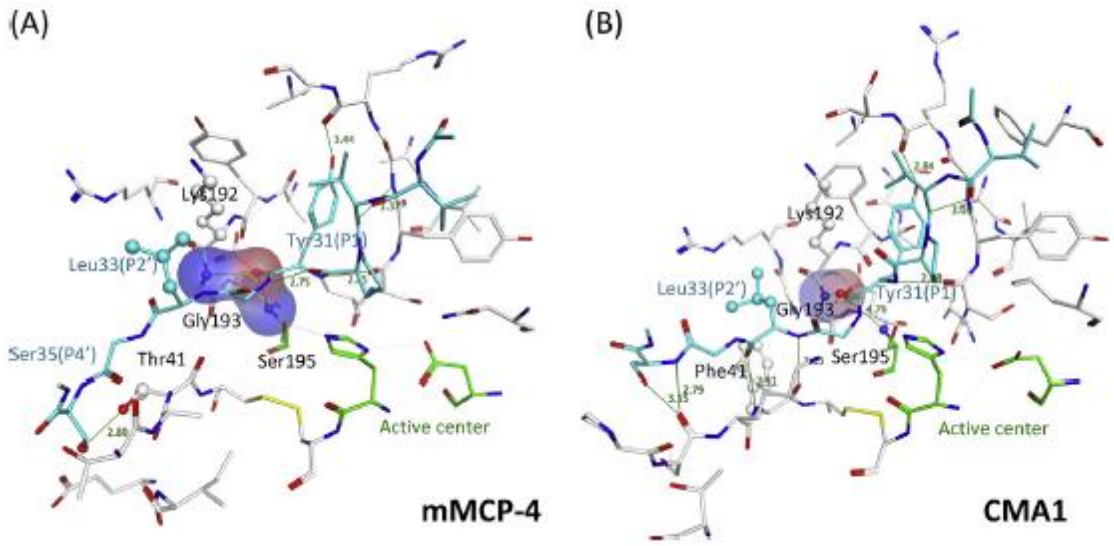

Figure 6: Predicted molecular structures of the complex of Big ET-1 with mMCP-4 or CMA1. Predicted molecular structures around the active sites of mMCP-4 and CMA1 interacting with Big ET-1 (28-35), CH3CO-Val-Val-Pro-Tyr-Gly-Leu-Gly-Ser-NHCH3, are shown in (A) and (B), respectively. Each chymase and its active center residues are described by a white and light-green stick model, and the Big ET-1 fragment is colored in light blue. Oxygen, nitrogen and sulfur atoms are colored in red, blue and yellow, and hydrogen atoms were omitted. Hydrogen bond distance between chymases and Big ET-1 is colored in green. The molecular surface of atoms related to the interaction of 'anion hole' is shown transparently. In the active site of CMA1 (B), the hydrogen bond between Ser^{195} –NH and Big ET-1– Tyr^{31}(P1) >C5O was lost (interatomic distance = 4.79 A˚). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

Our main results support the concept that both recombinant murine and human chymases are able to generate ET-1 (1–31) from its precursor Big ET-1 and that a specific chymase inhibitor, TY-51469, abolishes this process. The kinetics of either recombinant enzymes to produce ET-1 (1–31) or Ang-II from Ang-I, in terms of K_M and V_{max} are similar. In addition, the ET-1 (1–31) producing capacities of recombinant mMCP-4 and CMA1 are extended to chymase-like activity derived from mouse or humanmast cells as well as from human blood vessels. The results shown in the present study with recombinant mMCP-4 support our previous report identifying mMCP-4 as the sole murine chymase isoform involved in the genesis of exogenous and endogenous ET- 1 *in vivo* [16]. Our results for the conversion of Ang-I to Ang-II by rCMA1 and rmMCP-*4 in vitro* are also in accord with the literature [5,9].

In our experimental conditions, mMCP-4 was found to be more efficient than CMA1 to generate ET-1 (1–31) from its precursor, based on the K_{cat}/K_M of each enzyme. The *in silico* analyses presented in this study suggest that the peptide link between Tyr^{31}(P1) and Gly^{32}(P10) of Big ET-1 would be closer to the active center of mMCP-4 than that of CMA1, thus explaining the higherefficiency of the murine chymase when compared to its human counterpart. Worthy of mention, *in silico* analysis were performed with the octapeptide chain susceptible to chymase hydrolytic activity rather than the entire 38 amino acid sequence of Big ET-1.

Interactions of either mMCP-4 or CMA1 binding and/or catalytic sites with N- and C-terminal chains and to the two disulfide bridges of the 38 amino acid precursor are unaccounted for in the present computer assisted modeling.

Our group has previously shown that soluble extracts derived from mouse organs such as the heart, lungs, kidney and aorta hydrolyze the fluorogenic peptide Suc-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (AMC), a chymotryptic activity which is only partly inhibited by TY-51469 or in tissues derived from mMCP-$4^{-/-}$ mice, thus suggesting that other proteases are involved in the process [16]. In contrast, we show in the present study that the TY- 51469-sensitive hydrolysis of a fluorogenic peptide by extracts from mouse mast cells is totally dependent on the presence of mMCP-4. Furthermore, the extracts derived from mouse mast cells show the

exclusive contribution of mMCP-4 in the processing of Big ET-1 to ET-1 (1–31). Our data further suggest that mMCP-4 isthe predominant isoform involved in the conversion of Big ET-1 to ET-1 (1–31) in these cells since no more than 20% of the latter peptide were found in soluble extracts derived from mMCP-4$^{-/-}$ mice or from WT extracts pre-treated with TY-51469. Whether the mMCP-4-dependent hydrolysis of Big ET-1 reported in our study in peritoneal mast cells can be reproduced in bone marrow derived mastocytes, remains to be investigated.

We previously reported lower endogenous ET-1 levels in the lungs of mMCP-4$^{-/-}$mice [16], in concordance with results from ECE-1$^{-/-}$ECE-2$^{-/-}$ mouse embryos which show only a 45% reduction in whole body ET-1 levels [28]. Furthermore, anesthetized mMCP-4$^{-/-}$mice, when compared to WT mice, produced half the plasma ET-1 and ET-1 (1–31) levels as well as pressor responses, when challenged with intravenous Big ET-1 [16]. In concordance with those results, we show here for the first time in freely moving, conscious mice that Big ET-1 is less potent as a vasoconstrictor in mMCP-4$^{-/-}$ mice than in their WT congeners whereas in contrast, both murine strains respond equally to Ang-I and Ang-II. This suggests that albeit purified or recombinant chymases (of mouse or human origins) can convert Ang-I to Ang-II in vitro as previously reported [8,9,27], it is not as biologically significant as for Big ET-1 in the murine systemic circulation given the abundance of angiotensin converting enzyme *in vivo*. This study also addressed the capacity of human tissues to generate ET- 1 (1–31) in a chymase inhibitor-sensitive fashion. The LUVA cells used in the present study originate from the first human mast cell line derived from an individual without a clonal mast cell disorder, as indicated by normal serum tryptase levels in the patient and on the presence of metachromatic granules in those mastocytes [20,29]. This is also the first human mast cell line expressing a functional chymase [29]. Similarly to the recombinant enzymes and the extracts derived from mouse peritoneal mast cells, a TY- 51469-sensitive and chymase-dependent conversion of Big ET-1 to ET-1 (1–31) was observed in LUVA cells albeit less efficiently than with pure enzymes or mouse cells. The latter characteristic of LUVA cells may be explained by the heterogeneous expression of chymase in this particular cell line as reported initially by Laidlaw and colleagues [29]. Interestingly, in stark contrast to mouse peritoneal mast cell granular extracts, significant quantities of fluorescence and ET-1 (1–31) produced from Big ET-1 in LUVA granular extracts were TY-51469 insensitive, suggesting that yet to be identified enzymes other than chymase, perhaps such as

cathepsin G [30], have chymotrypsin-like activity and generate the 31 amino acid intermediate in this particular cell model.

Finally, similarly to pulmonary tissue investigated previously [14,16], soluble extracts of human aortic biopsies were found to generate ET-1 (1–31) with no further conversion to ET-1 in a TY-51469-sensitive fashion. In contrast to experiments with LUVA cells, conversion of Big ET-1 to ET-1 (1–31) by human aortic homogenates was fully inhibited by TY-51469 at 10 μM, in agreement with our results from the murine system and when using human recombinant CMA1. Thus, the present study suggests for the first time that the production of ET-1 (1–31) requires a chymase-like activity in human blood vessels. Interestingly, Mawatari and colleagues [31] reported ET-1 (1–31) reactivity in all vascular layers in the hamster aortic arch, which is increased in the intimal lesions of hypercholesterolemic animals. However, chymase-dependent generation of ET-1 (1–31) remains to be confirmed as a relevant factor in human atherosclerosis.

The present study did not address the ECE/chymase conversion ratio in murine and human tissue extracts. However, it is of interest to note that no ECE-dependent synthesis of ET-1 from exogenous Big ET-1 was detected by the LC–MS/MS approach. This could perhaps be due to the experimental conditions imposed throughout the present study, such as the removal of non-soluble proteins. It is possible that the ECE-dependent production of ET-1 would have been measurable in intact tissues or organs, particularly in conditions where the vascular endothelium would have been maintained intact. We suggest that, similarly to the angiotensin converting enzyme/chymase paradigm in the production of angiotensin-II from angiotensin-I [32], the ECE plays an important role in the production of the circulating levels of ET-1 whereas chymase-like activity may be predominantly involved in perivascular and tissue-derived genesis of the latter vasoactive peptide.

Taken together, the present study supports an important role for both murine and human chymase in the production of the intermediate ET-1 (1–31). Our results further suggest that the use of chymase inhibitors in clinical settings may be proven useful not only for inhibiting intramural angiotensin II but also by reducing tissue levels of endothelin-1, particularly in cardiovascular diseases of fibrotic origin in which infiltration of activated mast cells is well documented [1].

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Discussion

The main conclusion of this work is that the human chymase CMA1 and its murine homolog mMCP-4 be it recombinant, extracted from mast cells or from the soluble fractions of the human aorta (in the case of CMA1 only) convert Big ET-1 into ET-1 (1-31) in a TY-51469 sensitive fashion *in vitro*. Moreover, the importance of the chymase pathway in the conversion of Big ET-1 into ET-1 and its paramount role in hypertensive responses was confirmed in conscious mice *in vivo*.

The initiation of our experiments was based on the fact that our group has demonstrated that the mMCP-4 has the capacity to cleave Big ET-1 into ET-1 (1-31) *in vivo* and *in vitro* (Houde *et al.*, 2013). In the mouse model, there are 4 isoforms of chymase, 1 α-chymase and 3 β-chymases whereas in humans there is only 1 α-chymase identified. Several studies have been performed to identify the murine homolog of the human chymase and its implication in the endothelin system. The murine mMCP-5 is the only α-chymase; however it does not have chymase properties but elastase ones (Kunori *et al.*, 2002) which indicate that it is less likely to be involved in the genesis of the endothelin system. Of the 3 β-chymases, the mMCP-4 was demonstrated to be the homolog of human chymase since both enzymes are activated by heparin in a similar way and they are both able to generate Ang II from its precursor Ang I (Caughey *et al.*, 2000; Tchougounova *et al.*, 2003; Wu *et al.*, 2005).

Very few studies have attempted to characterize recombinant chymases (murine or human) vis-à-vis the Big ET-1. The literature shows a chymostatin, a non-specific chymase inhibitor, dependent characterization of CMA1 in regards to generation of Ang II from Ang I (Murakami *et al*., 1995) and different characteristics (regarding enzyme kinetics) of mMCP-4 and CMA1 with Ang I or Big ET-1 as substrates (Takai *et al.*, 1997, 1999; Caughey *et al.*, 2000; Kunori *et al.*, 2005). Regarding mMCP-4, Kinetic constants were established for the tissue purified enzyme using Ang I as a substrate (Caughey *et al.*, 2000; Kunori *et al.*, 2005). However no study to date was found to have used Big ET-1 as a substrate to characterize the enzyme; moreover, recombinant mMCP-4 was never tested in the literature. On the other hand, more studies involving CMA1 were published. In 1997, the group of Takai has characterized CMA1 purified from gastroepiploic arteries of donors suffering either from gastric cancer or gastric ulcers; although the donors did not have hypertension or diabetes mellitus, they were not healthy donors.

In this study, the authors have used Ang I as a substrate. In 1999, the group of Takao has used purified vascular CMA1 and has characterized the enzyme using Ang I and Big ET-1 (Takao *et al*., 1999). In that study the effect of deglycosylation of Asn-89 of CMA1 on its affinity and efficiency in processing Ang I and Big ET-1 were tested. Takao and colleagues have established in 1999 a K_M and a K_{cat}/K_M for the purified CMA1. It is important to note that in the two above mentioned studies, a purified enzyme was experimented. This purified enzyme was identified as chymase since it was inhibited by SBTI, PMSF and chymostatin but not inhibited by EDTA, pepstatin and aprotinin. As previously mentioned in the introduction, chymostatin is not a specific chymase inhibitor and it has shown to inhibit elastase II, an enzyme involved as well in the production of Ang II from its precursor Ang I (Becari *et al.*, 2005). This drives us to the conclusion that the purified enzyme utilized in the studies might contain other enzymes or impurities able to cleave Ang I. This pushed us to use a more specific enzyme that will inhibit chymase solely.

In 1999, the group of Takai has published another study comparing enzyme kinetics of the purified CMA1 to the recombinant CMA1. The authors have used Ang I and Big ET-1 as substrates. The authors have established a K_M value for CMA1 (purified and recombinant), showing their affinities to Ang I and Big ET-1. In addition they have established a K_{cat}/K_M value for both enzymes using Ang I as a substrate; however the K_{cat}/K_M value for CMA1 with Big ET-1 as a substrate was not published.

To date, there is no study comparing the human and murine enzymes in the same paper, using the same experimental conditions. Furthermore, the efficiency of recombinant CMA1 in regards to cleaving Big ET-1 was not characterized in the literature, hence the important and innovative aspect of our study that compares CMA1 and mMCP-4 and establishes an affinity and efficiency of both enzymes. This comparison is valuable since mMCP-4 was shown to be the murine homolog of CMA1. Thus understanding its relative characteristics *in vitro* and *in vivo* can help with testing inhibitors (at different concentrations) in mice which can give an idea about the inhibitor's concentrations needed in humans for an adequate response specially when developing new drugs targeting CMA1.

In a first series of experiments, we have used a fluorogenic substrate that is cleaved by chymotypsin-like enzymes and verified the effect of TY-51469, a specific chymase inhibitor, on

CMA1 and mMCP-4 cleavage activity of the substrate. The inhibition of the fluorogenic activity was complete, confirming that our recombinant chymases (CMA1 and mMCP-4) are pure. This total inhibition was not noted in the work of Houde and colleagues in 2013, there was an important however partial inhibition of the murine chymase activity. The incomplete inhibition of the cleavage of the fluorogenic substrate was because our laboratory has used the soluble fractions of tissues of different organs which contain mMCP-4 among other proteases that have chymotrypsin-like activity and might have cleaved the fluorogenic substrate (Houde *et al.*, 2013) whereas in the current study we have used recombinant enzymes.

The same activity of cleavage of the fluorogenic substrate observed with the soluble fractions of organs of WT mice pre-incubated with TY-51469, was seen in the soluble fractions of organs of mice mMCP-4 KO in the study of Houde and colleagues explaining that this remaining activity of cleavage is not due to chymase.

However this partial inhibition was not noted in the generation of ET-1 (1-31) from Big ET-1 incubated with soluble fractions of organs of WT mice, detected by HPLC; the inhibition was total. This suggests that the enzymes that have chymotrypsin-like activity and have the ability to cleave the fluorogenic substrate do not have any role in the cleavage of Big ET-1 into ET-1 (1-31). Moreover, both recombinant chymase incubated with ET-1 (1-31) and/or ET-1 did not cleave these peptides.

In another series of experiments, we have demonstrated that TY-51469 can inhibit the production of ET-1 (1-31) when pre-incubated with the recombinant enzymes mMCP-4 and CMA1; confirming once more that the recombinant enzymes are pure up to 99%. The concentrations of ET-1 (1-31) generated with mMCP-4 seemed to be more elevated than the ones generated by CMA1 indicating that mMCP-4 might be more efficient in cleaving Big ET-1.

Furthermore, mMCP-4 extracted from murine peritoneal mast cells was able to cleave the fluorogenic substrate. An action totally inhibited by TY-51469 demonstrating that in the mast cells mMCP-4 are the only enzymes that have a chymotrypsin-like activity.

The ability of mMCP-4 extracted from murine peritoneal mast cells to generate ET-1 (1-31) was also confirmed. The total inhibition of the formation of ET-1 (1-31) by TY-51469 proves that the

only chymase able to generate ET-1 (1-31) from Big ET-1 in mast cells is the mMCP-4. Similar reinforcing results were seen with peritoneal mast cells of mice mMCP-4 KO.

Studies on human mast cells, on the other hand, have been limited by the rarity of functional human cell lines. In our experiments, we have used the LUVA cells which are an immortalized human mast cell line. The cells were derived from mononuclear cells of a donor with Aspirin exacerbated respiratory disease. The importance of these cells is that they are the first human mast cell line derived from a donor without a clonal mast cell disorder, as indicated by normal serum tryptase levels in the donor and the presence of metachromatic granules in those cells. In addition, they are the first human mast cell line expressing a functional chymase and they are maintained without stem cell factor and present high levels of c-kit and FcεRI (Laidlaw *et al.*, 2011).

LUVA cells were utilized as a human model of mast cells. The human chymase extracted from these cells was able to cleave the fluorogenic substrate indicating that a chymotrypsin-like activity is present in this enzyme. Pre-incubation with TY-51469 was not sufficient to totally inhibit the chymotrypsin-like activity of the extract of LUVA cells. However the percentage inhibition was proportional to the concentration of TY-51469. The experiment was performed with concentrations of 10, 25 and 50 μM of TY-51469; an inhibition of around 60% was observed with the highest concentration of the inhibitor; indicating the possibility of the presence of other enzymes in the extract of LUVA cells that have a chymotrypsin-like activity.

The human chymase extracted from LUVA cells was able to generate ET-1 (1-31) from its precursor. As we would have expected, pre-incubation with TY-51469 did not inhibit totally the production of ET-1 (1-31). A concentration of 10 μM of the specific chymase inhibitor decreased the generation of ET-1 (1-31) by about 60%. This incomplete inhibition suggests the presence of other enzymes in LUVA cells responsible for the conversion of Big ET-1 into ET-1 (1-31) such as cathepsin G (Caughey, 2007).

Although the cleavage of the fluorogenic substrate and the conversion of Big ET-1 into ET-1 (1-31) occurred with chymase extracted from LUVA cells, this enzymatic activity however was less efficient when compared to chymase extracted from murine peritoneal mast cells (Laidlaw *et al.*, 2011).

Our group has shown that the soluble fractions of organs (lungs, heart, aorta and kidneys) of WT mice were able to generate ET-1 (1-31) from its precursor. Pre-incubation with TY-51469 inhibited totally the formation of ET-1 (1-31). This response was also seen in the soluble fractions of organs of mMCP-4 KO mice (Houde *et al.*, 2013). These results indicate that although several enzymes with chymotrypsin-like activity are present in the soluble fractions of organs (as shown with the partial inhibition of cleavage of the fluorogenic substrate by TY-51469 and/or with mMCP-4 mice), mMCP-4 is the only enzyme present in these fractions capable of processing Big ET-1 into ET-1 (1-31). Of note, no conversion to ET-1 was detected which can be explained by the fact that ECE and NEP are both membrane bound enzymes, contrary to mMCP-4 (Houde *et al.*, 2013).

With the same thought process, the soluble fractions of human aorta, taken from middle-aged brain-dead individuals with no histological signs of atherosclerosis, were able to generate ET-1 (1-31) from Big ET-1; a process, contrarily to LUVA cells, totally abolished by TY-51469. This finding came in concurrence with the murine results of our laboratory and with the results of recombinant CMA1. Therefore these results demonstrate for the first time that ET-1 (1-31) production in the human blood vessels is chymase dependent. Recent studies have shown an increase in ET-1 (1-31) levels in atherosclerotic lesions in the aortic arch of hamsters which could indicate a yet to be determined role of chymase in human atherosclerosis hence a possible preventative method in arterial diseases (Mawatari *et al.*, 2004).

In another series of experiments, a characterization of the recombinant enzymes, rmMCP-4 and rCMA1 was done. The kinetic constants were determined for both enzymes using a fluorogenic substrate, Big ET-1 and Ang I as substrates.

In the literature, the kinetic constants were determined for mMCP-4 purified from tissue extraction, using Ang I as a substrate and for recombinant human chymase using Ang I and Big ET-1 as a substrate. We wanted to compare our enzymes to the ones found in the literature. The hypothesis was that our recombinant enzymes have a chymotrypsin-like activity and is as efficacious in producing ET-1 (1-31) from Big ET-1 as Ang II from Ang I.

Our results showed that the rmMCP-4 has similar affinities to Ang I and Big ET-1, with a slightly higher affinity for Ang I; a K_M of 19.31 μM for Ang I versus a K_M of 23.43 μM for Big

ET-1. These results did not concur with the literature, where it was shown that the affinity of the murine enzyme is about 34 times less (Caughey *et al.*, 2000; Kunori *et al.*, 2005). In addition the cleavage efficacy of the substrates was different. Our enzyme (rmMCP-4) has shown a higher efficacy in cleaving Ang I than Big ET-1. This was determined by the higher value of K_{cat}/K_M 7.7 x 10^{-3} $\mu M^{-1}.s^{-1}$ (for Ang I) compared to 2.189 x 10^{-3} $\mu M^{-1}.s^{-1}$ (for Big ET-1). The efficacy of our enzyme was higher than that reported in the literature for Ang I, as shown in table V.

The difference observed when comparing our results to the literature may be attributed grossly to the nature of the enzymes utilized. The groups of Kumori and Caughey have used a chymase extracted and purified from Connective tissue Mast cells isolated from the skin of mice. This method compared to our recombinant method leads to a much less pure enzyme. The lack of purity of the enzymes implies that there are other enzymes or co-enzymes present in the purified extract that may affect the affinity of the enzyme to its substrate and/or affect the efficacy of the enzyme, rendering it either more or less efficacious.
In addition, considering that some β-chymases are known to degrade Ang II by cleaving the Tyr 4-Ile 5 bond and that mMCP-4 is able to generate and to degrade Ang II, the group of Caughey established the kinetic constants using the rate of degradation of Ang I rather than measuring the formation of the product Ang II (Lundequist *et al.*, 2004) . Moreover the group of Kumori used Ang (5-10) instead of using Ang I as a substrate which may alter the results by either increasing or decreasing the affinity and the efficacy of the enzyme.

Regarding CMA1, our results have shown a higher affinity to Big ET-1 than to Ang I; K_M of 12.55 μM and 37.53 μM respectively. These results show that our enzymes had a greater affinity for both substrates than what has been published in the literature. In addition, the affinity of CMA1 to the fluorogenic substrate was higher in our study than what has been published (Nakakubo *et al.*, 2000). The group of Takai has published in 1999 a study characterizing the recombinant human chymase. The recombinant human chymase showed an affinity of 23.5 μM for Big ET-1 and 62.5 μM Ang I. Regarding the efficacy of the enzyme, the recombinant enzyme in Takai's paper was more efficacious in cleaving Ang I, with a K_{cat}/K_M of 0.86 $\mu M^{-1}.s^{-1}$ whereas it was 1.8 x 10^{-4} $\mu M^{-1}.s^{-1}$ with our CMA1. This difference in affinity and efficacy can be attributed to the possible difference in the purity of the enzymes or the experimental conditions

in which the experiments were done. Other studies have published kinetics constants of CMA1 which were different than ours. However these studies (Takai *et al.*, 1997; Nakakubo *et al.*, 2000) have used a purified chymase from tissues rather than a recombinant enzyme which explains the difference.

In a comparison of the murine and human enzyme, we noticed that mMCP-4 is more efficient than CMA1 in processing Big ET-1 into ET-1 (1-31), as shown in table IV. In an attempt to explain this particular result, we performed in-silico studies using the X-ray structure of CMA1 and a substrate analog as the template homology modeling of mMCP-4. Big ET-1 (28-35) was used as a substrate model. This analysis, as mentioned in our study (Semaan *et al.*, 2015), suggested that the Tyr 31-Gly 32 (P1-P1') bond of Big ET-1 would be closer to the active center of mMCP-4 then CMA1 which can explain the higher Kcat/Km value of the murine enzyme compares to the human one. It is important to mention, however, that the *in-silico* studies were performed using an octapeptide as a substrate whereas Big ET-1 is a peptide constituted of 38 peptides.

Table IV. Enzyme Kinetics of rmMCP-4 and rCMA1 against Big ET-1 and Ang I

	mMCP-4		**CMA1**	
	Big ET-1	Ang I	Big ET-1	Ang I
K_M (μM)	23.43	19.31	12.55	37.53
K_{cat} (s^{-1})	5.13×10^{-2}	0.149	8.25×10^{-4}	6.75×10^{-3}
K_{cat}/K_M ($\mu M^{-1}.s^{-1}$)	2.19×10^{-3}	7.7×10^{-3}	6.57×10^{-5}	1.8×10^{-4}

Table V. Enzyme Kinetics of mMCP-4 against Big ET-1 and Ang I compared to the literature

	mMCP-4			
	Big ET-1	Ang I	Ang I	
			Caughey *et al.*, 2000	Kumori *et al.*, 2005
K_M (μM)	23.43	19.31	698	673
$K_{cat}(s^{-1})$	5.13×10^{-2}	0.149	3.1	0.33
$K_{cat}/K_M(\mu M^{-1}.s^{-1})$	2.19×10^{-3}	7.7×10^{-3}	4.4×10^{-3}	4.9×10^{-4}

In the third part of experiments, we wanted to confirm the role of chymase in the hypertensive response induced by administration of exogenous Big ET-1 *in vivo*. In the literature, several hemodynamic studies were performed on anesthetized mice. It is known that anesthetics can have different impacts on the heart rate and blood pressure. In the present study we have measured hemodynamic changes in conscious, freely moving, anesthesia-free mice.

Several techniques are used to measure hemodynamic parameters in conscious mice: The fluid filled catheter system, the tail cuff, the millar probe and the telemetry probe implantation. Each technique has its pros and cons; we have used the telemetry probe implantation technique since it is less cumbersome on mice without jeopardizing results. After the implantation of the probe, the mice would feel some discomfort since they will be in a post-operative phase. On the other hand, once the post operative phase is over, the mice can move freely while their blood pressure is being measured in a highly sensitive manner over a long period of time, which is a big advantage of this technique. In addition, this technique can detect small variations in the blood pressure (Van Vliet *et al.*, 2000; 2006). In order to help minimize the variation of blood pressure and heart rate related to pain or post operative discomfort, we have used Buprenorphine to control post-op pain in the first 24 hours and we started recording blood pressure and heart rate values 10 days after the surgery. Our group has shown that chymase (mMCP-4 specifically) is implicated in the hypertensive response induced by the administration of exogenous Big ET-1 in anesthetized mice (Simard *et al,.* 2009; Houde *et al.*, 2013). As mentioned earlier, anesthetic agents have several effects on the heart rate and blood pressure which may be a confounding variable. In the present study we have started recording blood pressure and heart rate measurements in conscious, free moving mice, 10 days after the surgical implantation of the telemetry probe, allowing the mice to recover from surgical stress and for the anesthetic agent to be metabolized from the body. We have shown that exogenous Big ET-1 could increase MAP by 56.6 mmHg in WT mice; whereas its administration in mMCP-4 KO mice has increased MAP by 34.4 mmHg. This 40% difference in the increase in MAP can be attributed to the conversion of Big ET-1 to ET-1 (1-31) by mMCP-4 and subsequently to ET-1 by the ubiquitous NEP. This attribution was supported by the results of our group in 2013, when plasma levels of ET-1 (1-31) and ET-1 where shown to be higher in WT mice compared to mMCP-4 KO after administration of exogenous Big ET-1 (Houde *et al.*, 2013). Furthermore ET-1 levels in plasma were similar in WT and mMCP-4 KO mice after administration of exogenous ET-1 (1-31) (Houde *et al.*, 2013).

The effect of exogenous Big ET-1 on MAP was not observed with Ang I or Ang II. WT and mMCP-4 mice had the same increase in MAP with administration of exogenous Ang I or Ang II. This may be due to the abundance of ACE *in vivo*.

There are few limitations to the present study. First regarding the recombinant enzymes, they were cloned as prochymases requiring activation by Cathepsin C and Heparin. We did not verify whether the solution that stops the activation process is able to inhibit/or enhance the activity of cathepsin C. Despite the fact that Cathepsin C is a DPPI, it might not be the ideal enzyme to activate our prochymases. Furthermore, the cathepsin C used was a murine one; so when it came to CMA1 activation, interspecies variations might have interfered in this activation. Second regarding MC, LUVA cells were not as efficacious as murine MC in the conversion of Big ET-1 into ET-1 (1-31) and this conversion was not completely inhibited TY-51469. These cells do not necessarily represent the ideal human MC but they are the best available model. Third, the *in silico* analysis was performed on an octapeptide rather than the whole Big ET-1 (38 aa) not taking into account a possible interaction between the N or C terminal and/or the disulfide bridges of Big ET-1 with both chymases binding and/or catalytic sites. Fourth, regarding *in vivo* studies of MAP, external stimuli might have led to fluctuations in the blood pressure, however these external stimuli would exist in both groups studied (WT and mMCP-4 KO). To take into account these minimal stress related fluctuations, we have calculated areas under the curve to determine MAP.

Conclusion

In conclusion, this work has shown an ability of mMCP-4 and CMA1 to generate ET-1 (1-31) from its precursor Big ET-1 *in vitro*. CMA1 extracted from healthy human aortas had a role in the production of ET-1 (1-31). Further studies are needed to establish its role in cardiovascular pathologies. For example, the use of aortas of hypertensive patients or atherosclerotic aortas with the comparison of the production of ET-1 (1-31) in these models is of high value.

Chymase inhibitors are still in the preclinical level regarding cardiovascular diseases. Promising studies and results have shown a role for TY-51469 in preventing the development of hypertension, heart failure and bowel disease in rats (Palaniyandi *et al.*, 2007; Takai *et al.*, 2014; Liu *et al.*, 2016). On the other hand, some chymase inhibitors are currently in clinical trials such as SUN 13834 for atopic dermatitis (Ogata *et al.*, 2011) and BAY 1142524 for heart failure (phase II) (Zarin and Tse, 2016) . The success of BAY 1142524 can open the doors to clinical trials involving other cardiovascular diseases targeting both the angiotensin and the endothelin system.

The hemodynamic studies suggested that although chymase is able to convert Ang I to Ang II *in vitro,* its significance is of less importance intravascularly *in vivo* which is not the case with the endothelin system where a KO of the chymase gene was associated with a lower increase in MAP after Big ET-1 administration.

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