Experimental Autoimmune Encephalomyelitis Potentiates Mouse Mast Cell Protease 4–Dependent Pressor Responses to Centrally or Systemically Administered Big Endothelin-1

Louisane Desbiens, Catherine Lapointe, Louis Gendron, Marjan Gharagozloo, Laurence Vincent, Gunnar Pejler, Denis Gris, and Pedro D’Orléans-Juste

Department of Pharmacology and Physiology, Medical School, Université de Sherbrooke, Sherbrooke, Québec, Canada (L.D., L.G., M.G., L.V., D.G., P.D.’O.J.); Department of Medical Biochemistry and Microbiology, Uppsala University BMC, Uppsala, Sweden (G.P.); and Department of Anatomy, Physiology and Biochemistry, Swedish University of Agricultural Sciences, Uppsala, Sweden (G.P.)

Received December 20, 2018; accepted June 25, 2019

ABSTRACT

Multiple sclerosis is a neurodegenerative disease affecting predominantly female patients between 20 and 45 years of age. We previously reported the significant contribution of mast cell protease 4 (mMCP-4) in the synthesis of endothelin-1 (ET-1) in healthy mice and in a murine model of experimental autoimmune encephalomyelitis (EAE). In the current study, the cardiovascular effects of ET-1 and big endothelin-1 (big-ET-1) administered systemically or intrathecally were assessed in the early preclinical phase of EAE in telemetry instrumented/conscious mice. Chymase-specific enzymatic activity was also measured in the lung, brain, and mast cell extracts in vitro. Finally, the impact of EAE immunization was studied on the pulmonary and brain mRNA expression of different genes of the endothelin pathway, interleukin-33 (IL-33), and monitoring of immunoreactive tumor necrosis factor-α (TNF-α). Systemically or intrathecally administered big-ET-1 triggered increases in blood pressure in conscious mice. One week post-EAE, the pressor responses to big-ET-1 were potentiated in wild-type (WT) mice but not in mMCP-4 knockout (KO) mice. EAE triggered mMCP-4–specific activity in cerebral homogenates and peritoneal mast cells. Enhanced pulmonary, but not cerebral preproendothelin-1 and IL-33 mRNA were found in KO mice and further increased 1 week post-EAE immunization, but not in WT animals. Finally, TNF-α levels were also increased in serum from mMCP-4 KO mice, but not WT, 1 week post-EAE. Our study suggests that mMCP-4 activity is enhanced both centrally and systemically in a mouse model of EAE.

Introduction

Multiple sclerosis (MS), mostly caused by complex cellular interactions (Duffy et al., 2014; Cheng et al., 2017; Luo et al., 2017; Ponath et al., 2018), is a chronic autoimmune and degenerative disease of the central nervous system (CNS) associated with inflammation, tissue insult, and neuromotor dysfunction (Lublin, 2005; Compton and Coles, 2008; Huang et al., 2016). In MS patients, albeit vascular and endothelial integrities are unaffected (Mincu et al., 2018), several cardiovascular comorbidities such as hypertension, heart diseases, and cardio-metabolic disorders have been reported (Ewanthuk et al., 2018).

Experimental autoimmune encephalomyelitis (EAE) reproduces the major cardinal signs of neurodegeneration and associated spinal cord lesions occurring in MS (Kipp et al., 2012; Ben-Nun et al., 2014). In EAE, a significant contribution of mast cell (MC) degranulation to the etiology of the neurodegenerative disease was initially reported by Secor et al. (2000). However, this concept was later challenged since complete removal of MCs in a Kit-independent MC-deficient strain had no significant impact on the development of the disease (Feyerabend et al., 2011). Directly targeting MCs in MS thus remains an open question.

Among several pro- and anti-inflammatory factors, MCs secrete chymase, a serine protease that hydrolyzes endothelin-1 (ET-1) from its precursor, big endothelin-1 (big-ET-1), in vitro (Petrie et al., 2001; Borland et al., 2005; Simard et al., 2009). Mouse MCs express several chymases, of which mouse mast cell protease 4 (mMCP-4) is the most functionally similar to its

Received December 20, 2018; accepted June 25, 2019

ABSTRACT

Multiple sclerosis is a neurodegenerative disease affecting predominantly female patients between 20 and 45 years of age. We previously reported the significant contribution of mast cell protease 4 (mMCP-4) in the synthesis of endothelin-1 (ET-1) in healthy mice and in a murine model of experimental autoimmune encephalomyelitis (EAE). In the current study, the cardiovascular effects of ET-1 and big endothelin-1 (big-ET-1) administered systemically or intrathecally were assessed in the early preclinical phase of EAE in telemetry instrumented/conscious mice. Chymase-specific enzymatic activity was also measured in the lung, brain, and mast cell extracts in vitro. Finally, the impact of EAE immunization was studied on the pulmonary and brain mRNA expression of different genes of the endothelin pathway, interleukin-33 (IL-33), and monitoring of immunoreactive tumor necrosis factor-α (TNF-α). Systemically or intrathecally administered big-ET-1 triggered increases in blood pressure in conscious mice. One week post-EAE, the pressor responses to big-ET-1 were potentiated in wild-type (WT) mice but not in mMCP-4 knockout (KO) mice. EAE triggered mMCP-4–specific activity in cerebral homogenates and peritoneal mast cells. Enhanced pulmonary, but not cerebral preproendothelin-1 and IL-33 mRNA were found in KO mice and further increased 1 week post-EAE immunization, but not in WT animals. Finally, TNF-α levels were also increased in serum from mMCP-4 KO mice, but not WT, 1 week post-EAE. Our study suggests that mMCP-4 activity is enhanced both centrally and systemically in a mouse model of EAE.

Introduction

Multiple sclerosis (MS), mostly caused by complex cellular interactions (Duffy et al., 2014; Cheng et al., 2017; Luo et al., 2017; Ponath et al., 2018), is a chronic autoimmune and degenerative disease of the central nervous system (CNS) associated with inflammation, tissue insult, and neuromotor dysfunction (Lublin, 2005; Compton and Coles, 2008; Huang et al., 2016). In MS patients, albeit vascular and endothelial integrities are unaffected (Mincu et al., 2018), several cardiovascular comorbidities such as hypertension, heart diseases, and cardio-metabolic disorders have been reported (Ewanthuk et al., 2018).

Experimental autoimmune encephalomyelitis (EAE) reproduces the major cardinal signs of neurodegeneration and associated spinal cord lesions occurring in MS (Kipp et al., 2012; Ben-Nun et al., 2014). In EAE, a significant contribution of mast cell (MC) degranulation to the etiology of the neurodegenerative disease was initially reported by Secor et al. (2000). However, this concept was later challenged since complete removal of MCs in a Kit-independent MC-deficient strain had no significant impact on the development of the disease (Feyerabend et al., 2011). Directly targeting MCs in MS thus remains an open question.

Among several pro- and anti-inflammatory factors, MCs secrete chymase, a serine protease that hydrolyzes endothelin-1 (ET-1) from its precursor, big endothelin-1 (big-ET-1), in vitro (Petrie et al., 2001; Borland et al., 2005; Simard et al., 2009). Mouse MCs express several chymases, of which mouse mast cell protease 4 (mMCP-4) is the most functionally similar to its
human counterpart, chymase 1 (Semaan et al., 2015). Indeed, recombinant mMCP-4 efficiently converts big-ET-1 to an intermediate precursor, ET-1 (1–31), which is then readily hydrolyzed to ET-1 via the ubiquitous neutral endopeptidase 24/11 (Fecteau et al., 2005; Semaan et al., 2015).

Antagonism of endothelin A (ET_A) receptors for ET-1 reduces the severity of EAE in rats (Shin et al., 2001), whereas blocking the endothelin B (ET_B) receptors indirectly inhibits oligodendrocyte progenitor cell differentiation as well as remyelination in mice (Hammond et al., 2015). Plasma levels of ET-1 are elevated in patients with MS (Hausfild et al., 2001; Pache et al., 2003) and the use of a mixed ET_A/ET_B antagonist, bosentan, normalizes cerebral blood flow in these patients (D’haeseleer et al., 2013). On the other hand, ET-1 administered either systemically in the conscious mouse (Semaan et al., 2015) or centrally in the nonanesthetized rat (Poulat et al., 1994), triggers a trademark protracted increase in blood pressure in vivo. To our knowledge, there is no evidence in the literature indicating that cardiovascular responses are prompted by centrally administered endothelins (ETs) in the conscious mouse model.

mMCP-4 is also involved in early neuromotor disabilities associated with EAE (Desbiens et al., 2016). We have previously shown that genetic repression of mMCP-4 improved clinical signs and reduced spinal cord damage afforded by EAE in the mouse model (Desbiens et al., 2016). Furthermore, EAE triggers a significant increase in mMCP-4 mRNA levels in the CNS as well as production of cerebral immune-reactive ET-1. However, an open question remains: does EAE trigger an increase in mMCP-4 activity outside the CNS as well? MC degranulation products are mediators of the allergic response to EAE outside the CNS (Tanzola et al., 2003). In addition, the lungs have been shown to be the niche in which self-reactive T cells are activated, allowing them to enter the target tissues such as the CNS where they trigger pathogenic events associated with EAE (Odoardi et al., 2012).

Inflammation in MS is associated with a cytokine storm within the CNS (Link, 1998). With regard to the present study, interleukin-33 (IL-33) reduces neuronal damage in the EAE model by suppressing interleukin-17 and interferon γ (Jiang et al., 2012). Furthermore, repression of IL-33 increases neuronal damage in EAE mice with specific impact on immune and neural cells (Xiao et al., 2018). In addition, another cytokine, tumor necrosis factor-α (TNF-α), increases the expression of IL-33 in human epidermal keratinocytes as well as in psoriasis (Balato et al., 2014), and nonselective TNF-α blockers enhance demyelination in MS patients (van Oosten et al., 1996; Dreyer et al., 2016).

Of relevance to the present study, Piliponsky et al. (2012) have shown that TNF-α is a substrate for mMCP-4 in a mouse model of sepsis and Waern et al. (2013) showed that IL-33 is a preferred substrate for chymase in allergic airway inflammation. Thus, in conditions of chronic repression of the murine chymase, one would expect an increase in the levels of IL-33 as well as TNF-α, particularly in inflammatory settings.

The aim of the present study was to investigate, in the mouse model of MS, whether mMCP-4 activity is modulated solely in the CNS or if it can be extended to systemic circulation. Our central hypothesis is that EAE enhances mMCP-4-dependent conversion and thus biologic activity of the ET-1 precursor, big-ET-1, within and outside the central nervous system.
and SuperScript III buffer with dithiothreitol, RNaseOUT, and SuperScript III (Invitrogen). Quantitative polymerase chain reaction was performed for actin, preproendothelin-1 (prepro-ET-1), endothelin-converting enzyme (ECE) 1a, ET<sub>A</sub> and ET<sub>B</sub> receptors, and IL-33 by monitoring in real time the fluorescence increase of the SYBR Green using Perfecta SYBR Green SuperMix, Low ROX (Quanta Biosciences, Gaithersburg, MD) and the MX3000P Multi

plex Quantitative PCR System (Agilent Technologies, Santa Clara, CA). Primers (IDT, Coralville, IA) were used at final concentration of 300 nM per primer and sequences were designed as follows:

**Prepro-ET-1:** Forward 5'-GCA CAA CCG AGC ACA TTG-3';
**Prepro-ET-1:** Reverse 5'-GCC AGC ATG GAG AGT-3';
**ECE1a:** Forward 5'-GGG GGG TC CCT CTG GGG GAA-3';
**ECE1a:** Reverse 5'-CTG CAG GGA AGA AAG GGG-3';
**ET<sub>A</sub>:** Forward 5'-CAA CCA TTA GGC CAC AGA-3';
**ET<sub>A</sub>:** Reverse 5'-CAG GAA AGA CCC ATT GCC-3';
**ET<sub>B</sub>:** Forward 5'-TCA ACA CCG GAT ATA TCC AGG GCC-3';
**ET<sub>B</sub>:** Reverse 5'-GCT CTG CTG AAG TGC TGA CTA AGA-3';
**IL-33:** Forward 5'-TGA GCC GCC GTT GTG GCC GC-3';
**IL-33:** Reverse 5'-GTA CAT TGG GTT TGC TGA A-3';
**β-actin:** Forward 5'-GAT CAA GAT CAT TGC TCA TCC TGA GC-3';
**β-actin:** Reverse 5'-GCA GCT CAG TAA CAC TAG GCC TAG GC-3'.

The cycle profile was: 10 minutes at 95°C, followed by 40 cycles of denaturation for 30 seconds at 95°C, annealing for 1 minute at 55°C, and extension for 1 minute at 72°C, followed by final denaturation for 1 minute at 95°C, 30 seconds at 55°C, and 30 seconds at 95°C for prepro-ET-1, ECE1a, and IL-33. The annealing time for ET receptor genes was 30 seconds at 60°C. The fluorescence was measured after each annealing period during the 40 cycles and the complete dissociation curve was constructed between the 30-second period at 55°C and the final 30 seconds at 95°C.

Since β-actin levels were stable between healthy and immunized mice, the latter mRNA was used as the internal control for normalization and relative expressions of prepro-ET-1, ECE1a, ET<sub>A</sub> and ET<sub>B</sub> receptors, and IL-33 were calculated using the 2<sup>-ΔΔCT</sup> method.

**Hemodynamic Studies in Conscious Mice.** Mice were implanted with radio telemetry transmitters (TA11PA-C10; Data Science International, St. Paul, MN). Mice were anesthetized with ketamine/xylazine (87/13 mg/kg, i.m.). The left common carotid artery was implanted with radio telemetry transmitters (TA11PA-C10; Data Science International, St. Paul, MN). Mice were anesthetized with ketamine/xylazine (87/13 mg/kg, i.m.). The left common carotid artery was subsequently placed back in contention cages and a first dose of ET-1 and subsequently released in their respective cages. Three days later, mice were EAE induced for a period of 7 days prior to intrathecal administration of the agonist followed by a second intravenous injection done 2 days later. Recording of hemodynamic parameters (mean arterial pressure (MAP), systolic blood pressure, diastolic blood pressure, and heart rate (HR)) was initiated 5 minutes before the contention of the animal for the drug administration, to determine the basal pressure, and up to 30 minutes post agonist injections. Data points were recorded every 30 seconds with the acquisition software Dataquest ART 4.33 (DSI, St. Paul, MN). Each mouse was administered only one agonist. All data were analyzed with the Dataquest ART 4.33 analysis software. Blood pressure variations (Δ) obtained after the injection of each agonist were calculated as the difference between blood pressure increases postinjection and basal pressure recorded prior to administration of each agent.

**Measurement of Endogenous IL-33, ET-1, and TNF-α Levels.** For the measurement of TNF-α levels, frozen left-brain hemispheres or left lung lobes were weighed and homogenized in 1 ml for lung tissues or 2 ml for brain tissues of ice-cold lysis buffer (Cell Signaling Technology, Beverly, MA) supplemented with protease inhibitor cocktail (Cell Signaling Technology) using a glass-Teflon homogenizer. Tissue lysates were centrifuged at 13,000g and 4°C for 20 minutes and supernatants were transferred to new tubes. Serum was obtained by full-speed centrifugation of blood samples for 20 minutes. Serum or tissue homogenate TNF-α levels were determined using the Mouse TNF-α Quantikine ELISA Kit (R&D Systems, Minneapolis, MN), according to the manufacturer’s instructions. The concentrations of TNF-α are expressed in picograms per milliliter. On the other hand, for measurement of ET-1 and IL-33 levels the frozen left-brain hemispheres of left lung lobes were weighed and homogenized in two volumes of 500 μl of a chloroform:methanol (1:4) solution and then purified on a DSC-18 solid phase extraction column (Supelco, Bellefonte, PA) and eluted in acetoni-trile:water:trifluoroacetic acid (ACN 60%H<sub>2</sub>O 40%TF A 0.1%). The collected eluates were then speed vacuum-dried overnight before reconstitution in PBS supplemented with 1/100 mouse plasma, and then endogenous ET-1 or IL-33 was measured using the Quantikine ELISA kit from R&amp;D Systems according to the manufacturer’s instructions. The concentrations of IL-33 and ET-1 are expressed in picograms per milliliter and picograms per milligrams, respectively.

**Peritoneal Mast Cell Isolation.** MCs from the peritoneal cavity were isolated from healthy and 1 week post-EAE mice and used for measurement of chymase activity. Mice anesthetized with ketamine/xylazine (87/13 mg/kg, i.m.) were injected with 5 ml of isolation buffer (PBS pH 7.4) containing 1 mg/ml of bovine serum albumin and 0.5 mg/ml of heparin) in the peritoneal cavity. The peritoneal fluids were collected following 1-minute abdominal massage and centrifuged at 200g for 5 minutes. Pellets were suspended in 1 ml RPMI 1640 medium containing 2 mM of L-glutamine, 100 U/ml of penicillin, and 50 μg/ml of streptomycin.

**Fig. 1.** Timeline regarding the treatments for hemodynamic studies in conscious mice.
penicillin, and 1 mg/ml of bovine serum albumin, and subsequently incubated for 1 hour at 37°C in tissue culture dishes (35 x 10 mm) to sediment and let the macrophages and leukocytes adhere to the plastic surface of the dish, thus allowing purification of the MCs. RPMI 1640 medium and nonadherent cells were collected and centrifuged at 200g for 5 minutes. Pellets were suspended in 1 ml isolation buffer and cells were counted according to the Moore and James (1953) method with toluidine blue. A final centrifugation was performed at 200g for 5 minutes and pellets were suspended in PBS (pH 8) in a concentration of 10,000 MCs/μl and used as described subsequently.

**Cerebral Protein Extraction.** Chymase-containing homogenates were first extracted from the left brain hemisphere of healthy or 1 week post-EAE mice as described previously (Kakizoe et al., 2001). The brain was homogenized in 10 volumes (v/w) of 20 mM sodium phosphate buffer (pH 7.4) using a glass-Teflon homogenizer. The homogenate was then centrifuged at 18,000g for 30 minutes at 4°C. The supernatant was discarded since it contained soluble proteins that might interfere with the chymase activity. These three steps were repeated twice. Following the last centrifugation, the pellet was suspended in five volumes (v/w) of 10 mM sodium phosphate buffer (pH 7.4), 2 M KCl, and 0.1% Triton X-100. The homogenate was agitated overnight at 4°C and subsequently centrifuged at 18,000g for 30 minutes at 4°C. The resulting supernatant was kept as the final chymase extract at −80°C until used. Protein concentrations were determined by the Bradford method with γ-globulin (Sigma-Aldrich) as the standard.

**Pulmonary Protein Extraction.** Chymase-containing homogenates were extracted from the left lobes of the lung from healthy or 1 week post-EAE mice as previously described (Houde et al., 2013). Tissues were homogenized in two volumes (200 μl) of PBS (pH 7.4) using a glass-Teflon homogenizer. The homogenates were then centrifuged at 18,000g for 20 minutes at 4°C. The resulting supernatants were collected and kept at −80°C until used. Protein concentrations were determined by the Bradford method with γ-globulin as the standard.

**Measurement of Chymase Enzymatic Activity.** The brain chymase extracts were diluted to obtain a final concentration of 1 mg protein/ml and incubated for 20 minutes at 37°C, with vortex mixing every 5 minutes with cathepsin G inhibitor I (Cayman Chemical, Ann Harbor, MI) at a final concentration of 10 μM to discriminate the chymase from cathepsin G activity. Lung chymase extracts were diluted to obtain a final concentration of 1 mg protein/ml and incubated for 20 minutes at 37°C, with vortex mixing every 5 minutes. These solutions or peritoneal MC extracts (100,000 MCs) were placed in 96-well plates. The chymase activity was determined by the hydrolysis rate of 10 μM of the substrate, succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Peptide Institute inc., Osaka, Japan), at 37°C. The fluorescence of the released 7-amino-4-methylcoumarin molecule was measured with an Infinite M1000 spectrophotometer (Tecan Group Ltd., Männedorf, Swiss) with λex = 370 nm and λem = 460 nm for 15 minutes.

**Statistical Analyses.** All data are presented as mean ± S.E.M. All statistical analyses were conducted using GraphPad Prism 7 software (GraphPad Software, La Jolla, CA). Statistical significance was reached when the P value was below 0.05 and was determined using one-way ANOVA and multiple Student’s t tests.

**Results**

**EAE-Induced Potentiation of Intravenously or Intrathecally Administered Big-ET-1.** The impact of EAE immunization on basal hemodynamic parameters was investigated in telemetry-instrumented and conscious mice. First, prior to EAE treatments basal blood pressure parameters were similar in wild-type (WT) and mMCP-4 KO mice (MAP: 91.39 ± 5.27 and 92.96 ± 6.15 mm Hg; systolic blood pressure: 101.67 ± 6.19 and 102.76 ± 5.32 mm Hg; diastolic blood pressure: 81.59 ± 6.07 and 80.39 ± 5.18 mm Hg; and HR: 659.44 ± 19.34 and 677.15 ± 14.97 beats/min, respectively).

As shown in Fig. 2A, the induction of EAE did not affect the basal parameters. Figure 2B represents the typical time-course profile of the MAP and HR responses to an intravenous or intrathecal injection of ET-1 (1 fmol/kg or 0.1 pmol/mouse, respectively) in healthy WT mice. In our experiments, we did not observe any variation in HR after injection of big-ET-1 or ET-1. We suggest that this is caused by the particularly elevated basal HR in conscious mice. Another explanation would be that the doses of agonists used in the present study were too low to induce increases in HR.

Figure 3A shows the maximal increase of the MAP after intravenous administration of big-ET-1 in conscious and unrestrained mice. Intravenous administration of big-ET-1 (10 or 100 fmol/kg) prompted dose-dependent increases in MAP in healthy WT mice that were significantly potentiated in congeners 1 week post-EAE immunization (P < 0.01). In healthy mMCP-4 KO mice, we observed a significant reduction of the MAP increases when compared with WT congeners (P < 0.001) and no potentiation of the response to big-ET-1 post-EAE induction. Finally, intravenous administration of ET-1 (1 fmol/kg) prompted similar blood pressure responses in healthy or 1 week post-EAE immunized WT and mMCP-4 KO mice (Fig. 3B).

Similarly, intrathecally administered big-ET-1 (0.5 or 5 pmol/mouse) increased MAP in healthy WT mice in a dose-dependent fashion. Conversely, a decreased MAP was observed in healthy mMCP-4 KO mice (P < 0.05). The increases in MAP were significantly potentiated 1 week post-EAE in WT (P < 0.05) but not in EAE-induced mMCP-4 KO mice (Fig. 3C). In contrast, intrathecal administration of ET-1 (0.1 pmol/mouse) induced a similar blood pressure response in healthy and EAE-immunized WT or mMCP-4 KO mice (Fig. 3D). Figure 3 E and F shows the time-course profile of MAP increases in response to intrathecal administration big-ET-1 (0.5 pmol/mouse) in WT or mMCP-4 KO mice, either under healthy conditions or 1 week post-EAE.

**mMCP-4 Specific Activity in Mast Cells, Brains, and Lungs of EAE-Induced Mice.** The hydrolytic activity on the fluorogenic substrate succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin was measured in brain homogenates (Fig. 4A). We observed a linear increase in the fluorescence in brain extracts of healthy WT mice and to a lesser extent in EAE-induced WT congeners (P < 0.05 when compared with healthy WT mice). Total hydrolytic activity was also significantly reduced in extracts from healthy or EAE-induced mMCP-4 KO mice when compared with healthy WT mice (P < 0.05).

Figure 4B shows the hydrolytic activity of the same substrate in peritoneal MCs. As was the case with brain extracts, we observed a linear increase in the fluorescence in brain extracts of healthy WT mice and to a lesser extent in EAE-induced WT congeners (P < 0.05 when compared with healthy WT mice), whereas no effects of EAE induction on fluorescence were seen in MCs derived from healthy or EAE-induced mMCP-4 KO mice.

Hydrolitic activities were also measured in lung extracts (Fig. 4C). The maximal fluorescence, detected 15 minutes after initiation of the reaction, was not significantly different between homogenates derived from the two WT groups (healthy or EAE). In contrast, a significant decrease in maximal fluorescence was seen in extracts from healthy mice.
mMCP-4 KO mice versus WT congeners \((P < 0.05)\), with no influence of EAE on the hydrolytic activity in lung extracts from the KO strain.

**Upregulation of Prepro-ET-1 mRNA in the Lungs of EAE-Induced KO Mice.** We previously showed a significant increase in immunoreactive ET-1 in cerebral tissues of WT mice 1 week post EAE but not 2 weeks postimmunization (Desbiens et al., 2016).

Figure 5A shows the relative expression of cerebral and pulmonary prepro-ET-1 in WT and mMCP-4 KO mice. Lungs and brain mRNA samples were extracted from healthy or 1 week post-EAE mice and quantified using the \(2^{-\Delta\Delta CT}\) calculation method and \(\beta\)-actin as the housekeeping gene. The levels of prepro-ET-1 mRNA in the brain did not differ among the four groups of mice studied. In pulmonary extracts, albeit no difference in the expression of prepro-ET-1 mRNA was detected in WT mice 1 week post-EAE immunization, an increase in prepro-ET-1 mRNA levels was found in healthy mMCP-4 KO mice when compared with WT congeners \((P < 0.05)\). Prepro-ET-1 mRNA levels were further increased 1 week post-EAE immunization in the KO congeners \((P < 0.001\) compared with healthy WT mice; \(P < 0.001\) compared with healthy mMCP-4 KO mice).

We also measured the cerebral and pulmonary expression of ECE1a with no differences measured in either healthy or post-EAE immunization WT or mMCP-4 KO mice (brain: healthy WT = 1.00 ± 0.36, WT EAE = 1.17 ± 0.41, healthy mMCP-4 KO = 1.24 ± 0.23, and mMCP-4 KO EAE = 1.64 ± 0.54; lungs:

---

**Fig. 2.** (A) Effect of EAE in the preclinical phases on basal MAP in conscious mice. Each point corresponds to the mean ± S.E.M. of WT \((n = 8)\) and mMCP-4 KO \((n = 7)\) mice. (B) Typical time course profile of MAP variation (upper panels) and HR (lower panels) in healthy WT mice in response to exogenous intravenous administration of ET-1 (1 fmol/kg) (left panels) or intrathecally administered ET-1 (0.1 pmol/mouse) (right panels). Each point corresponds to the mean ± S.E.M. of the average of at least four different experiments.
healthy WT = 1.00 ± 0.58, WT EAE = 0.76 ± 0.38, healthy mMCP-4 KO = 0.93 ± 0.70, and mMCP-4 KO EAE = 0.79 ± 0.61) (data not shown).

We also evaluated the mRNA expression of the ETA or ETB receptors in lung and brain extracts. In the brain, we did not observe any change in the expression of these two receptors (ETA: healthy WT = 1.00 ± 0.39, WT EAE = 1.28 ± 0.44, healthy mMCP-4 KO = 1.18 ± 0.28, and mMCP-4 KO EAE = 1.07 ± 0.46; ETB: healthy WT = 1.00 ± 0.07, WT EAE = 1.09 ± 0.12, healthy mMCP-4 KO = 1.01 ± 0.06, and mMCP-4 KO EAE = 1.02 ± 0.17). Furthermore, no significant differences were found in the pulmonary expression of ET-1 receptors between WT and mMCP-4 KO mice or between these two groups after EAE immunization (ETA: healthy WT = 1.00 ± 0.60, WT EAE = 1.04 ± 1.25, healthy mMCP-4 KO = 1.34 ± 0.34, and mMCP-4 KO EAE = 1.55 ± 0.71; ETB: healthy WT = 1.00 ± 0.50, WT EAE = 1.62 ± 1.10, healthy mMCP-4 KO = 1.20 ± 0.45, and mMCP-4 KO EAE = 1.74 ± 0.60) (data not shown).

**Increased mRNA Expression of Pulmonary IL-33 in mMCP-4 KO Mice.** We also investigated if EAE has an impact on mRNA levels of IL-33. As seen in Fig. 5B, no significant differences were found in cerebral IL-33 mRNA levels in the WT EAE-immunized group or in mMCP-4 KO mice when compared with baseline levels in healthy WT mice. In contrast, increased expression of IL-33 mRNA in the lung was measured in healthy mMCP-4 KO (P < 0.01) as well as in mMCP-4 KO 1 week post-EAE mice when compared with baseline levels in healthy WT mice (P < 0.001). Notably, we observed greater expression of IL-33 in the healthy WT brain compared with healthy WT lungs (3167-fold) (data not shown).

**EAE Has No Influence on ET-1 and IL-33 Tissue Levels but Increases Serum TNF-α in mMCP-4 KO Mice.** Albeit basal levels of ET-1 (in pulmonary tissues) and IL-33 (in brains and lungs) were measured, no significant changes were prompted by EAE in the same type of tissues of WT or mMCP-4 KO mice (Supplemental Tables 1 and 2).
In addition, TNF-α in brain or lung tissue homogenates from healthy WT or mMCP-4 KO mice was undetectable (data not shown). TNF-α was also nondetectable in serum from healthy WT mice (Fig. 6). Finally, albeit low serum levels of TNF-α were measured in WT mice 1 week post-EAE and healthy mMCP-4 KO congeners (5.96 ± 4.69 and 3.65 ± 3.11 pg/ml, respectively) a 4-fold increase in TNF-α was observed in serum samples of mMCP-4 KO 1 week post-EAE immunization versus healthy mMCP-4 KO congeners (21.21 ± 7.82 pg/ml; P < 0.05) (Fig. 6).

**Discussion**

In the present study, we show that both systemic and intrathecal administration of big-ET-1 and ET-1 trigger a sustained increase in blood pressure with little effect on heart rate in conscious mice. In addition, induction of EAE selectively potentiates the pressor response to the precursor big-ET-1 in an mMCP-4-sensitive fashion and prompts MC degranulation and loss of chymase activity in the brain but not in the lungs. Finally, at very early stages of EAE, increases in the expression of IL-33 and serum levels of TNF-α were detected in the periphery but not in the CNS of mMCP-4 KO mice.

These results suggest that mMCP-4–driven events prompted by EAE occur within and outside the CNS in the mouse model. Previous studies demonstrated that EAE, actively induced in C57Bl/6 mice with myelin oligodendrocyte glycoprotein-35–55, was associated with perivascular inflammation, activation of T cells, CD4+ and mononuclear cells, and subsequent axonal demyelination leading to progressive/ ascending hind limb paralysis (Miller et al., 2007). Relevant to
In the present study, Odoardi et al. (2012) were the first to demonstrate that in MS transiently airway-residing T cells reprogram their gene expression and alter their surface expression of adhesion molecules responsible for subsequent migration in cerebral blood vessels. Based on the previously described observations, EAE may have a significant impact on non-CNS inflammatory processes.

Since it is established that mMCP-4 is involved in the production of ET-1 from big-ET-1 in vitro and in vivo (Fecteau et al., 2005; Simard et al., 2009; Houde et al., 2013; Semaan et al., 2015), we investigated the influence of EAE on the acute activity of this particular enzyme, both centrally and in the periphery. We have previously reported that mMCP-4 is responsible for 50% of the pulmonary content of ET-1 (Houde et al., 2013). Furthermore, we also reported a reduction in clinical signs and brain levels of ET-1 in mMCP-4 KO mice subjected to EAE, suggesting a role for this protease in the early phases of the neurodegenerative disease (Desbiens et al., 2016).

Interestingly, EAE triggered an increase in mMCP-4 activity in the CNS as well as in the periphery since the dose-dependent responses to big-ET-1, but not to ET-1 (administered intrathecally or intravenously), triggered significant increases in mean arterial pressure in conscious WT but not mMCP-4 KO mice. To our knowledge, this is the first study comparing the central versus systemic pressor properties of vasoactive peptides in conscious mice. As previously shown in the rat model (Poulat et al., 1994), ganglionic blockade with pentolinium significantly reduced basal mean arterial blood pressure and heart rate, but did not alter the pressor responses to ET-1 (data not shown); a possible indication that the blood pressure and heart rate were maintained by vagal reflex through the sympathetic nervous system.

Albeit, the characterization of central ET receptors has previously been reported in EAE mice, antagonists were not used in the present study since the expression of these receptors was not affected in our model and the pressor responses to ET-1 (administered centrally or intravenously) were not affected under any of the conditions tested. It is also of interest that mRNA levels for the prepro-ET-1 but not for any other components of the ET pathway, were enhanced in the lungs but not the cerebral tissues of EAE mice knocked out for the mMCP-4 gene. In line with these findings, we have previously shown that repression of the chymase gene abolished the increase in brain immunoreactive ET-1 after 1 week post-EAE (Desbiens et al., 2016). In addition, mMCP-4 KO mice show reduced basal levels of the same peptide in the lungs (Houde et al., 2013), suggesting that the increased expression of the precursor of ET-1 reflects a compensatory mechanism for the alternate production of ET-1 in proinflammatory conditions in the respiratory tract.

We have previously shown that MCs of murine or human origin release hydrolytically active chymase generating the intermediate ET-1 (1–31) from its precursor big-ET-1 (Semaan et al., 2015). Therefore, it is of interest that EAE prompts release of mMCP-4 from peritoneal MCs as well as in the brain but not in the lungs. These results support a role for MC-derived
chymase both centrally and in the periphery in the MS mouse model. The absence of chymase-specific hydrolysis in lungs suggests that EAE-promoted differentiation of T cells in the airways has little impact on MC-derived mMCP-4 in the pulmonary system. Thus, the source of big-ET-1 hydrolysis to ET-1 in the systemic circulation may be found elsewhere than in resident MCs located in the airways. Thus, our results suggest that the inflammatory process triggered by EAE enhances mMCP-4-like activity in the compartments more involved in the control of vascular resistance rather than in pulmonary vessels. Notwithstanding the absence of detectable pulmonary chymase activity post-EAE, it is of interest that enhanced expression of IL-33 mRNA in lungs derived from mMCP-4 KO mice when compared with WT congeners; a phenomenon further enhanced by EAE in our study. To our knowledge, there is no reported evidence of a direct effect of this particular chymase isoform on the expression of IL-33. However, it was shown in a previous study that IL-33 protein is a preferred substrate for mMCP-4 proteolytic activity (Waern et al., 2013).

Among several other cytokines, TNF-α upregulates the expression of IL-33 via extracellular signal-regulated kinase and p38 pathways in primary nasal epithelial and A549 cells (Park et al., 2016) and via phosphoinositide-3-kinase and c-Jun N-terminal kinase pathways in nasal fibroblasts (Nomura et al., 2012), leading in both cases to activation of nuclear factor-κB (Nomura et al., 2012; Park et al., 2016). In addition, TNF-α is a substrate for the chymase mMCP-4 (Piliponsky et al., 2012). Interestingly, TNF-α was increased significantly in serum derived from mMCP-4 KO versus WT mice subjected to EAE. We suggest that the latter cytokine is involved in the increased expression of IL-33. This mechanism, in conditions of EAE, appears to be limited to the respiratory tract as opposed to the CNS. Finally, we suggest that 1 week of EAE is too short a period to trigger significant increases in IL-33 or ET-1 proteins in the lungs and the former in the brain as well. However, it is of interest that cardiovascular phenotypes triggered by EAE were identified in this study even at such an early stage of the disease.

One limitation of the present study is that the direct impact of anti-TNF-α blockers on the expression of IL-33 in EAE mice was not assessed. However, considering that TNF-α blockers have recently been counterindicated in MS patients due to the demyelination properties of these molecules (van Oosten et al., 1996, The Lenorect Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group, 1999; Probert, 2015), the present study provides mechanistic insights into why TNF-α should be left intact in this particular neurodegenerative disease.

We have previously reported a significant role for mMCP-4 in EAE (Desbiens et al., 2016). The results from the present study now suggest that mMCP-4-dependent hydrolysis of vasoactive and neurogenic peptides such as ETs is enhanced in the periphery and CNS at least in this murine model of MS. If our results in the mouse model are translatable to the human disease, chymase inhibitors may be useful in reducing neurogenic decay as well as overall systemic inflammation in MS patients.

Acknowledgments

We thank Dr. Réjean Couture (Université de Montréal, Quebec, Canada) for useful discussions as well as Dr Jean-Bernard Denault (Université de Sherbrooke, Quebec, Canada) for the use of the Infinite M1000 spectrophotometer.

Authorship Contributions

Participated in research design: D’Orléans-Juste, Desbiens, Gris, Ghargalozo.

Conducted experiments: Desbiens, Lapointe, Gendron, Ghargalozo, Vincent.

Provided data analysis: Desbiens, Lapointe, Vincent.

Wrote or contributed to the writing of the manuscript: D’Orléans-Juste, Desbiens, Gris, Pejler, Gendron, Ghargalozo, Lapointe, Vincent.

References


SUPPLEMENTARY DATA

JPET/2018/256016
Experimental autoimmune encephalomyelitis potentiates mouse mast cell protease 4-dependent pressor responses to centrally or systemically administered big endothelin-1 (Louisane Desbiens et al.)

Supplement Table 1

Tissue quantification of immunoreactive- IL-33 (pg/mL of homogenate) in healthy (n = 4) or 1 week post-EAE WT and mMCP-4 KO mice (n = 5 for brains and n = 6 for lungs).

<table>
<thead>
<tr>
<th>Animals</th>
<th>Brain</th>
<th>Lungs</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>232.1 ± 13.0</td>
<td>245.2 ± 71.6</td>
</tr>
<tr>
<td>WT + EAE 1 wk</td>
<td>189.2 ± 27.0</td>
<td>168.5 ± 39.9</td>
</tr>
<tr>
<td>mMCP-4 KO</td>
<td>169.8 ± 10.8</td>
<td>341.2 ± 77.0</td>
</tr>
<tr>
<td>mMCP-4 KO + EAE 1 wk</td>
<td>214.0 ± 34.0</td>
<td>246.5 ± 20.7</td>
</tr>
</tbody>
</table>

Supplement Table 2

Tissue quantification of lung-extracted immunoreactive ET-1 (pg/mg of tissue) in healthy (n = 4) or 1 week after EAE immunisation WT and mMCP-4 KO mice (n = 6).

<table>
<thead>
<tr>
<th>Animals</th>
<th>ET-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>WT + EAE 1 wk</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>mMCP-4 KO</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>mMCP-4 KO + EAE 1 wk</td>
<td>0.4 ± 0.2</td>
</tr>
</tbody>
</table>