CLINICAL STUDY

Adrenal endothelin-1 levels are not associated with aldosterone secretion in primary aldosteronism

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Abstract

Background: Endothelin-1 (ET-1) may function as an aldosterone secretagogue and, in turn, aldosterone can upregulate ET-1 expression. Hence, the existence of a feedforward loop involving ETs and aldosterone has been speculated in primary aldosteronism (PA). In the present study, we sought to examine ET-1 secretion from the adrenal glands in patients with PA.

Design: We determined ET-1 levels in blood samples obtained during adrenal venous sampling of patients affected by PA ($n=17$). Furthermore, we examined the mRNA expression of the ET system in tissue samples from aldosterone-producing adenomas (APAs, $n=9$) and control normal adrenals ($n=3$).

Methods: Blood ET-1 levels were determined by RIA. Tissue mRNA expression of the ET system was assayed with Affymetrix microarrays.

Results: ET-1 levels did not differ between inferior vena cava and adrenal vein blood in both bilateral adrenal hyperplasia and APA patients. Moreover, cortisol-normalized ET-1 levels did not show lateralized adrenal ET-1 secretion in APAs. Through gene expression profiling with microarray performed in a distinct set of APA individuals ($n=9$), we confirmed the adrenal expression of a complete ET system, but we did not detect a significant upregulation of ET components within the APA tissue compared with normal adrenals.

Conclusions: The present data argue against the hypothesis of increased ET-1 secretion from APAs and do not support a general role for adrenal ET-1 in the vascular pathophysiology of PA.

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Introduction

Endothelins (ET-1, ET-2, and ET-3) are potent vasoactive peptides with biological activity in several cardiovascular diseases, including pulmonary hypertension (1, 2), systemic arterial hypertension (3), and heart failure (4). Several lines of evidence have suggested that there is a functional link between ETs and aldosterone. First, ETs (in particular ET-1 and ET-2) have been shown to act via ET(A) and ET(B) receptors as aldosterone secretagogues *in vitro* and *in vivo* (5–11). Secondly, ETs may promote activation and proliferation of adrenal cells (12). Recent studies have also shown that aldosterone can, in turn, upregulate ET-1 expression (13–15). Since normal adrenal tissue and adrenal adenomas express components of the ET system (16–18), a positive feedback loop between ETs and aldosterone may be hypothesized. Moreover, any ET spillover from the adrenal gland may have pathophysiological and diagnostic implications.

Primary aldosteronism (PA) is the most common secondary form of hypertension and accounts for 5–10% of all hypertensive patients (19). PA is

characterized by inappropriate aldosterone secretion, mostly from an aldosterone-producing adrenal adenoma (APA) or from idiopathic bilateral adrenal hyperplasia (BAH) (20). Our group and others have shown in previous studies that ET-1 levels in peripheral blood are not elevated in PA patients when compared with matched essential hypertensive patients (21, 22). However, peripheral blood ET-1 levels may not reflect adrenal blood levels. In order to assay the adrenal activation of the ET system in PA directly, we have measured ET-1 in samples obtained from adrenal venous blood and we have compared the expression levels of ETs, endothelin-converting enzymes (ECE-1, ECE-2), and ET receptors ET(A) and ET(B) in tissue samples from APAs and control normal adrenal tissue.

Materials and methods

Patients

The study protocol and procedures were approved by our institution's responsible committee and all patients

provided written informed consent. The study involved two different groups of patients with PA (outlined in Table 1). PA and its subtypes, APA and BAH, were diagnosed according to widely accepted diagnostic criteria (20, 23). Briefly, patients were screened using the aldosterone plasma aldosterone concentration (PAC)/plasma renin activity (PRA) ratio (ARR). The ARR cut-off level considered to be ‘positive’ was (40 ng/dl per ng×ml⁻¹ h⁻¹; 4000 pmol/l per ng×l⁻¹ s⁻¹) together with an aldosterone level of > 15 ng/dl (416 pmol/l). Blood samples were obtained in the sitting position between 0800 and 1000 h. Patients discontinued all anti-hypertensive drugs at least 3 weeks before PAC and PRA measurements were taken. Diuretics were stopped at least 6 weeks before and spironolactone stopped at least 8 weeks before testing. Patients were advised to maintain a diet of normal and constant sodium intake (120 mmol sodium and 60 mmol potassium per day). Patients who strictly required drug therapy were permitted to take an α-blocker (doxazosin) and/or a calcium channel blocker (verapamil or amlodipine). This therapy was maintained throughout screening to final diagnosis.

The confirmatory test was an i.v. saline load (2 l of 0.9% NaCl infused over 4 h), which was considered positive if post-test aldosterone levels were > 5 ng/dl (138.7 pmol/l) (24). Fine-cut CT scanning (2.5 mm) of the adrenal glands was performed in all patients with a positive confirmatory test. Adrenal vein cannulation was considered successful if the adrenal vein/inferior vena cava (IVC) cortisol gradient was at least 2 (catheterization ratio); lateralization was considered when the aldosterone/cortisol ratio (A/C) from one adrenal was at least four times the ratio from the other adrenal gland (lateralization ratio, (LR)) or if it was three times the contralateral together with an A/C in the contralateral lower than the A/C in the peripheral vein (contralateral ratio). All patients underwent adrenal vein sampling (AVS) without ACTH stimulation. The presence of an adenoma was confirmed after adrenalectomy. Finally, all patients with PA were screened for glucocorticoid-remediable

aldosteronism using a long PCR technique (25). PRA, cortisol, and aldosterone were measured as described previously (25).

A final diagnosis of APA was considered ‘proven’ only when all of the following conditions were satisfied: i) histological demonstration of adenoma, ii) normalization of hypokalemia if present, iii) cure (i.e., normal blood pressure levels without treatment) or improvement (i.e., normal blood pressure achieved with a reduced number of drugs compared with before adrenalectomy) of hypertension, and iv) normalization of ARR and suppressibility of aldosterone levels below 5 ng/dl under saline load. All patients with a diagnosis of APA underwent adrenalectomy and had the above conditions satisfied. In particular, all patients had an ARR < 20 and a PRA > 1 after adrenalectomy.

To determine the ET-1 levels in adrenal vein blood, 17 adult patients with confirmed PA undergoing AVS were included. Based on AVS, seven were diagnosed with APA and ten with BAH. For our gene expression assays, we obtained normal adrenal tissue samples (n = 3) from patients undergoing monolateral nephrectomy and consensus ipsilateral adrenalectomy for localized (T1-2, N0, M0) renal carcinoma and who were otherwise healthy and normotensive. APA tissue samples (n = 9) were obtained from patients undergoing therapeutic monolateral adrenalectomy. All tissue samples were collected at the time of adrenalectomy, briefly washed with cold saline, immediately submerged in RNAlater solution (which preserves RNA integrity until extraction), and stored at -80 °C until further use.

Endothelin-1 measurement

Only samples obtained from successful and diagnostic adrenal vein cannulation were used, as demonstrated by the concomitant measurement of cortisol levels (20, 23). Adrenal vein blood samples were collected in chilled EDTA-treated test tubes during routine AVS. Plasma was separated by centrifugation, added with aprotinin (0.6 TIU/ml blood), immediately snap frozen, and stored at -80 °C until further use. Plasma ET-1 was measured by RIA (DRG Inc., Mountainside, NJ, USA) after extraction with SPEC C18 cartridges, according to the manufacturer’s protocol. Assays were run in duplicate, and values were expressed in picograms per milliliter (pg/ml). The intra- and interassay coefficients of variation were respectively 4 and 9%. To account for cannulation variability, raw ET-1 concentrations in adrenal blood samples were normalized by local cortisol concentrations (cortisol-normalized ET-1, calculated as ET-1 concentration divided by cortisol concentration in the same sample, pg ET-1/μg cortisol). Finally, an ET-1 LR was determined (ET1-LR, cortisol-normalized ET-1 on side 1 divided by cortisol-normalized ET-1 on side 2).

Table 1 Clinical and anthropometric parameters of enrolled individuals (adrenal blood endothelin-1 (ET-1) study and adrenal microarray study).

	Adrenal blood ET-1 study	ET system gene expression study
N	17	9
M/F	13/4	5/4
Age (years)	54±7	54±8
BMI (kg/m ²)	28.4±4.6	26.5±2.9
Creatinine (mg/dl)	1.0±0.2	0.9±0.3
Potassium (mEq/l)	3.6±0.7	3.1±0.9
PRA (ng Ang l/ml per hour)	0.3±0.3	0.2±0.1
Aldosterone (pg/ml)	419±228	530±279

RNA extraction and microarray analysis

After adrenalectomy, the adrenal glands were first carefully dissected, removing the surrounding connective tissues and separating the adrenal cortex from the medulla. For both APA and control samples, RNA was extracted from 100 mg of adrenal tissue. In the case of APAs, the tissue samples for RNA extraction were obtained within the adenoma, discarding the capsule. For control adrenal glands, the adrenal cortex was cut on its transversal axis and the tissue sample for RNA extraction was obtained from the outer part of the cortex. In preliminary studies, we verified on histological sections that this area spanned across the zona glomerulosa and part of the fasciculata zone of the adrenal cortex. Total RNA was extracted with TRIzol reagent and precipitated in isopropanol, following standard protocols (26). All RNA samples were assayed for purity and quality, using an Agilent Bioanalyzer, according to the manufacturer's instructions. For gene expression profiling studies, we chose the oligonucleotide Affymetrix GeneChip Human Genome U133 Plus 2.0 Array, which allows analysis of more than 47 000 transcripts. We performed cRNA synthesis and labeling, hybridization, and scanning, following the manufacturer's protocols as previously described (27).

CEL files were analyzed using dChip 2006 Software (9). Briefly, the data underwent invariant set normalization in order to adjust for differences in overall array brightness. Expression levels of probe sets were then computed using the model-based expression indexes. Next, we queried for further analysis all the probe sets ($n=20$) exploring the gene transcripts of the endothelin system (ET-1, ET-2, ET-3, ECE-1, ECE-2, ET(A), and ET(B)). Only probe sets producing a presence (P) call in $> 50\%$ of normal adrenal samples and in $> 50\%$ of APA samples were considered. For each filtered probe set, a fold change value was determined as the ratio of average expression level in the APA and in the normal adrenal group.

Microarray results were validated using quantitative real-time PCR (qRT-PCR). qRT-PCR was performed in a two-step fashion. RT was performed in the presence of 50–100 ng/ μ l total RNA, 10 ng/ μ l random primers, 500 nM dNTPs, 0.1 M dithiothreitol, and 10 U/ μ l SuperScript II Reverse Transcriptase (Invitrogen) in first-strand buffer. Quantitative PCR was subsequently performed using TaqMan chemistry (ABI) on an Applied Biosystems ABI 7500 instrument, following the standard protocols. Primers and probes were pre-designed assays by ABI. Gene expression levels were analyzed using the $2^{-\Delta\Delta C_t}$ relative quantification system, using β -actin as the endogenous control gene.

Statistical analysis

Values are expressed throughout as average $\pm$ S.E.M. Differences between groups were analyzed by one-way

ANOVA or Student's *t*-test, using $P < 0.05$ as statistically significant.

Results

ET-1 levels in AVS samples

We measured ET-1 levels in blood samples obtained from the right and left adrenal veins and from the IVC during AVS performed in 17 patients with PA, as described above. The clinical and anthropometric parameters of patients are reported in Table 1.

Overall, average ET-1 levels in the adrenal veins ($n=17$) were similar to ET-1 levels in the IVC (5.2 ± 0.5 vs 4.3 ± 0.5 pg/ml, $P=0.27$). Also, in APA patients ($n=7$), no difference was observed in the ET-1 levels in the adrenal veins when compared with those in the IVC (5.0 ± 1.2 vs 4.1 ± 0.9 pg/ml, $P=0.54$). Furthermore, raw ET-1 levels in adrenal vein blood were similar ipsilaterally and contralaterally to the APA (6.3 ± 2.2 vs 3.7 ± 0.7 pg/ml, $P=0.27$). Even after normalization for local cortisol levels (to account for sampling variability related to adrenal vein cannulation), normalized adrenal blood ET-1 levels did not differ ipsilaterally and contralaterally to the APA (4.3 ± 2.5 vs 7.2 ± 3.4 pg ET-1/ μ g cortisol), a finding that rules out clear-cut ET-1 lateralization in APAs (Fig. 1A). Average ET1-LR in APAs was 1.5 ± 0.7 (range 0.1–5.3), with only one APA showing lateralized ET-1 secretion (ET1-LR 5.3) and all others presenting ET1-LR lower than 1 ($n=3$) or between 0 and 2 ($n=3$).

In BAH patients ($n=10$), ET-1 levels were similar in adrenal veins and IVC (right adrenal vein 5.6 ± 0.4 pg/ml, left adrenal vein 5.2 ± 0.5 pg/ml, IVC 4.5 ± 0.6 pg/ml, $P=0.28$). No difference was detected between right and left cortisol-normalized adrenal blood ET-1 levels (Fig. 1B). ET1-LRs ranged between 0.1 and 17.8, with most individuals ($n=7$) presenting an ET1-LR between 0.5 and 2.0.

Gene expression of the ET system in adrenal samples

To validate these results using a different technological platform and a different set of subjects, we examined the gene expression fingerprint of the ET system in adrenal tissue samples obtained from patients with APA ($n=9$) and subjects with normal adrenals ($n=3$). Microarray data analysis was performed as described in 'Materials and methods' section. The clinical and anthropometric parameters of patients and controls are reported in Table 1.

Like other investigators (17, 18), we detected a significant expression of ET-1, ECE-1, ECE-2, ET(A), and ET(B) in all adrenal samples. Based on signal/noise criteria, however, the signal we obtained from probes exploring ET-2 and ET-3 was non-significant, and we

Figure 1 Scatter plots of cortisol-normalized adrenal blood ET-1 levels in enrolled individuals. (A) Cortisol-normalized ET-1 levels (see Materials and methods section) in adrenal blood samples of APA patients ($n=7$), pg ET-1/ μ g cortisol. APA side, adrenal vein ipsilateral to the APA; other side, adrenal vein contralateral to the APA. (B) Cortisol-normalized ET-1 levels in adrenal blood samples of BAH patients ($n=10$), pg ET-1/ μ g cortisol. Right and left refer to the side of the adrenal vein.

considered ET-2 and ET-3 to be absent in the study samples. The average normalized probe intensities (which represent expression levels and allow for inter-array comparison) of ET system mRNAs are shown in Fig. 2. No significant difference was observed between ET-1 mRNA levels in APAs and in normal adrenal samples (fold change: -1.06 , $P=0.84$). Similarly, ECE-1 (fold change: -1.01), ECE-2 (fold change: -1.31), ET(A) (fold change: -1.02), and ET(B) (fold change: -1.04) were all similarly expressed in APA samples when compared with normal adrenals ($P>0.05$). Microarray findings were further confirmed by quantitative real-time PCR (data not shown).

Discussion

ETs are peptides involved in cardiovascular physiology and disease. ETs can also stimulate aldosterone secretion from adrenal cells (5–11), and aldosterone may, in turn, upregulate ET-1 expression (13–15).

Figure 2 mRNA expression of ET-1, ECE-1, ECE-2, ET(A), and ET(B) in APA ($n=9$) and normal adrenal ($n=3$) tissue samples. Expression is based on the microarray signal intensity of corresponding probes.

Several components of the ET system (ET-1, ECE-1, ET(A), and ET(B)) have been detected in normal adrenals and in APAs (16–18), a prototypical cause of PA. Overall, these findings have led investigators to speculate that a feedforward loop of ETs and aldosterone may be involved in the pathogenesis of PA. Although ET-1 levels in the peripheral blood PA patients are similar to the levels in controls (21, 22), peripheral blood levels may not be informative about local adrenal ET secretion. To address this issue, we have evaluated ET-1 levels in blood samples obtained from the adrenal veins of patients with PA. These vessels collect venous blood arising from the adrenal glands. Their cannulation and sampling for the measurement of local aldosterone and cortisol levels is the gold standard in the diagnosis workup of PA (23). In this study, we did not detect a significant difference in the ET-1 levels between adrenal vein and IVC samples. Also, in BAH, which is characterized by excessive bilateral aldosterone secretion, the levels of ET-1 in adrenal veins and IVC were similar. ET-1 levels were also similar in the adrenal vein samples both ipsilaterally and contralaterally to APAs.

In addition to the above studies, we quantitatively assessed mRNA expression of the various ET system components in a group of APA samples when compared with normal control adrenals. Our microarray assay confirmed the expression of ET-1, ECE-1, and both ET receptor in adrenal tissues (normal and adenomatous), as reported in the previous studies (16–18). ET-2 and ET-3 mRNA was undetectable. We also found significant levels of adrenal ECE-2 in normal adrenals and APA. Overall, the ET system appears to be fully expressed in the adrenal tissue. However, we could not detect significant differences in ET-1, ECEs, and ET receptor expression levels in APA samples when compared with normal adrenal controls. These last data, obtained in a

different set of patients and confirmed by qRT-PCR, corroborate our ET-1 immunoassay findings.

Previous studies have reported significant heterogeneity in the gene expression fingerprints and overall phenotypes of APAs (28, 29). We did detect significantly higher levels of ET-1 in one subject ipsilaterally to the APA. We also found that in one of the APA tissue samples, ET-1 mRNA was 1.64-fold upregulated when compared with normal adrenal tissue samples. Hence, we cannot rule out that some APAs may have ET-1 overproduction and secretion in the bloodstream. Further studies of ET expression and adrenal blood levels in a larger number of subjects will be necessary to address the present issue.

Overall, our data undermine the hypothesis that adrenal gland ET-1 secretion is enhanced in PA and argue against a general role for adrenal ETs in the vascular pathophysiology of PA. However, a number of questions remain open. First, our data do not rule out a paracrine role for the ET system in the adrenal biology of PA. In fact, it is possible that a vascular–adrenal cell feedforward loop between ET-1 and aldosterone may be locally active without affecting ET-1 levels in the collecting venous vessels. Furthermore, a longer feedforward loop between aldosterone and ET-1 could be active in other tissues. In fact, it is possible that in PA the high levels of circulating aldosterone may lead to a local overexpression of ET-1 in vessels and tissues, hence contributing to the vascular pathophysiology and target organ damage of PA. Finally, although circulating ET-1 levels of PA patients are similar to those of control hypertensive individuals, it is also conceivable that aldosterone and ET-1 may work in biological synergy, leading to increased ET-1 and/or aldosterone actions in PA.

Declaration of interest

The authors declare no conflicts of interest that could be perceived as prejudicing the impartiality of the research.

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