PIVOTAL ROLE OF MOUSE MAST CELL PROTEASE 4 IN THE CONVERSION AND PRESSOR PROPERTIES OF BIG-ENDOTHELIN-1

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Abstract

The serine protease chymase has been reported to generate intra-cardiac angiotensin-II (Ang-II) from Ang-I as well as an intermediate precursor of endothelin (ET)-1, ET-1 (1-31) from Big-ET-1. While humans possess only one chymase, several murine isoforms are documented, each with its own specific catalytic activity. Among these, mouse mast cell protease 4 (mMCP-4) is the isoform most similar to the human chymase for its activity. The aim of this study was to characterize the capacity of mMCP-4 to convert Big-ET-1 into its bioactive metabolite, ET-1, in vitro and in vivo in the mouse model. Basal mean arterial pressure did not differ between WT and mMCP-4(-/-) mice. Systemic administration of Big-ET-1 triggered pressor responses and increased blood levels of immunoreactive ET-1 (1-31) and ET-1 that were reduced by more than 50 % in mMCP-4 knockout (-/-) mice when compared to WT controls. Residual responses to Big-ET-1 in mMCP-4(-/-) mice were insensitive to the enkephalinase/neutral endopeptidase inhibitor, thiorphan, and the specific chymase inhibitor, TY-51469 (2-[4-(5-fluoro-3-methylbenzo[b]thiophen-2-yl)sulfonamido-3-methanesulfonylphenyl]thiazole-4-carboxylic acid). Soluble fractions from the lungs, left cardiac ventricle, aorta and kidneys of WT but not mMCP-4(-/-) mice generated ET-1 (1-31) from exogenous Big-ET-1 in a TY-41569-sensitive fashion as detected by HPLC/MALDI-mass spectrometry. Finally, pulmonary endogenous levels of immunoreactive-ET-1 were reduced by more than 40 % in tissues derived from mMCP-4(-/-) mice when compared to WT mice. Our results show that mMCP-4 plays a pivotal role in the dynamic conversion of systemic Big-ET-1 to ET-1 in the mouse model.
Introduction

In the human cardiovascular system, mast cell-derived serine protease chymase generates the vasoconstrictor peptide angiotensin II (Ang-II), especially in the heart and the vascular wall (Urata et al., 1993; Mangiapane et al., 1994). Chymase, similarly to the angiotensin converting enzyme, cleaves the precursor angiotensin-I to yield the biologically active Ang-II (Urata et al., 1990). Pivotal roles of chymase have also been demonstrated in several animal models of cardiovascular diseases, such as atherosclerosis, many of them in relation to its Ang-II producing activity (Fleming, 2006). For instance, chymase presence is increased in the atherosclerotic plaque (Kaartinen et al., 1994) and the inhibition of chymase reduces the size of Ang-II-induced abdominal aneurysms in the mouse (Inoue et al., 2009).

Endothelin-1 (ET-1), on the other hand, is a 21 amino acid peptide (Yanagisawa et al., 1988) that exerts its actions via two receptors, ET_A and ET_B (Arai et al., 1990; Sakurai et al., 1990). ET-1 is derived from pro-endothelin-1, which is cleaved by furin to yield a 38 amino acid intermediate, Big-ET-1 (Denault et al., 1995). Big-ET-1 is then hydrolysed at the tryptophan^{21}-valine^{22} bond to yield the bioactive ET-1 by an endothelin converting enzyme (ECE) (McMahon et al., 1991; D’Orleans-Juste et al., 2003).

Mice knocked out for both ECE genes do not survive the late gestational stage, yet embryonic tissues of these mice still retain two thirds of total endothelin peptides measured in wild type (WT) congeners (Yanagisawa et al., 2000). Thus, other proteases are involved in the overall production of mature ET-1 in the mouse.

The first report of non-ECE dependent synthesis of ET-1 from Big-ET-1 showed that chymostatin, a non-specific inhibitor of chymotrypsin-like proteases, efficiently blocked the processing of Big-ET-1 into its active metabolite in perfused rat lungs (Wypij et al., 1992).
Chymase has subsequently been reported to hydrolyze Big-ET-1 to a 31 amino acid peptide, ET-1 (1-31) (Hanson et al., 1997; Nakano et al., 1997). Initially reported as a direct ETA receptor agonist (Yoshizumi et al., 1998), further in vitro (Hayasaki-Kajiwara et al., 1999) and in vivo studies (Fecteau et al., 2005) have shown that ET-1 (1-31) must first be converted by the neutral endopeptidase 24.11 (NEP) to normal-length ET-1 to exert biological activities. Interestingly, Matawari and colleagues (2004) reported high concentrations of ET-1 (1-31) in the atheromas of atherosclerotic patients. More recently, our laboratory demonstrated that specific chymase inhibition markedly reduces the synthesis of ET-1 from exogenous Big-ET-1 in the mouse model in vivo (Simard et al., 2009).

Whereas a single human chymase isoform has been reported, several have been identified in the mouse, each with a distinct activity (Pejler et al., 2010). Among those isoforms, studies on the role of chymase in the synthesis of Ang-II suggest that mMCP-4 is the murine isoform having the most similar proteolytic activity to that of human chymase (Caughey, 2007; Andersson et al., 2008; D’Orléans-Juste et al., 2008). Whether mMCP-4 is also involved in the generation of ET-1 from its precursor Big-ET-1 has yet to be determined.

Therefore, using mice genetically deficient for mMCP-4 (mMCP-4(-/-)) (Tchougounova et al., 2003) as well as the specific chymase inhibitor TY-65149 (Koide et al., 2003; Palaniyandi et al., 2007), we studied the role of this chymase isoform in the biological activity of Big-ET-1 in vitro and in vivo. Our results suggest a pivotal role for mMCP-4 in the cardiovascular properties of Big-ET-1.
Methods

See supplementary material online for additional information

Animals

C57BL/6J mice were purchased from Charles River (Montréal, QC, Canada) and housed in our facilities. Genitor mMCP-4(-/-) mice (Tchougounova et al., 2003) were bred in our facilities and their genotype was confirmed by PCR (See supplemental data). All animals were kept at constant room temperature (23 °C) 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 Université de Sherbrooke in respect of 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 male mice under general anaesthesia, with a mixture of ketamine/xylazine (87/13 mg/kg, intra-muscular) (Vetalar©, Bioniche, Belleville, ON, CAN and Xylamax©, Bimeda, Cambridge, ON, CAN). Maintenance anaesthesia was performed with a third of the initial dose, every 30 minutes. Complete anaesthesia was assumed when no withdrawing reflex was found during a pressure on any paw of the mouse. After procedure completion and prior to tissue sampling, anaesthetized mice were sacrificed by cervical dislocation.

RNA extraction and RT-qPCR

Pulmonary, cardiac, aortic and renal tissues from C57BL/6J and mMCP-4(-/-) mice were homogenized using a tissue dispersing apparatus ULTRA-TURRAX T8 (IKA Works, Wilmington, NC). The purification of total RNA was carried out on RNeasy columns for fibrous
tissues (Quiagen, Toronto, ON). The protocol was performed as recommended by the manufacturer except for DNAse treatment. Quantitative PCR for actin, ECE-1a, NEP, mMCP-1, carboxypeptidase-A1 (CPA1) was performed by monitoring in real time increase in fluorescence of SYBR Green incorporation (Perfecta SYBR Green SuperMix, low ROX (Quanta Biosciences, Gaithersburg, MD, USA)) using the MX3000P Multiplex Quantitative PCR System (Stratagene). Levels of actin were constant between organs of WT and mMCP4(-/-) mice and used as internal controls for normalisation. Fold changes from the WT to mMCP-4(-/-) is determined using the \(2^{-\Delta\Delta CT}\) method (Pfaffl, 2001).

Specific chymotrypsin-like activity in vitro

WT and mMCP-4(-/-) mice were sacrificed and the lungs, left cardiac ventricle, aorta and kidneys were homogenized, centrifuged and the supernatants were collected for the assay. Using the non-fluorescent substrate Suc-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (AMC) (Peptide Institute, Osaka, Japan), fluorescence AMC-forming activity, as chymotrypsin-like activity, was then measured with a fluorescence spectrophotometer (\(\lambda_{ex}: 370\) nm; \(\lambda_{em}: 460\) nm) (Molecular Devices, Sunnyvale, CA). Some experiments were performed with pre-treatment of the soluble extracts with the specific chymase inhibitor TY-41569 (10 µM) (graciously provided by Toa Eiyo Ltd, Osaka, Japan).

In vitro conversion of Big-ET-1 to ET-1 (1-31)

Soluble fractions from the lungs, left cardiac ventricle, aorta and kidneys were collected from WT and mMCP-4(-/-) mice as described above.
The soluble fractions were incubated with Big-ET-1 (7.5 µM), either with vehicle or TY-51469 (10 µM), after which samples were filtered through a 30K Amicon centrifugal filter unit (Millipore Corporation, Billireca, MA, USA) for High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) analysis. Filtrates were collected and purified by reversed phase HPLC (1100 series) with a Zorbax C-18 analytical column (Agilent Technologies, Santa Clara, CA). Quantification was assessed with absorbance at a wavelength of 214 nm and 30 sec fractions at the Big-ET-1 and ET-1 (1-31) elution peaks were collected for MALDI mass spectrometry analysis. The MALDI-Time of Flight (TOF) was performed on a ProteinChip system (Bio-Rad Laboratories Inc, Mississauga, ON, Canada) using a gold plate as support and α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich, St-Louis, MO, USA) as the matrix.

Telemetric hemodynamic recording in conscious mice

Telemetry probe implantation was achieved in accordance to Carlson and Wyss (2000) and Butz and Davisson (2001). Briefly, anaesthetized WT and mMCP-4(-/-) mice were implanted with a catheter-tipped transmitter (TA11PA-C20, Data Sciences International, St-Paul, MN) into the aortic arch. The transmitter was placed subcutaneously along the right flank of the animal. A buprenorphine protocol of 0.1 mg/kg at every 9 h for 24 h post-operation was conducted to control surgical pain. A 14-day recovery period was then allowed to the mice before recording of hemodynamic data. The mice were then monitored at 15 min intervals for 24 h, via Acquisition Dataquest 2.3 (Data Sciences International).
Hemodynamic recording *in vivo*

Anaesthetized WT and mMCP-4(-/-) mice were cannulated *via* the left jugular vein (for intra-venous (i.v.) administration of pharmacological agents) and the right carotid artery (for hemodynamic recording and blood sampling) with a 10-gauge polyethylene catheter.

The right carotid artery catheter was then connected to a Blood Pressure Analyzer 200A (Micro-Med, Tustin, CA) for arterial pressure measurements. The mice were allowed a 15 min stabilization period before the intra-venous injection of Big-ET-1, ET-1 (1-31) or ET-1 (Peptide Institute, Osaka, Japan) at doses ranging from 0.001 nmol/kg to 10 nmol/kg. Data was then collected for 20 min before mice were sacrificed.

In another series of experiments, specific inhibitors of the ECE (CGS 35066; 0.1 mg/kg i.v.) (Tocris Bioscience, Bristol, UK), chymase (TY-51469; 20 mg/kg i.v.) or NEP (thiorphan; 2.5 mg/kg i.v.) (Sigma-Aldrich, Saint-Louis, MI, USA) were administered to the mice 20 min prior the injection of Big-ET-1 or ET-1 (1-31) (1 nmol/kg).

Measurement of plasmatic immunoreactive (IR)-ET-1 (1-31) and IR-ET-1 following Big-ET-1 administration *in vivo*

WT and mMCP-4(-/-) mice were cannulated as described above. After a stabilization period, Big-ET-1 (1 nmol/kg) or ET-1 (1-31) (0.1 nmol/kg) was administered. A pre-treatment of WT mice with CGS 30566 (0.1 mg/kg, i.v.) was also performed 20 minutes prior to Big ET-1 (1 nmol/kg) administration. After a 1 min time lapse, blood was collected and plasma was prepared by centrifugation.

The plasma samples were purified by HPLC as described above. As the concentrations are not high enough for direct HPLC detection, high concentration pure standards were used for
each experiment to select the appropriate elution fractions for subsequent Enzyme Immuno
Assay (EIA) dosages.

The collected fractions were then dried overnight in a vacuum concentrator and the measuring of immunoreactive-ET-1 (IR-ET-1) and IR-ET-1 (1-31) was made using EIA kits from Immuno-Biological Laboratories Co (Fujioka, Japan) according to the manufacturer’s instructions.

Measurement of endogenous tissue IR-ET-1

The lungs, heart, aorta and kidneys from WT and mMCP-4(-/-) mice were collected, homogenized, then purified on a DSC-18 solid phase extraction column (Supelco, Bellefonte, PA, USA) and eluted with acetonitrile. The IR-ET-1 was measured by EIA as described above.

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Statistical significance was reached when the value of $p$ was below 0.05, and based on the Student’s t-test performed with the GraphPad Instat 3.0 software.
Results

Expression of ET-1 generating enzymes in mMCP-4(-/-) mice

We evaluated the mRNA levels of endothelin-converting enzyme (ECE)-1a, nepriylyn (NEP), mouse mast cell protease (mMCP)-1 and carboxypeptidase A1 (CPA)1 in the lungs, left cardiac ventricle, aorta and kidneys of WT and mMCP-4(-/-) mice by real-time RT-PCR (Supplemental data, Table S4). The mRNA expression of ET-1 generating enzymes, ECE-1a and NEP, was not significantly different in mMCP-4(-/-) mice compared to their WT controls. The same was true for another mast cell protease with chymase activity, mMCP-1. In contrast, the mRNA expression of CPA1, a protease involved in ET-1 degradation, was elevated in mMCP-4(-/-) mice in the left cardiac ventricle (1.63 ± 0.17, p = 0.014) and the aorta (2.44 ± 0.37, p = 0.012), but not in the lungs (0.72 ± 0.66, p = 0.689) or the kidneys (1.27 ± 0.55, p = 0.644).

Reduced chymase-like activity in mMCP-4(-/-) mice tissue homogenates.

The chymase-like activity in soluble fractions of tissue homogenates derived from WT and mMCP-4(-/-) mice is shown in Figure 1. In all four organs tested, the basal chymase-like activity was significantly lower in the tissues of mMCP-4(-/-) mice than from their WT congeners. Moreover, the specific chymase inhibitor TY-51469 significantly reduced the chymase-like activity in WT mice down to the level of that found in corresponding samples from mMCP-4(-/-) mice, but had no effect on residual chymase-like activity in extracts from tissues of mMCP-4(-/-) mice.
Absence of *in vitro* conversion of Big-ET-1 to ET-1 (1-31) in mMCP-4(-/-) mice

Figure 2 shows a typical experiment of the *in vitro* conversion of Big-ET-1 to ET-1 (1-31) in soluble fractions from lung homogenates derived from WT (with either vehicle or TY-51469) and mMCP-4(-/-) mice. The result of the conversion was purified by HPLC, and the Big ET-1 and ET-1 (1-31) peaks were isolated and verified by MS. Figure 2 shows that conversion of Big ET-1 to ET-1 (1-31) occurred in homogenates from WT lungs, but not homogenates treated with TY-51469 or from mMCP-4(-/-) mice. Supplemental Figure S6 shows the HPLC traces of these experiments. Similar results were obtained from the left cardiac ventricle, the aorta and the kidneys (data not shown).

Figure 3 shows the HPLC quantification of the conversion in the lung, left heart ventricle, aorta and kidney homogenates. In soluble fractions from all 4 organs, conversion of Big-ET-1 to ET-1 (1-31) occurred in tissues from WT mice but not in tissues from WT mice pre-treated with TY-51469 or in tissues derived from mMCP-4(-/-) mice. Big-ET-1 levels were similar in all conditions tested, indicating that the non-specific degradation of Big-ET-1 in the soluble fraction of organs was not significantly affected by the absence of chymase activity in our experimental settings.

No difference in basal hemodynamic parameters in WT and mMCP-4(-/-) mice

Prior to drug injection, the basal hemodynamic parameters were monitored in all cannulated mice. There was no difference in mean arterial pressure or heart rate between anaesthetized WT and mMCP-4(-/-) mice (data not shown). Furthermore, the radio-telemetry experiment revealed that the mean arterial pressure was similar in both groups (103.2 ± 0.6 mmHg vs. 108.7 ± 4.9 mmHg). The mean, systolic and diastolic arterial pressures, along with
the arterial pulse pressure and the heart rate were similar in both groups and are reported in Supplementary data, Figure S5.

Reduced potency of Big-ET-1 in mMCP-4(-/-) mice.

We compared the \textit{in vivo} dose-response curves for mean arterial pressure increase following the i.v. administration of Big-ET-1, ET-1 (1-31) or ET-1 in WT and mMCP-4(-/-) mice. ET-1 (1-31) and ET-1 elicited similar responses in WT and mMCP-4(-/-) mice at each dose used (Figure 4(B) and 4(C)). However, at doses of 0.1, 0.5 and 1.0 nmol/kg, the pressor response to Big-ET-1 was significantly reduced in mMCP-4(-/-) mice when compared to WT mice (Figure 4(A)).

The residual response to Big-ET-1 is insensitive to chymase and NEP inhibition in mMCP-4(-/-) mice

Figure 5 shows the increase of mean arterial pressure induced over time by Big-ET-1 or ET-1 (1-31) (1 nmol/kg) in WT and mMCP-4(-/-) mice following systemic administration of either vehicle, CGS 35066, TY-51469 or thiorphan. The chymase inhibition by TY-51469 and the NEP inhibition by thiorphan effectively blunted the response to Big-ET-1 in WT mice but were ineffective in mMCP-4(-/-) mice, while ECE inhibition by CGS 35066 reduced the pressor response to Big ET-1 by 45 % in WT mice and abolished the residual response to the precursor in mMCP-4(-/-) mice. In contrast, TY-51469 did not affect the response to ET-1 (1-31) in WT or mMCP-4(-/-) mice, and thiorphan blocked the pressor response to ET-1 (1-31) in both types of mice.
Reduced *in vivo* generation of IR-ET-1 (1-31) and IR-ET-1 in mMCP-4(-/-) mice after Big-ET-1 administration.

Figure 6 shows the plasma levels of immunoreactive (IR) ET-1 and ET-1 (1-31) following i.v. administration of Big-ET-1 (1 nmol/kg) or ET-1 (1-31) (0.1 nmol/kg). Significantly lower levels of IR-ET-1 (1-31) were detected in plasma of mMCP-4(-/-) mice compared to their WT controls following Big-ET-1 administration. However, no difference in the plasma levels of IR-ET-1 (1-31) was detected following administration of ET-1 (1-31). Furthermore, a similar reduction was observed in the plasma levels of IR-ET-1 in mMCP-4(-/-) mice compared to their WT congeners. ECE inhibition reduced the plasma levels of IR-ET-1 by more than 40% following Big ET-1 administration in WT mice (without CGS 35066: 833.9 ± 91.9 fmol/ml; in presence of CGS 35066: 434.5 ± 46.5 fmol/ml, p < 0.05, n = 5-7 experiments) but not in mMCP-4(-/-) mice (468.8 ± 73.9 fmol/ml, n = 6).

Reduced endogenous IR-ET-1 levels in the lungs of mMCP-4(-/-) mice

Finally, Figure 7 shows tissue IR-ET-1 levels in soluble fractions derived from lungs, left cardiac ventricle, aorta and kidneys of WT or mMCP-4(-/-) mice. No significant differences in endogenous IR-ET-1 levels were observed in the left cardiac ventricle, the aorta or the kidneys. However, significant reductions in IR-ET-1 levels were observed in lungs of mMCP-4(-/-) mice when compared to lungs from WT controls. Furthermore, no significant differences were detected in baseline plasma IR-ET-1 levels between WT and mMCP-4(-/-) mice (5.2 ± 0.7 fmol/ml, n = 7 vs. 6.8 ± 1.8 fmol/ml, n = 8).
Discussion

The present study demonstrates a pivotal role for mMCP-4 in the pressor properties of Big-ET-1, in the conversion of the latter to the intermediate peptide ET-1 (1-31) \textit{in vitro} and \textit{in vivo} as well as in the pulmonary production of endogenous ET-1.

These results suggest that in addition to the endogenous ECE (McMahon et al., 1991), mouse mast cell chymase (mMCP-4) significantly contributes to the overall production of the potent pressor peptide ET-1 in the mouse model. mMCP-4(-/-) mice, when compared to WT controls, show a 50% reduction in the pressor response, as well as a marked decrease in plasma levels of both ET-1 (1-31) and ET-1, following systemic Big-ET-1 administration. Yet, in accordance with Groschwitz and colleagues (2009), we show that the basal blood pressure does not differ between conscious WT and mMCP-4(-/-) mice. Accordingly, the baseline plasma ET-1 levels did not vary between WT and mMCP-4(-/-). In addition, the specific inhibition of chymase (with TY-51469) and of NEP 24.11 (with thiorphan), reduced the pressor response to Big-ET-1 in WT mice but did not affect the residual response in mMCP-4(-/-) mice. Specific ECE inhibition with CGS 35066 reduced the pressor response to Big ET-1 in a similar fashion in both groups and further inhibited the residual response to the 38 amino acid precursor in mMCP-4(-/-) mice, while both groups responded equally to ET-1 (1-31) administration. Our group previously showed the dual contribution of both chymase and NEP in the \textit{in vivo} conversion of Big-ET-1 to ET-1 in WT mice (Simard et al., 2009).

In the present study we also demonstrate for the first time that the genetic interruption of the mMCP-4 gene is sufficient to reduce the dynamic production of ET-1 (1-31) and ET-1 following systemic administration of Big-ET-1, and that ECE specific blockade reduced the plasma level increase of ET-1 by 50% in WT mice but could not further reduce ET-1 levels in...
mMCP-4(-/-) mice. These results are concordant with previously reported data from our laboratory showing that a chymase inhibitor, Suc-Val-Pro-Phe\textsuperscript{P}(O\textsuperscript{Ph})\textsubscript{2}, inhibited by over 60 % the pressor response of Big ET-1 and reduced the plasma level increases of ET-1 by close to 90 % in WT mice in vivo (Simard et al., 2009). While this does not prove conclusively of a more important role of chymase vs ECE in ET-1 biosynthesis, as plasma ET-1 is mostly a spillover from endothelial basolateral metabolism, it still highlights an important role for chymase in the processing of Big ET-1 in the mouse model. One cannot exclude, in addition, adaptation mechanisms afforded by the genetic deletion of the mMCP-4 gene in the mouse model.

Pharmacological inhibition of chymase in WT mice with systemically administered Suc-Val-Pro-Phe\textsuperscript{P}(O\textsuperscript{Ph})\textsubscript{2} or in the present study with TY-51469, or via the genetic deletion of mMCP-4, reduced the pressor responses to Big ET-1 to the same extent. In addition, we also show that the genetic deletion of mMCP-4 reduced the increase of plasmatic ET-1 by more than 60 % following the systemic administration of Big ET-1. We therefore suggest that the residual ET-1 plasma concentrations measured in mMCP-4(-/-) mice following Big-ET-1 administration represents a spillover produced by other enzymes with no currently identified significance in the overall cardiovascular effects of Big-ET-1 in the mouse in vivo. For example, matrix metalloproteinase-2 (MMP-2) can also cleave Big-ET-1 to generate ET-1 (1-32) (Fernandez-Patron et al., 1999). Whether the MMP-2-generated ET-1 (1-32) requires further C-terminal amino acid hydrolysis to elicit pharmacological effects remains to be investigated. Thus, pathways other than mMCP-4 may be involved in ET-1 (1-31) generation in vivo, but it is yet to be defined whether they have any significant role in the endothelin system in the mouse under normal conditions.
In addition, an HPLC/MALDI-MS approach demonstrated that the soluble fraction of either lung, cardiac, aortic or renal tissue extracts derived from mMCP-4(-/-) mice do not have, in contrast to those from WT mice, the capacity to generate ET-1 (1-31) from Big-ET-1. It is also of interest that in this series of experiments, the specific chymase inhibitor TY-51469 (Koide et al., 2003; Palaniyandi et al., 2007) abolished the ET-1 (1-31) producing capacity of soluble fractions of several organs (lungs, left cardiac ventricle, aorta, kidney) derived from WT mice. These data strongly argue in favour of a pivotal role for mMCP-4 in the in vitro production of ET-1 (1-31) in the lungs, left cardiac ventricle, aorta and kidney, all organs with significant ET-1 and chymase activity (Thorin and Clozel, 2010; Takai et al., 2011). Noteworthy, no conversion to mature ET-1 was detected by either HPLC or MALDI-MS in any of the samples tested. This lack of processing of either Big-ET-1 or ET-1 (1-31) to ET-1 in these experimental settings can be explained by the fact that both ECE and NEP, unlike mMCP-4, are membrane bound entities (McMahon et al., 1991) and therefore should be found in insignificant quantities in soluble fractions. The loss of Big-ET-1 conversion capacity of soluble extracts from vascular and non-vascular tissues of mMCP-4(-/-) mice is correlated with the absence of chymase inhibitor sensitive hydrolysis of the fluorogenic substrate Suc-Leu-Leu-Val-Tyr-AMC, in the same preparations. We have previously shown that soluble extracts from aorta, left ventricle and lungs of WT mice hydrolyze the fluorogenic peptide via a chymase-sensitive but not an ECE sensitive pathway (McMahon et al., 1991; Simard et al., 2009).

Finally, the total pulmonary content of endogenous ET-1 is markedly reduced in mMCP-4(-/-) mice when compared to WT controls. It is well established that the pulmonary system accounts for 40% of all vascular endothelial cells, which are the main source of vascular ET-1 (Milnor, 1989). We therefore suggest that, in the NEP-rich pulmonary system (Baraniuk et al.,
1995), mMCP-4-dependent synthesis of ET-1 accounts for a significant part of the overall endothelin production in the mouse. This particular state of events may explain the relative discrepancy of these results with the mMCP-4/ECE mRNA ratio between the lungs, heart and aorta, where our previous study showed a smaller mMCP-4/ECE ratio in the lungs compared to the heart and the aorta (Simard et al., 2009).

In agreement with Yanagisawa and colleagues, who suggested that proteases other than ECE may account for the genesis of ET-1 in the mouse (Yanagisawa et al., 2000), our data advocates for a pivotal contribution of the mMCP-4 in the non-ECE dependent tissue production of ET-1 in the murine model. Our data are also in line with the finding that chymostatin is more efficient than phosphoramidon in blocking the extracellular conversion of Big-ET-1 to ET-1 in perfused rat lung (Wypij et al., 1992). As such, the pharmacological inhibition of chymase might provide an interesting alternative to ECE inhibitors and ET receptors antagonists. It would also have the added beneficial effects of inhibiting both ET-1 and angiotensin-II production directly at mast cell-infiltrated sites. The ET-1 and Ang-II systems are complimentary and potent inducers of each other and both play crucial roles in a large number of biological systems, such as the vasculature, heart, lungs and the kidneys (Montanari et al., 2003; Davenport and Maguire, 2006; Paul et al., 2006). ET-1 and Ang-II can also induce the expression of fibrogenic factors such as TGF-β1 (Leask, 2010) and MMP-9 (Rouet-Benzineb et al., 2000; Ergul et al., 2003), which are themselves enzymatically activated by chymase (Takai et al., 2010). ET-1 can itself induce mast cell degranulation, thereby releasing chymase in the interstitium in a positive feedback loop (Walsh et al., 2009). Hence, chymase inhibition could have a tremendous effect on the ET-1 and Ang-II systems and their activities.
In vitro studies show that human chymase cleaves Big ET-1 to ET-1 (1-31) (Hanson et al., 1997; Nakano et al., 1997) but there is no data regarding the relevance of chymase-dependent synthesis of ET-1 in humans. However, recent studies show the beneficial impact of chymase inhibitors, or the genetic deletion of mMCP-4, in a number of animal models of disease (Jin et al., 2003; Takai et al., 2003; Tsunemi et al., 2004; Sun et al., 2009; Pejler et al., 2010), suggesting that study of chymase-dependent synthesis of ET-1 in human physiopathology warrants investigation. Finally, it is of interest that, unlike the genetic deletion of the ECE (Yanagisawa et al., 2000), that of the mMCP-4 gene is not as deleterious as mMCP-4(-/-) mice breed and develop normally, with only minor intestinal anomalies (Tchougounova et al., 2003; Groschwitz et al., 2009).

In conclusion, our study demonstrates that the serine protease mMCP-4 is importantly involved in the conversion and thus biological activity of Big-ET-1 in the mouse. In cardiovascular diseases, in which inflammatory processes and mast cell degranulation occur, chymase inhibitors may ultimately lead to the reduction of intramural production of not only angiotensin-II but of endothelin-1 as well and thus reduce many of the downstream pathological consequences.
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Authorship Contributions

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 Contributed new reagents or analytical tools: Pejler, Gurish, Takai, D’Orléans-Juste.

Performed data analysis: Houde, Labonté, D’Orléans-Juste

Wrote or contributed to the writing of the manuscript: Houde, Labonté, Pejler, Gurish, Takai, D’Orléans-Juste.
References


Milnor WR (1989) *Hemodynamics*. Williams & Wilkins, Baltimore. MD.


Footnotes

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Legends for figures

Figure 1: Chymase-like enzymatic activity in the soluble fraction of tissue homogenates from WT or mMCP-4(-/-) mice, treated with either vehicle (PBS) or a specific chymase inhibitor (TY-51469). ***: p < 0.001. n = 6.

Figure 2: In vitro conversion of Big-ET-1 to ET-1 (1-31) in soluble fractions from lung homogenates, with HPLC-MS identification of conversion results from WT (A), mMCP-4(-/-) (B) and WT tissues treated with TY-51469 (C).

Figure 3: Quantification of the in vitro conversion of Big ET-1 to ET-1 (1-31) in homogenates from the lungs (A), left heart ventricle (B), aorta (C) and kidneys (D), from WT (treated with vehicle or TY-51469) or mMCP-4(-/-) mice, using HPLC area under the curve arbitrary units. n=5.

Figure 4: Maximal variation in mean arterial pressure following the i.v. administration of (A) Big-ET-1, (B) ET-1 (1-31) and (C) ET-1 in WT and mMCP-4(-/-) mice. *: p < 0.05, **: p < 0.01, n = 6.

Figure 5: Time course of the variation of the mean arterial pressure in response to i.v. administration of Big ET-1 (1 nmol/kg) (in A: WT mice; in B: mMCP-4(-/-) mice) or ET-1 (1-31) (1 nmol/kg) (in C: WT mice; in D: mMCP-4(-/-) mice) in mice pre-treated with either a
chymase (TY-51469) or a NEP (thiorphan) inhibitor. *: p < 0.05 vs PBS; #: p < 0.05 Thiorphan vs. PBS. n = 5-9.

**Figure 6:** *In vivo* conversion of Big-ET-1 into ET-1 (1-31) and ET-1 in WT and mMCP-4(-/-) mice. Quantification of the plasma levels of immunoreactive endothelin-1 (1-31) (A) and IR-ET-1 (C), following the i.v. administration of Big-ET-1 1.0 nmol/kg. In (B), the plasma levels of IR-ET-1 (1-31) were determined following the i.v. administration of ET-1 (1-31) (0.1 nmol/kg). *: p < 0.05, **: p < 0.01. n=5-6.

**Figure 7:** Endogenous tissue levels of IR-ET-1 in the lungs, left cardiac ventricle, the aorta and the kidneys. *: p < 0.05. n = 6-7.
PIVOTAL ROLE OF MOUSE MAST CELL PROTEASE 4 IN THE CONVERSION AND PRESSOR PROPERTIES OF BIG-ENDOTHELIN-1

Martin Houde, Marc-David Jamain, Julie Labonté, Louisane Desbiens, Gunnar Pejler, Michael Gurish, Shinji Takai, Pedro D'Orléans-Juste.

Journal of Pharmacology and Experimental Therapeutics

Supplementary Figures and Tables
Supplementary Table 1: Primers for the genotyping of mMCP-4(-/-) mice.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Amplicon length (bp)</th>
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<td>mMCP4 F</td>
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Supplementary Table 1: Primers for the genotyping of mMCP-4(-/-) mice.
Supplementary Table 2: Primers used for the quantitative RT-PCR. The accession number and the gene bank identification are in the National Center for Biotechnology Information (NCBI) Gene Bank.

<table>
<thead>
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<th>Targeted mRNA</th>
<th>Accession number</th>
<th>Gene bank identification</th>
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<td>Reverse: 5'-GCT CCG GGC ATC CCA AAG GC-3'</td>
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Supplementary Figure 1: Genotyping of WT and mMCP-4(-/-) mice for the mMCP-4 gene. The top row indicates from which genotype the samples tested come from (MPM stands for the ladder).
Supplementary Table 3: Quantification of the Neutral Endopeptidase 24.11 (NEP), Endothelin-Converting-Enzyme-1a (ECE-1a), mouse Mast Cell Protease 1 (mMCP-1) and Carboxypeptidase A1 (CPA) mRNA levels by RT-qPCR in the lungs, left cardiac ventricle, aorta and kidneys. Versus their WT congeners, no changes were detected in the expression of the endothelin-1 generating enzymes NEP and ECE-1a in the organs of mMCP-4(-/-) mice. No changes were detected either for another mouse β-chymase, mMCP-1. However, a statistically significant increase in the mRNA expression in mMCP-4(-/-) mice in another mast cell protease, CPA, which is responsible for the degradation of ET-1, was detected in the left cardiac ventricle and the aorta. N = 8.

<table>
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<tr>
<th>Gene</th>
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<th>Aorta</th>
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<td>NEP</td>
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<td>0.771</td>
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<td>CPA</td>
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Supplementary Figure 2: Basal hemodynamic parameters from conscious, free-moving WT and mMCP-4(-/-) mice. MAP: mean arterial pressure, SAP: systolic arterial pressure, DAP: diastolic arterial pressure, PP: pulse pressure, HR: heart rate. n=9-10.

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Supplementary Figure 3: HPLC traces of the *in vitro* conversion of Big-ET-1 into ET-1 (1-31) in the soluble extracts from the lungs of WT (A) and mMCP-4(-/-) mice (C). In (B), the samples were treated with the chymase inhibitor TY-51469. The traces are representative of n=5. They are also representative of data obtained from the left cardiac ventricle, aorta and kidneys (data not shown).
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Supplementary Methods
DNA extraction and genotyping

The end tip of the tail of WT and mMCP-4 KO mice was collected and the DNA was isolated and purified using the E.Z.N.A Tissue DNA Kit (Omega Bio-Tek Inc, Norcross, GA, USA) according to the manufacturer’s instructions. The genotype of the mice was then confirmed by PCR using increasing annealing temperatures in the following protocol: 2 min at 94°C, followed by 40 cycles of denaturation for 2 min at 94°C, annealing for 2 min 30 sec at 61 °C (+ 0.2 °C for each cycle), and extension for 6 min at 72°C, ending with a final 10 min at 72°C. The PCR contained 0.4 mM of dNTPs (Fermentas Canada Inc, Burlington, ON, Canada), 04 mM of MgCl₂, 1µM of each primer, 4 µl of the isolated DNA and 0.04 U/µl of recombinant Taq DNA polymerase (Life Technologies Inc, Grand Island, NY, USA). The primers used are presented in Table S1.

RNA extraction and RT-qPCR

Pulmonary, cardiac, aortic and renal tissues from C57BL/6J and mMCP-4 KO mice were homogenized using a tissue dispersing apparatus ULTRA-TURRAX T8 (IKA Works, Wilmington, NC). The purification of total RNA was carried out on RNeasy columns for fibrous tissues (Quiagen, Toronto, ON). The protocol was performed as recommended by the manufacturer except for DNAse treatment. DNASE 1 20 U/ml (Omega Bio-Tek, Norcross, GA) was applied directly on RNeasy spin column membranes, and placed on the benchtop for 30 min. 1 µg of RNA were reverse transcribed using 200 U of SuperScript III in addition of oligo(dT)$_{12-18}$ primer (Invitrogen, Burlington, ON) at 42 °C for 60 min. Each PCR contained 2 µl (هائي 100 ng) of cDNA and 300 nM primers in 25 µl of reactive mixture with 12.5 µl of Perfecta SYBR Green SuperMix, low ROX (Quanta Biosciences, Gaithersburg, MD). Quantitative PCR for actin, ECE-
1a, NEP, mMCP-1, carboxypeptidase-A1 was performed by monitoring in real time, measuring
the fluorescence increase of SYBR Green (Perfecta SYBR Green SuperMix, low ROX (Quanta
Biosciences, Gaithersburg, MD, USA) using the MX3000P Multiplex Quantitative PCR System
(Stratagene). Levels of actin were constant between organs of WT and mMCP4 KO mice and
used as internal controls for normalisation. The cycle profile was: 2 min at 95°C, followed by 40
cycles of denaturation for 30 s at 95°C, annealing for 1 min at 60°C, and extension for 30 s at
72°C. The primers used are detailed in Table S2.

The mean of the cycle threshold values ($C_T$) was used for subsequent analysis. Data were
analyzed according to fold changes for relative quantification. $C_T$ values of candidate mRNAs
were normalized by subtracting the Ct of reference gene. The resulting $\Delta C_T$ values provided
relative gene expression levels for each sample. A calibrator sample was defined by calculating a
$C_T$ (i.e. arithmetic mean of control group $C_T$) and was normalized by the selected combination of
control genes to obtain the $\Delta C_{Tcalibrator}$.

Finally, $\Delta \Delta C_T$ values were calculated ($C_T$ target – Ct actin) mMCP4 KO - (Ct target-Ct
actin) WT and converted into fold changes from WT to mMCP-4 KO using the $2^{-\Delta \Delta C_T}$ method.

**Hemodynamic recording in vivo**

Anaesthetized WT and mMCP-4(-/-) mice were cannulated via the left jugular vein (for
substance intra-venous (i.v.) administration) and the right carotid artery (for hemodynamic
recording and blood sampling) with a polyethylene catheter, gauge 10.

The right carotid artery catheter was then connected to a Blood Pressure Analyzer 200A
(Micro-Med, Tustin, CA) for mean arterial pressure measurements. The mice were allowed a 15
min stabilization period before the intra-venous injection of either Big-ET-1, ET-1 (1-31) or ET-1
(Peptide Institute, Osaka, Japan) with doses ranging from 0.001 nmol/kg to 10 nmol/kg. Data was then collected for 20 min before the mice were sacrificed.

In another series of experiments, the specific ECE inhibitor CGS 35066 (0.1 mg/kg i.v.), the specific chymase inhibitor TY-51469 (40 mg/kg i.v.) (Toa Eiyo, Osaka, Japan) or the specific NEP inhibitor thiorphan (3 mg/kg i.v.) (Sigma-Aldrich, Saint-Louis, MI, USA) were administered to the mice 20 min prior the injection of Big-ET-1 or ET-1 (1-31) (1 nmol/kg), after the stabilization period.

**Hemodynamic recording in conscious mice**

Telemetry probes were opened 24 hours and offset recorded before use. The mice were anaesthetized with ketamine/xylazine (87/13 mg/kg, i.m). A buprenorphine protocol of 0.1 mg/kg at every 9 h for 24 h post-operation was conducted to control surgical pain. A skin incision of about 15-25 mm was done along the shaved and disinfected ventral neck area and the common left carotid artery was isolated and ligated to stop blood circulation toward the head. Blood circulation from the heart was temporary occluded and the catheter-tipped transmitter (TA11PA-C20, Data Science International, St-Paul, MN) was inserted into the artery through a little incision to be positioned in the aortic arch. The catheter was then secured with a thread, the transmitter placed subcutaneously along the right flank of the mouse through a pocket made with blunt scissors and the incision was sutured with absorbable thread. The whole procedure was performed under sterile conditions and the animals were kept under a warm lamp until full recovery from anaesthesia. A minimum of ten days recovery period was allowed before recording of data and the beginning of either normal or high salt diets. After each experimentation period and euthanasia of the mice, the location of the catheter tip into the aortic arch was validated by autopsy.
Specific chymotrypsin-like activity \textit{in vitro}

Male WT and mMCP-4(-/-) mice were sacrificed and the lungs, left cardiac ventricle, aorta and kidneys were taken and frozen at -80 °C. On the day of the experiment, the organs were homogenized in a potassium phosphate buffer (0.25 g/l, pH 8.0, 4 °C) with a tissue grinder. The homogenates were then centrifuged (14 000 g, 20 minutes, 4 °C) and the supernatants were collected for use in the assay. Using the non-fluorescent substrate Suc-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (AMC) (Peptide Institute, Osaka, Japan) at 10 µM, their AMC-forming activity (fluorescence), as chymotrypsin-like activity, was then measured. Some experiments were performed with pre-treatment of the soluble extracts with a specific chymase inhibitor, TY-41569 (10 µM). The chymase-like activity of the homogenates was measured for 1 hour at 37 °C with a fluorescence spectrophotometer (ex: 370 nm; em: 460 nm) (Molecular Devices, Sunnyvale, CA).

1.6 \textit{In vitro} conversion of Big-ET-1 to ET-1 (1-31)

Soluble fractions from the lungs, left cardiac ventricle, aorta and kidneys were collected from WT and mMCP-4 KO mice as described above.

The soluble fractions were incubated with Big-ET-1 7.5 µM at 37 °C for 20 min, either with vehicle or TY-51469 10 µM, after which they were diluted in a PBS-acetonitrile (80:20) solution and filtered through a 30K Amicon centrifugal filter unit (Millipore Corporation, Billireca, MA, USA). The filtrate was collected and purified by reverse phase HPLC (1100 series) with a Zorbax C-18 analytical column (Agilent Technologies, Santa Clara, CA). The flow was 1 ml/min, the buffer was trifluoroacetic acid (0.1%), the acetonitrile gradient from 28% to 40% over 35 min. Quantification was performed with absorbance at a wavelength of 214 nm and
30 sec fractions at the Big-ET-1 and ET-1 (1-31) peak times were collected for MALDI mass spectrometry. The MALDI-TOF was performed on a ProteinChip SELDI system (Bio-Rad Laboratories Inc, Mississauga, ON, Canada) using a gold plate as support and -cyano-4-hydroxycinnamic acid (Sigma-Aldrich, St-Louis, MO, USA) as the matrix.

**Measurement of plasmatic IR-ET-1 (1-31) and IR-ET-1 following Big-ET-1 administration in vivo**

WT and mMCP-4(-/-) mice were cannulated as described above. After the stabilization period, Big-ET-1 (1 nmol/kg) or ET-1 (1-31) (0.1 nmol/kg) was administered. After a 1 min time lapse, 100 µl of blood was collected in 10 µl sodium citrate 3.5 %. Plasma was prepared by centrifugation for 1 min at 20 000 g, after which 50 µl of plasma was collected then frozen at -80 C until further use.

On the day of the extraction, the plasma samples were thawed and purified by HPLC as described above. As the concentrations are not high enough for direct HPLC detection, high concentration pure standards were used for each experiment to select the right fractions for dosage.

The collected fractions were then dried overnight and the measuring of immunoreactive-ET-1 (IR-ET-1) and IR-ET-1 (1-31) was made using EIA kits from Immuno-Biological Laboratories Co (Fujioka, Japan) according to the manufacturer’s instructions, with the following modification: plasma from untreated mice was diluted in the EIA buffer (1:32) prior to use to help sample dissolution.

**Measurement of endogenous tissular IR-ET-1**
The lungs, heart, aorta and kidneys from WT and mMCP-4(-/-) mice were collected and frozen at -80°C until further use. On day of measurement, the samples were thawed homogenized in a chloroform-methanol solution (1:4), then centrifuged at 2500 g. The aqueous phase was then collected, acidified with three volumes of TFA 0.2%. The samples were then purified on a DSC-18 solid phase extraction column (Supelco, Bellefonte, PA, USA) and eluted with 60% ACN-0.1% TFA. The resulting samples were then dried overnight and the IR-ET-1 was measured by EIA as described above with the lungs diluted 25X compared to other tissues.