

Research Article

Open Access

Lucia Monti*, Umberto Arrigucci, Alessandro Rossi

Insights into Endothelin-3 and Multiple Sclerosis

<https://doi.org/10.1515/bmc-2020-0012>

received January 8, 2020; accepted February 21, 2020.

Abstract: Endothelins are powerful vasoconstrictor peptides that play numerous other roles. Endothelin-1 (ET1) is the principal isoform produced by the endothelium in the human cardiovascular system. Endothelin-3 (ET3) and its rPptor affinity have been demonstrated to support neuronal repair mechanisms throughout life. In multiple sclerosis (MS), the role of vasoactive peptides are not well defined. Here we focus on ET3, specifically the plasma levels between MS patients and healthy subjects. Furthermore, we evaluated the changes in ET1 and ET3 plasma levels during different disease phases, the correlation between ET3 and cerebral circulation time, and the relationship between ET1 and ET3. In MS patients, the ET3 plasma levels were altered in a time-dependent manner. These results could support a putative role of ET3 in neuroprotection and/or neuroimmune modulation over time.

Keywords: multiple sclerosis (MS); endothelin-3; blood-brain barrier; neuroimmune modulation; cerebral circulation time.

Introduction

Endothelin peptides (ETs) comprise of three structurally similar 21-amino acid vasoactive peptides. Endothelin-1 (ET1) and endothelin-2 (ET2) activate two G-protein

coupled receptors ETA and ETB, with similar affinity, whereas endothelin-3 (ET3) has a high affinity for the ETB subtype. ET3 is the most abundant ET peptide in the rat brain, mainly localized to neurons and glia of the neostriatum, hypothalamic nuclei, hippocampus, and Purkinje cells of the cerebellum and medulla oblongata [1]. This led to the proposal that ET3 is the “brain” ET [2]. High expression of brain ETB receptors is observed in astrocytes [3]. Expression of astrocytic ETB receptors is upregulated after brain injury and is known to be a potent mitogen of astrocytes [4]. Furthermore, astrocytes produce and release various bioactive substances, for example, neurotrophic factors, cytokines, chemokines, nitric oxide, and vascular permeability factors, through which they interact with neurons and brain microvessels [4]. Production of astrocyte-derived molecules is stimulated by the activation of astrocytic ETB receptors [5].

The biosynthetic pathway of ETs is considered to be similar: 1. a large precursor designated preproET is formed; 2. after enzymatic removal of a signal peptide, proET is cleaved by a protease leading to the formation of big ET, finally 3. big ET is hydrolyzed into the small, mature ET1 by an endothelin-converting enzyme (ECE). At present, the mammalian ECE family consists of three known forms (ECE-1, ECE-2, ECE-3), which are classified into the neutral endopeptidase group of proteins [6]. Each member of this family shows homology to the others, mainly in the C-terminal part of the sequence that is responsible for catalytic activity. In addition, there are structural similarities in that all neutral endopeptidase members, for example, ECE-1, Kell glycoprotein can proteolytically cleave ET1, ET2, and ET3, with a marked preference for ET3 [7].

While the primary source of circulating ET1 is the vascular endothelial cells [8], these cells do not synthesize ET3. One possible source of circulating big ET3 could be the adrenal gland [2]. Although this peptide has been visualized in secretory cells of the medulla using selective antisera, mature ET3 could not be detected [9,10]. Changes associated with the disease have not been extensively investigated, but concentrations of ET3 increased significantly in hemodialysis patients (together

***Corresponding author: Lucia Monti**, Unit of Diagnostic and Functional Neuroimaging, Dpt. of Neurology and Human Movement Sciences, University Hospital of Siena, Santa Maria alle Scotte, Viale Bracci 2, 53100 Siena, Italy, E-mail: l.monti@ao-siena.toscana.it
Umberto Arrigucci, Unit of Diagnostic and Functional Neuroimaging, Dpt. of Neurology and Human Movement Sciences, University Hospital of Siena, Santa Maria alle Scotte, Viale Bracci 2, 53100 Siena, Italy

Alessandro Rossi, Neurology and Neurophysiology Unit, Dpt. of Neurology and Human Movement Sciences, University Hospital of Siena, Santa Maria alle Scotte, Viale Bracci 2 53100 Siena, Italy

with big ET1 and big ET2) but with only a moderate rise in corresponding active peptides [11]. If released, further processing of big ET3 could occur within the vasculature by smooth muscle cell ECE [12,13]. Interestingly, the pituitary was observed to have higher levels of ET3 than ET1. Many studies have identified dysregulation of the hypothalamic-pituitary-adrenal axis in patients with MS [14,15]. Indeed, pituitary-ovary axis and ovarian reserve have been investigated, and lower corticotropin like intermediate lobe peptide has been demonstrated. Generally, the hypothalamic-pituitary-adrenal axis has been demonstrated to be hyperactive in MS patients, and putative involvement of plasmatic active ET3 and hypothalamic-pituitary-adrenal axis dysregulation could be hypothesized [15].

This study aims to investigate the possible differences between ET3 plasma levels between MS patients and healthy subjects. Furthermore, we investigate the potential relationship between circulating vasoactive ET3 and cerebral circulation time and the relationship between circulating ET3 and ET1 in the same MS patient cohort.

Materials and Methods

Pregnant or nursing women, heavy smokers, and patients within 30 days of previous therapy were excluded from the study.

A total of 43 patients with proven MS have been included. Blood samples with hemolysis were discarded, and a total of 29 MS patients were considered. The 29 MS patients (11 men and 18 women) had the relapsing-remitting form of MS.

A group of 37 age and sex-matched healthy control subjects were included in the study. Seven subjects were excluded due blood sample hemolysis; therefore a total of 30 healthy subjects (11 men and 19 women) were considered.

In 29 MS patients, peripheral blood samples were collected, and ET3 and ET1 titration were quantified. Peripheral blood samples were collected after 30 minutes of rest in a supine position from the brachial vein and circulating ET3 and ET1 levels were measured. Cerebral circulation time was calculated in all MS patients recruited by digital subtracted angiography (DSA) [16].

Ethical approval: The research related to human use has been complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration, and has been approved

by ethical board of the AOUS General Hospital Santa Maria alle Scotte, Siena.

Informed consent: Informed consent has been obtained from all individuals included in this study

Biochemical assessment

Only one peripheral blood sample was obtained from each patient or healthy control subject. After blood collection, samples were centrifuged at 700G-force 2500 rpm for 15 minutes. Samples that showed evidence of hemolysis were discarded. Plasma was aliquoted into 500 μ L/vials and stored at -80°C until required for the quantification of ET1 and ET3. Residual samples were stored at -80°C for further evaluation if required. Commercially available enzyme-linked immunosorbent assay kits for the measurement of vasoactive mediators (ET1, ET3) were run according to the manufacturers' instructions (R&D Systems, Space Import Export Srl, Milan, Italy, and Alexis, Vinci-Biochem, Vinci, Firenze, Italy, respectively). Each sample was run in duplicate, and measurements were extrapolated using a reference compound standard curve. Data were expressed as pg/ml for ET3 and μ g/ml for ET1.

Statistical Analysis

The entire data set was initially analyzed by sex and by group (control vs. patients). The homogeneity of the sample composition by sex between the control group and the patient group, and between the MS forms were verified through the χ^2 test (with one degree of freedom). The assumptions of normal distribution were determined using the Shapiro-Wilk test. Accordingly, unpaired t-test (with Welch correction for unequal standard deviation [SD]) or Mann-Whitney test was used to compare two samples. The one-way analysis of variance test (or nonparametric Kruskal-Wallis test) was used to verify the null hypothesis of equal levels of variables. The Holm-Šidak multiple comparisons were used as a post hoc test. After testing whether the values come from a Gaussian distribution, analysis of correlation by two-tailed Pearson coefficients or nonparametric Spearman test was performed among the following variables: cerebral circulation time, ET1, ET3, and disease duration.

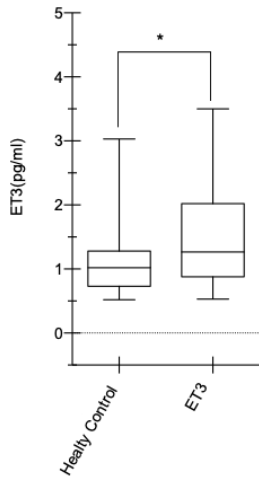


Figure 1: Endothelin-3 (ET3) plasma levels determined from healthy (control) subjects and multiple sclerosis patients (mean \pm standard deviation). Statistical significance was demonstrated using Mann-Whitney (two-tailed) $*p = 0.0329$.

Results and Discussion

Despite extensive research, there remains a critical gap in our understanding of the mechanisms that promote brain hypoperfusion in MS patients. The most reliable hypothesis in the literature is a vascular dysregulation resulting in increased brain vessel resistance mediated by ET1. However, although ET1 plasma levels are increased in MS with respect to healthy subjects, the relationship between ET1 and cerebral circulation time values remains elusive [17,18]. Here we focus on the ET3 vasoactive peptide, for which little is known. This study has demonstrated that in MS patients the ET3 plasma levels are higher than in healthy controls (Fig.1), but we found no correlation between ET3 levels and cerebral circulation time (Fig.2b). ET3 is positively correlated with ET1 plasma levels (Fig.2d), and both peptides correlate with disease duration (Fig.2a; Fig.2c).

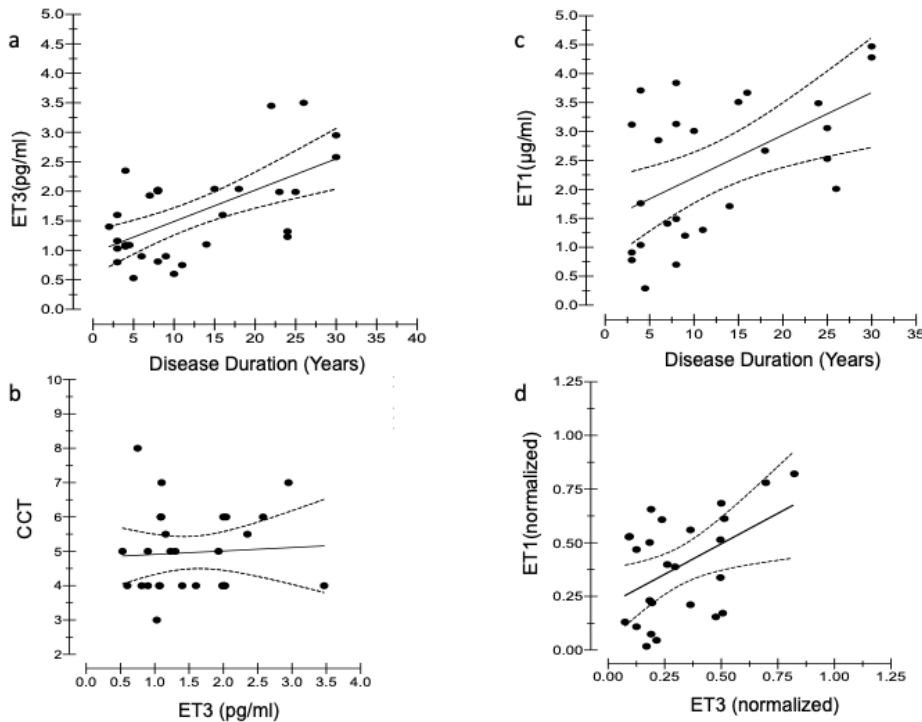


Figure 2: Analysis of correlation among the variables cerebral circulation time (CCT), endothelin-1 (ET1), endothelin-3 (ET3), and disease duration in MS patients. (a) Correlation between ET3 and disease duration. ET3 changes during the disease duration reached significant statistical difference using the Pearson correlation coefficient (two-tailed) $***p = 0,0002$. (b) Correlation between ET3 and CCT. The CCT delay was not statistically significantly correlated to increased ET3 titration. (c) Correlation between ET1 and disease duration. ET1 was statistically significantly correlated with disease duration using the Pearson correlation coefficient (two-tailed) $r = 0.54025$ with a 95% confidence interval between 0.19336 - 0.76708, $R^2 = 0.29187$, $**p = 0.0044$ ($\alpha = 0.05$). (d) Correlation between circulating ET3 and ET1. Increasing ET3 and ET1 plasmatic titration levels were statistically significantly correlated using Pearson correlation coefficient. ($r=0,54025$ with 95% confidence interval 0.19336 to 0.76708 and $R^2=0,29187$). The increasing ET3 plasmatic values are statistically correlated with increasing ET1 plasmatic values and the P (two-tailed) value results as 0,0044 (**)

Source and possible functional significance

The plasmatic ET3 titration source could be from smooth muscle cells in the vasculature [13] or from the medulla of the adrenal gland. However, the stimuli inducing increased ET3 plasmatic levels are still unknown.

ET3 has a high affinity for the ETB receptors subtype. In an animal model of ischemic stroke, it has been shown that ETA expression dominates in the acute phase (the first 24 hours), whereas the density of ETB receptors was significantly increased only in the sub-acute stage (seven days after). It could be deduced that microvascular damage, inflammation, and breakdown of the blood-brain barrier (BBB), occurring in the acute phase, are not causative factors for ET3 production. Instead, the latter could play a role in the recovery phase, when angiogenesis, neurogenesis, and neuroblast migration towards the ischemic boundary starts to manifest [19]. Translating these results to MS, the observation that ET3 plasma levels increased with increasing disease duration, could suggest a slow time-dependent neuroprotective mechanism mediated by ETB receptors. Furthermore, the possibility that the high ET3 plasmatic levels in MS patients may play a role in the immune response cannot be eliminated. Shinji Nakashima et al. [20] showed that ETB receptor expression rate is inversely correlated to tumor-infiltrating T lymphocytes, suggesting an immune escape mechanism in gliomas. Considering the ET3 high affinity for ETB receptors, a progressive attempt to inhibit the entry of T cells into the central nervous system by increasing ET3 production in MS could also be considered. Moreover, the strong significant correlation between ET3 and disease duration could underpin other MS features, such as increased BBB permeability. In an in vitro study, chronic endothelin exposure led to the alteration of astrocytic gap-junction and intercellular communication, therefore, chronic high ET3 titration could be involved in BBB permeability to, for example, lymphocytes or molecules in MS patients [21-23].

The present study failed to show any relationship between ET3 plasma levels and cerebral perfusion expressed by the cerebral circulation time delay. As previously observed for ET1 [18], ET3 and cerebral circulation time are independent variables.

Finally, the observed significant correlation between circulating ET3 and ET1 may be due to a common biosynthetic pathway. Most of the neutral endopeptidase group of proteins can proteolytically cleave big ET3 and ET1 into the small, mature ET3 and ET1.

Acknowledgments: The Authors would like to thank Prof. Elisabetta Rossi for revisions and comments and Prof. Lucia Morbidelli for biochemical assessment.

Conflict of interest: Authors state no conflict of interest

References

1. Fuxe K, Anggård E, Lundgren K, Cintra A, Agnati LF, Galton S, et al. Localization of [125I]endothelin-1 and [125I]endothelin-3 binding sites in the rat brain. *Acta Physiol Scand*. 1989 Dec;137(4):563–4.
2. Koseki C, Imai M, Hirata Y, Yanagisawa M, Masaki T. Autoradiographic distribution in rat tissues of binding sites for endothelin: a neuropeptide? *Am J Physiol*. 1989 Apr;256(4 Pt 2):R858–66.
3. Puppala B, Awan I, Briyal S, Mbachu O, Leonard M, Gulati A. Ontogeny of endothelin receptors in the brain, heart, and kidneys of neonatal rats. *Brain Dev*. 2015 Feb;37(2):206–15.
4. Bhalla S, Leonard MG, Briyal S, Gulati A. Distinct Alteration in Brain Endothelin A and B Receptor Characteristics Following Focal Cerebral Ischemia in Rats. *Drug Res (Stuttg)*. 2016 Apr;66(4):189–95.
5. Koyama Y. Endothelin systems in the brain: involvement in pathophysiological responses of damaged nerve tissues. *Biomol Concepts*. 2013 Aug;4(4):335–47.
6. Hasegawa H, Hiki K, Sawamura T, Aoyama T, Okamoto Y, Miwa S, et al. Purification of a novel endothelin-converting enzyme specific for big endothelin-3. *FEBS Lett*. 1998 May;428(3):304–8.
7. Maguire JJ, Davenport AP. Endothelin@25 - new agonists, antagonists, inhibitors and emerging research frontiers: IUPHAR Review 12. *Br J Pharmacol*. 2014 Dec;171(24):5555–72.
8. Endothelin-I- And Endothelin-3-Induced Vasorelaxation Via Common Generation Of Endothelium-Derived Nitric Oxide. Namiki A, Hirata Y, Ishikawa M, Moroi M, Aikawa J, Machii K. *Life Sci*. 1992;50(10):667–82.
9. Davenport AP, Hyndman KA, Dhaun N, Southan C, Kohan DE, Pollock JS, et al. Endothelin. *Pharmacol Rev*. 2016 Apr;68(2):357–418.
10. Davenport AP, Hoskins SL, Kuc RE, Plumpton C. Differential distribution of endothelin peptides and receptors in human adrenal gland. *Histochem J*. 1996 Nov;28(11):779–89.
11. Miyauchi Y, Sakai S, Maeda S, Shimojo N, Watanabe S, Honma S et al. Increased plasma levels of big-endothelin-2 and big-endothelin-3 in patients with end-stage renal disease. *Life Sci*. 2012 Oct 15;91(13-14): 729–32. <https://doi.org/10.1016/j.lfs.2012.08.008>.
12. Skovsted GF, Kilic S, Edvinsson L. Endothelin-1 and Endothelin-3 Regulate Endothelin Receptor Expression in Rat Coronary Arteries. *Basic Clin Pharmacol Toxicol*. 2015 Nov;117(5):297–305.
13. Davenport AP, Kuc RE, Mockridge JW. Endothelin-converting enzyme in the human vasculature: evidence for differential conversion of big endothelin-3 by endothelial and smooth-muscle cells. *J Cardiovasc Pharmacol*. 1998;31(31 Suppl 1):S1–3.

14. Takahashi K, Ghatei MA, Jones PM, Murphy JK, Lam HC, O'Halloran DJ, et al. Endothelin in human brain and pituitary gland: comparison with rat. *J Cardiovasc Pharmacol.* 1991;17 Suppl 7:S101–3.
15. Burfeind KG, Yadav V, Marks DL. Hypothalamic Dysfunction and Multiple Sclerosis: Implications for Fatigue and Weight Dysregulation. *Curr Neurol Neurosci Rep.* 2016 Nov;16(11):98.
16. Monti L, Donati D, Menci E, Cioni S, Bellini M, Grazzini I, et al. Cerebral circulation time is prolonged and not correlated with EDSS in multiple sclerosis patients: a study using digital subtracted angiography. *PLoS One.* 2015 Feb;10(2):e0116681.
17. Monti L, Morbidelli L, Rossi A. Impaired Cerebral Perfusion in Multiple Sclerosis: Relevance of Endothelial Factors. *Biomark Insights.* 2018 May;13:1177271918774800.
18. Monti L, Morbidelli L, Bazzani L, Rossi A. Influence of Circulating Endothelin-1 and Asymmetric Dimethylarginine on Whole Brain Circulation Time in Multiple Sclerosis. *Biomark Insights.* 2017 Jun;12:1177271917712514.
19. Bhalla S, Leonard MG, Briyal S, Gulati A. Distinct Alteration in Brain Endothelin A and B Receptor Characteristics Following Focal Cerebral Ischemia in Rats. *Drug Res (Stuttg).* 2016 Apr;66(4):189–95.
20. Nakashima S, Sugita Y, Miyoshi H, Arakawa F, Muta H, Ishibashi Y, et al. Endothelin B receptor expression in malignant gliomas: the perivascular immune escape mechanism of gliomas. *J Neurooncol.* 2016 Mar;127(1):23–32.
21. Grossman RI, Gonzalez-Scarano F, Atlas SW, Galetta S, Silberberg DH. Multiple sclerosis: gadolinium enhancement in MR imaging. *Radiology.* 1986 Dec;161(3):721–5.
22. Sobel RA, Ahmed AS. White matter extracellular matrix chondroitin sulfate/dermatan sulfate proteoglycans in multiple sclerosis. *J Neuropathol Exp Neurol.* 2001 Dec;60(12):1198–207.
23. van Horssen J, Bö L, Vos CM, Virtanen I, de Vries HE. Basement membrane proteins in multiple sclerosis-associated inflammatory cuffs: potential role in influx and transport of leukocytes. *J Neuropathol Exp Neurol.* 2005 Aug;64(8):722–9.