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Increased plasma levels of big-endothelin-2 and big-endothelin-3 in patients with end-stage renal disease

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ABSTRACT

Aims: Big endothelins (pro-endothelin; inactive-precursor) are converted to biologically active endothelins (ETs). Mammals and humans produce three ET family members: ET-1, ET-2 and ET-3, from three different genes. Although ET-1 is produced by vascular endothelial cells, these cells do not produce ET-3, which is produced by neuronal cells and organs such as the thyroid, salivary gland and the kidney. In patients with end-stage renal disease, abnormal vascular endothelial cell function and elevated plasma ET-1 and big ET-1 levels have been reported. It is unknown whether big ET-2 and big ET-3 plasma levels are altered in these patients. The purpose of the present study was to determine whether endogenous ET-1, ET-2, and ET-3 systems including big ETs are altered in patients with end-stage renal disease.
Main methods: We measured plasma levels of ET-1, ET-3 and big ET-1, big ET-2, and big ET-3 in patients on chronic hemodialysis (n = 23) and age-matched healthy subjects (n = 17).
Key findings: In patients on hemodialysis, plasma levels (measured just before hemodialysis) of both ET-1 and ET-3 and big ET-1, big ET-2, and big ET-3 were markedly elevated, and the increase was higher for big ETs (Big ET-1, 4-fold; big ET-2, 6-fold; big ET-3: 5-fold) than for ETs (ET-1, 1.7-fold; ET-3, 2-fold).
Significance: In hemodialysis patients, plasma levels of the inactive precursors big ET-1, big ET-2, and big ET-3 levels are markedly increased, yet there is only a moderate increase in plasma levels of the active products, ET-1 and ET-3. This suggests that the activity of endothelin converting enzyme contributing to circulating levels of ET-1 and ET-3 may be decreased in patients on chronic hemodialysis.

Introduction

Endothelin-1 (ET-1) has potent and extremely long-lasting vasoconstrictor effects (Yanagisawa et al., 1988). Several studies have demonstrated that mammals and humans produce three distinct members of the ET peptide families ET-1, ET-2 and ET-3, which may have different profiles of biological activity on vascular and non-vascular tissues (Inoue A et al., 1988; Yanagisawa and Masaki, 1989; Miyauchi and Masaki, 1999). Although ET-1 is produced by vascular endothelial cells, these cells do not produce ET-3 (Inoue et al., 1989; Yanagisawa and Masaki, 1989; Bloch et al., 1989; Miyauchi and Masaki, 1999). ET-3 was found to be located in the non-cardiovascular tissues including the porcine brain and the cells outside the neurologic systems, therefore these findings suggest that ET-3 plays several physiological roles including neuropeptide functions (Shinmi et al., 1989; Miyauchi and Masaki, 1999). Thus, ET-1 and ET-3 are considered to possess different physiological roles.

Big endothelin (pro-endothelin; the inactive precursor of endothelin) is converted to endothelin, the active form of the peptide with strong vasoconstrictor and vasoproliferative activity (Miyauchi and Masaki, 1999). ET-1 is produced by vascular endothelial cells, however, the vascular endothelium does not produce ET-3 (Inoue et al., 1989; Yanagisawa and Masaki, 1989; Bloch et al., 1989; Miyauchi and Masaki, 1999). There are two receptors for endothelins, the ET_A-receptor and the ET_B receptor (Miyauchi and Masaki, 1999). The affinity for ET_A receptors is greater for ET-1 than for ET-3, whereas the affinity of ET_B receptors is equivalent for ET-1and ET-3 (Miyauchi and Masaki, 1999). Therefore, the ET-1/ET_A system has been speculated to be more important for cardiovascular regulation than the ET-3/ET_B system. Interestingly, exogenously applied big ET-3 was reported to constrict forearm resistance vessels in humans via conversion to ET-3, the active form of the peptide (Ferro et al., 2000).

Currently, endothelin receptor antagonists, such as bosentan (endothelin $ET_{A/B}$ receptor blocker; i.e., non-selective antagonist) and ambrisentan (ET_A receptor selective antagonist) are clinically applied in patients with pulmonary hypertension (Shimojo et al., 2009;

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Baliga et al., 2011). At the doses of bosentan currently used for the treatment of pulmonary hypertension (up to 125 mg/day [b.i.d]; systemic arterial pressure does not decrease whereas pulmonary arterial pressure is lowered (Shimojo et al., 2009; Baliga et al., 2011). Furthermore, it has been reported that at higher doses endothelin antagonists are effective in reducing also systemic blood pressure in humans (Krum et al., 1998; Maeda et al., 2009). The pro-hypertensive effect of endothelin-1 is also supported by animal studies, since it also has been reported that endothelial-cell specific ET-1 over-expressing mice are hypertensive (Leung et al., 2011), whereas that the endothelial-cell specific ET-1 deficient mice are hypotensive (Kisanuki et al., 2010). This evidence suggests that endogenous ET-1 generated in the vascular endothelium may participate in the modulation of the systemic blood pressure in vivo.

In addition to systemic hypertension and ET-1, it has been suggested that overexpression of ET-1 aggravates increases in blood pressure under conditions such as a high salt diet (Amiri et al., 2004, 2010). Amiri et al. reported that the endothelial over-expression of ET-1 had no statistically significant effect on the total 24-hour blood pressure measurements of these mice (Amiri et al., 2004), whereas these mice showed differences in blood pressure at individual time points of the 24 h measurements (Amiri et al., 2004) and were shown to become hypertensive if placed on a high salt diet (Amiri et al., 2010). Moreover, Saleh et al. reported that chronic ET-1 infusion in rats does not increase blood pressure, yet promotes inflammation (Saleh et al., 2010).

It has been reported that ET-1 is involved in progression of renal diseases (Hocher et al., 1997; Shindo et al., 2002). Hocher et al. reported that ET-1 transgenic mice developed glomerulosclerosis, interstitial fibrosis, and renal cysts (Hocher et al., 1997). Shindo et al. reported that the renal damage and salt-dependent hypertension were observed in aged transgenic mice overexpressing ET-1 (Shindo et al., 2002). Therefore, it has been considered that the ET systems are involved in the progression of chronic renal failure. Indeed, it has reported that plasma ET-1 levels are elevated in patients with chronic hemodialysis (Koyama et al., 1989; Miyauchi et al., 1991; Ottosson-Seeberger et al., 1999). However, it is unknown whether plasma big ET-2 and big ET-3 levels are altered in patients on chronic hemodialysis. We have previously established sandwich-enzyme immunoassays (sandwich-EIAs) for ET-1 (Miyauchi et al., 1989), ET-3 (Miyauchi et al., 1991; Maeda et al., 1997) and big ET-1, big ET-2, and big ET-3 (Miyauchi et al., 1989; Suzuki et al., 1990, 1991). To study the potential pathophysiological role of the ET family in human end-stage renal disease, we measured plasma levels of ET-1, ET-3 and big ET-1, big ET-2, and big ET-3 in patients on chronic hemodialysis and in age-matched healthy control subjects using the sandwich-EIAs for each of the above peptides of the endothelin family.

Methods

Blood samples from normal subjects and patients

Blood samples were collected from antecubital veins of male patients undergoing chronic hemodialysis (n = 23, 46 ± 1.6 years of age) and age-matched male healthy subjects (n = 17, 45 ± 1.8 years of age). In hemodialysis patients, blood samples were drawn just before patients underwent hemodialysis. This study was reviewed and approved by the Institutional Review Board at the University of Tsukuba, and the subjects gave their written informed consent to participate in the study. Blood samples of hemodialysis patients were obtained from Sumiyoshi Clinic Hospital. Each blood sample was put in chilled tubes containing aprotinin (300 KIU/ml) and EDTA (2 mg/ml) and then centrifuged at 2000 × *g* for 15 min at 4 °C. The plasma was stored at −30 °C until used.

Extraction procedure

Plasma (1 ml) was acidified with 3 ml of 4% acetic acid, and immunoreactive ET-1, ET-3 or big ET-1, -2, and -3 were extracted with a Sep-Pack C-18 cartridge (Waters Associates, Milford, MA, USA) as previously described (Miyauchi et al., 1989; Suzuki et al., 1990; Maeda et al., 1997). The elutes were reconstituted with 0.25 ml of assay buffer and subjected to the EIAs.

Sandwich-EIAs

Sandwich-EIAs for ET-1 were carried out as previously described using immobilized mouse monoclonal antibody AwETN40, which recognizes the N-terminal portion of ET-1, and peroxidase-labeled rabbit anti-ET-1 C-terminal peptide (15-21) Fab' (Takeda Chemical Industry, Tsukuba, Japan) (Miyauchi et al., 1989). In the sandwich-EIA for ET-3, the monoclonal antibody AET-30, which recognizes the N-terminal loop domain of ET-3, was used as an immobilized capture antibody (Takeda Chemical Industry, Tsukuba, Japan) (Miyauchi et al., 1991; Maeda et al., 1997). An antibody against the C-terminal heptapeptide of ET-3, the sequence common to other endothelins, was elicited in rabbits by immunizing animals with ET-3 (15–21)-BSA conjugates (Takeda Chemical Industry, Tsukuba, Japan) (Miyauchi et al., 1991; Maeda et al., 1997). The Fab fragment of this rabbit antibody was used as an enzyme-labeled detector antibody after being coupled with horseradish peroxidase (Miyauchi et al., 1991; Maeda et al., 1997). Similarly, in the sandwich-EIA for big ET-1, immobilized AwET40 and peroxidase-labeled rabbit anti-human big ET-1 C-terminal peptide (22–38) Fab' was used (Takeda Chemical Industry, Tsukuba, Japan) (Suzuki et al., 1990). Similarly, plasma big ET-2 and big ET-3 levels were also measured by their sandwich EIAs according to previous reports (Suzuki et al., 1991; Matsumoto et al., 1994). The assay for ET-1 does not cross-react with ET-3 or big ET-1 (cross-reactivity: <0.1%). The assay for ET-3 does not cross-react with ET-1, big ET-1 or big ET-3 (cross-reactivity: <0.1%). The assay for big ET-2 does not cross-react with big ET-1 or big ET-3 (cross-reactivity: <1.0%). The assay for big ET-3 does not cross-react with big ET-1 or big ET-2 (cross-reactivity: <1.0%). Thus, these sandwich-EIAs are sensitive enough to detect 0.1–0.4 pg/well of the respective ETs or big ETs studied with a high specificity due to a cross-reactivity of less than 1% with other ETs and big ETs (Miyauchi et al., 1989; Suzuki et al., 1990, 1991; Matsumoto et al., 1994; Maeda et al., 1997).

Statistics

Data are expressed as mean ± S.E.M. Statistical analysis was carried out using the Student's *t*-test for unpaired comparisons. Differences were considered statistically significant at p values of <0.05.

Results

In patients on chronic hemodialysis, peptide plasma of both ET-1, ET-3, and big ET-1, big ET-2, and big ET-3-1 were significantly higher than those in healthy subjects (Fig. 1, Table 1) In patients on hemodialysis, plasma levels (measured just before hemodialysis) of both ET-1 and ET-3 and big ET-1, big ET-2, and big ET-3 were markedly elevated, and the relative increase was higher for big ETs (big ET-1, 4-fold; big ET-2, 6-fold; big Et-3, 5-fold, all $p<0.05$) than for ETs ((ET-1, 1.7-fold; ET-3, 2-fold), all $p<0.05$)). Also, the relative levels of the different big ET peptides were markedly different between healthy controls and renal disease patients, in which levels were approx. 17 pg/ml (big ET-1), approx. 40 pg/ml (big ET-2) and more than 70 pg/ml (big ET-3) (Fig. 1 and Table 1).

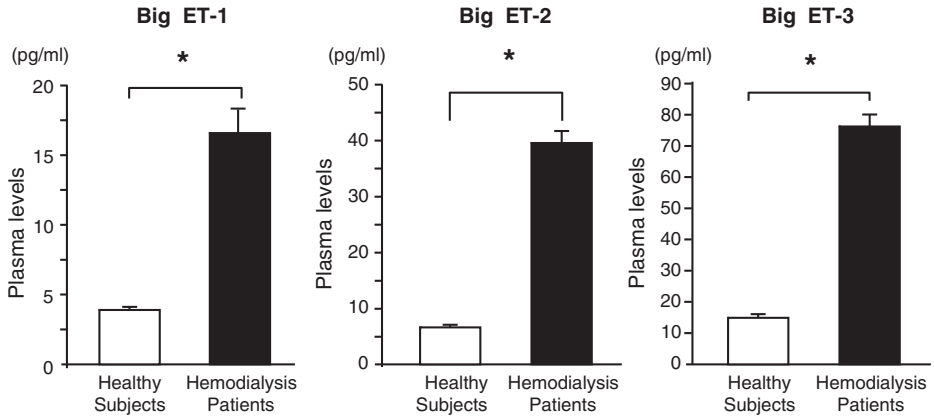

Fig. 1. Plasma levels of big endothelins (big ET-1, big ET-2, and big ET-3) in healthy subjects (n = 17; open columns) and patients on hemodialysis (n = 23; filled columns). Note different scales of the y axes in the three panels. Data are mean ± SEM, *p<0.05 vs healthy subjects.

Discussion

The present study demonstrates the novel finding that plasma levels of big ET-2, and big ET-3 are markedly increased in patients on hemodialysis, and therefore it was suggested that the elevation in these peptides might be partly attributed to the increase in production of mature ET peptides in hemodialysis patients that was also observed. Since vascular endothelial cells produce ET-1 and big ET-1 (Miyauchi and Masaki, 1999), it has been suggested that production of ET-1 in vascular endothelial cells was increased due the hemodynamic overload in hemodialysis patients. Since vascular endothelial cells do not produce ET-3 – but do produce ET-1 (Inoue et al., 1989; Yanagisawa and Masaki, 1989; Bloch et al., 1989; Miyauchi and Masaki, 1999) – it is possible that the elevation in plasma levels of big ET-3 and ET-3 in hemodialysis patients can be in part attributed to a decreased clearance of these peptides due to renal insufficiency. The present study demonstrates for the first time that the plasma levels of big ET-2 and big ET-3 are markedly increased in patients with hemodialysis, and also demonstrates that the relative intensity of the increase is higher for big ET-3 (the inactive precursor with a 5-fold increase) compared to ET-3 (mature active peptide, 2-fold increase). Therefore, it is possible that the production of big ET-3 is increased whereas the conversion of big ET-3 to ET-3 might be decreased in these patients. The data also shows a 6-fold increase in plasma big ET-2 in hemodialysis patients. The increase in plasma big ET-1 level in hemodialysis patients observed in the present study is in accordance with a previous report (Suzuki et al., 1990). Inasmuch as ET-3 may be a neuropeptide (Shinmi et al., 1989; Miyauchi and Masaki, 1999), the elevation in plasma ET-3 and big ET-3 levels in hemodialysis patients may suggest that ET-3-mediated neuronal responses are altered in hemodialysis patients. Moreover, the absence of ET-3 production in the vascular endothelium would suggest that the ET-1/ET_A system is likely to be more important for cardiovascular regulation than the ET-3/ET_B system. However, in this context an interesting finding has been reported, namely that exogenously applied big ET-3 causes constriction of forearm resistance vessels in humans via conversion to ET-3, its converted, active form (Ferro et al., 2000). Because it was reported that the high-dose bosentan (ET-A/B dual receptor antagonist) causes a significant decrease in systemic blood pressure in healthy middle-aged and older adults (Maeda et al., 2009), and in patients with essential hypertension (Krum et al., 1998), it has been considered that the endogenous endothelin systems is involved in regulation of the systemic blood pressure in humans. Therefore, it is likely that both the endogenous ET-1/ET_A system and endogenous ET-3/ET_B system may be differently involved in regulation of blood pressure in normal subjects and hemodialysis patients.

Plasma ET-1 levels are increased in various cardiovascular diseases (Miyauchi and Masaki, 1999; Shimojo et al., 2009). ET-1 has been implicated in the pathogenesis of arterial stiffening (McEniery et al., 2003). McEniery et al. (2003) reported that the administration of ET-1 increases arterial pulse wave velocity (PWV), a traditional index of regional arterial stiffness, and treatment with an ET receptor antagonist reduces PWV. We also reported that the directly-measured central arterial compliance increased with $ET_{A/B}$ dual receptor blockade in humans (Maeda et al., 2009). We found that arterial compliance increases significantly with the administration of $ET_{A/B}$ dual receptor blockade humans (Maeda et al., 2009). Taken together, these results suggest that endogenously produced ET-1 contributes to the regulation of arterial stiffness and arterial compliance in the systemic circulation of humans. We previously reported that exercise training reduces plasma ET-1 levels (Maeda et al., 2001, 2003, 2009). Therefore, exercise training possibly could be a useful therapy for reducing ET-1 levels, thereby improving cardiovascular diseases including chronic kidney disease (CKD).

In humans, plasma levels of ET-1 obtained from peripheral vessels are very low, much lower than those of ET-1 and ET-3 (Miyauchi et al., 1991; Suzuki et al., 1991; Matsumoto et al., 1994). However, the present study shows that apparently high circulating levels of big ET-2, a precursor of ET-2, are present in patients with hemodialysis. This suggests that the ET-2 system might play some pathophysiological roles of patients with chronic hemodialysis. The present study also demonstrates that the magnitude of increase in patients with hemodialysis was highest for big ET-2 (6-fold increase), followed by big ET-3 (5-fold increase), and big ET-1 (4-fold increase).

The observed changes of plasma levels of big endothelins in patients with chronic kidney disease suggest that ET-1 and ET-3 may play different roles in acute and chronic alterations of blood pressure in hemodialysis patients. Since plasma endothelin-like immunoreactivity has been reported to be elevated in various disease states in humans (Miyauchi et al., 1989; Masaoka et al., 1989; Cernacek and Stewart, 1989; Miyauchi and Masaki, 1999), the findings of the present study suggest that concentrations of each endothelin (i.e., separately measured levels of ET-1 and -3, and big ET-1, big ET-2, big ET-3) might be used as markers of disease in renal patients and when studying the physiological/pathophysiological roles of endothelin in human diseases.

Table 1
Plasma levels of endothelins (ET-1 and ET-3) in healthy subjects and patients on hemodialysis.

	ET-1	ET-3
Healthy subjects (n = 17)	1.5 ± 0.1	0.8 ± 0.1
Patients on hemodialysis (n = 23)	2.6 ± 0.3*	1.7 ± 0.2*

* p<0.05 vs healthy subjects. Values are pg/ml and given as mean ± SEM.

Conflict of interest statement

There are no financial, consultant, institutional and other relationships to disclose that might lead to bias or pose a conflict of interest.

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