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Downregulated serum 14, 15-EET is associated with abdominal aortic calcification in patients with primary aldosteronism

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Abstract

Patients with primary aldosteronism (PA) have increased risk of target organ damage, among which vascular calcification is an important indicator of cardiovascular mortality. 14, 15-epoxyeicosatrienoic acid (14, 15-EET) has been shown to have beneficial effects in vascular remodeling. However, whether 14, 15-EET associates with vascular calcification in PA is unknown. Thus, we aimed to investigate the association between 14, 15-EET and abdominal aortic calcification (AAC) in patients with PA. 69 patients with PA and 69 controls with essential hypertension, matched for age, sex, and blood pressure, were studied. 14, 15-dihydroxyeicosatrienoic acid (14, 15-DHET), the inactive metabolite from 14, 15-EET, was estimated to reflect serum 14, 15-EET levels. AAC was assessed by computed tomographic scanning. Compared with matched controls, the AAC prevalence was almost one-fold higher in patients with PA [27 (39.1%) *vs.* 14 (20.3%), $P = 0.023$], accompanied by significantly higher serum 14, 15-DHET levels [(7.18 ± 4.98) *vs.* (3.50 ± 2.07) ng/mL, $P < 0.001$]. Plasma aldosterone concentration was positively associated with 14, 15-DHET ($\beta = 0.444$, $P < 0.001$). Multivariable logistic analysis revealed that lower 14, 15-DHET was an independent risk factor for AAC in PA (odds ratio [95% confidence interval], 1.371 [1.145-1.640], $P < 0.001$), especially in young patients with mild hypertension and normal body mass index. In conclusion, PA patients exhibit more severe AAC, accompanied by higher serum 14, 15-DHET levels. On the other hand, decreased 14, 15-EET was significantly associated with AAC prevalence in PA patients, especially in those at low cardiovascular risk.

Keywords: 14, 15-epoxyeicosatrienoic acid; 14, 15-dihydroxyeicosatrienoic acid; primary aldosteronism; abdominal aortic calcification; inflammation.

Introduction

Primary aldosteronism (PA), characterized by autonomous aldosterone secretion and suppressed plasma renin activity (PRA), accounts for 5%-13% of resistant hypertension and accordingly is not common in the clinic. Patients with PA are at increased risk of target organ damage, especially cardiac and renal complication.[1,2] Cardiovascular diseases (CVD) are the leading causes of death in PA patients, which account for 50% mortality.[3] Hypertension is an important risk factor for CVD. Effective antihypertensive medication or surgery prevents CVD in PA patients.[4] However, some PA patients still exhibit severe cardiovascular complications even with treatments.[5] It indicates that some other nontraditional risk factors may be also involved in the development of CVD in PA patients.

Vascular calcification (VC), especially abdominal aortic calcification (AAC), an important nontraditional risk factor, is associated with high risk of CVD.[6] Findings from our studies and others show that hyperaldosteronism is significantly associated with increased VC.[7,8] It was reported that AAC served as independent risk factor of persistent hypertension in patients undergoing adrenalectomy.[9] Thus, AAC may be another important indicator of CVD in PA patients and understanding the mechanisms of AAC is critical. Many factors such as inflammatory cytokines are closely related to VC development.[10] Notably, vascular inflammation was commonly seen in PA patients and associated with pronounced vascular alterations.[11] Identifying the key factors underlying inflammation will facilitate the development of targeted therapies for reducing CVD in PA population.

Metabolized from arachidonic acids, epoxyeicosatrienoic acids (EETs) are important anti-inflammatory factors, which have protective effects on cardiovascular homeostasis. There are four types of EETs, 5, 6-, 8, 9-, 11, 12-, 14, 15-EET, among which 14, 15-EET is of high concentration in vasculature and have the closest relationship with CVD.[12] In patients with established coronary heart disease, increasing serum EET levels was associated with lower risk of CVD.[13,14] The polymorphism of the soluble epoxide hydrolase (sEH) gene was a significant predictor of coronary artery calcification status even after adjusting for traditional risk factors[15]. Accumulating preclinical and epidemiologic evidence suggest that inhibition of sEH-mediated EET hydrolysis has various cardiovascular protective effects including anti-inflammation.[16,17] As EETs are easily hydrolyzed and hard to detect directly, the levels of their metabolite, dihydroxyeicosatrienoic acids (DHETs), are commonly used to reflect EET levels, which are also reported to be closely associated with CVD.[18,19] Therefore, in our study, we assessed 14, 15-DHET as an indirect measure of 14, 15-EET levels.

Pre-clinical studies showed that the aldosterone infusion in rats increased sEH protein expression in renal cortex and microvasculature. Aldosterone treatment of endothelial cells also significantly increased mRNA expression of sEH.[20] In deoxycorticosterone acetate (DOCA)-salt treated mice, a model that mimics hyperaldoteronism, we demonstrated that the level of 20-hydroxyeicosatetraenoic acid was significantly reduced and further contributed to increased sodium retention and blood pressure.[21] This phenomenon indicated that excess aldosterone secretion

inhibited EET production. However, whether downregulation of 14, 15-EET is associated with vascular damage in patients with PA is unknown. Thus, we conducted a case-control clinical study to measure serum 14, 15-DHET and investigated its role in AAC in patients with PA.

Methods

The authors declare that all supporting data are available within the article.

Study population

This was a single-center, case-controlled study conducted in Sun Yat-sen memorial hospital of Sun Yat-sen University. From January 2013 to June 2015, a total of 117 patients suspicious of PA who were admitted to our unit, 20 patients who did not meet the following inclusion criteria or met the exclusion criteria were excluded. And we also excluded 28 patients missing the data of computerized tomography (CT) or serum biomedical tests. Baseline clinical and biochemical data were extracted from the hospital database.

Inclusion of PA cases and EH controls were those who met the diagnostic criteria as detailed below. Patients with clinical/or laboratory evidence of associated conditions were excluded from this study, such as: (1) administration of any antihypertensive drugs within two weeks before recruitment; (2) recent infection inflammatory disorders, or hormonal replacement therapies; (3) history of chronic kidney diseases, hepatic diseases, rheumatologic diseases or malignancy including adrenocortical carcinoma.

This study protocol conformed to the ethical guidelines of the 1975 Declaration of

Helsinki by the Ethics Committee of Sun Yat-sen University, and written informed consent was obtained from every study participants.

Our screening methods and diagnostic criteria for PA and EH were in accordance with the current guideline.[22] After withdrawal of medication influencing the renin-aldosterone system, patients were screened for PA using PAC to PRA [aldosterone-to-renin ratio (ARR), ng/L per ng/mL/h], with a cutoff of 25ng/L per ng/mL/h in the standing position. Diagnosis of PA was confirmed by the failure of aldosterone suppression after the oral sodium loading test (24-hour urinary aldosterone concentration≥10μg/24h) and captopril test (PAC≥130ng/L) as previously described.[22,23]

During the same period, patients with EH were included when meeting the following criteria: a known history of hypertension with anti-hypertensive drugs treatment; and/or three documented office systolic blood pressure (SBP) ≥ 140 mmHg and /or diastolic blood pressure (DBP) ≥ 90 mmHg at different days; secondary forms of hypertension were excluded by reviewing records for medical history, physical examination, and appropriate biochemical tests and imaging studies. Notably, only patients who had a normal ARR were included as EH controls. In the present study, patients with PA and EH controls were 1:1 individually matched for age (± 3 years), gender and blood pressure (± 5mmHg) were included as controls.

Multi-detector CT analysis of abdominal aorta

All patients underwent an adrenal CT scan to evaluate abdominal aortic plaques. The plaques occupied by calcified tissue more than 50% of the plaque area (an area ≥ 1

154 mm^2 with density of > 130 HU) were classified as calcified plaques (CPs).[24] All
155 imaging procedures were done on the same equipment using the same parameters. To
156 measure AAC, the CT images were reconstructed in a 35 cm field of view with a slice
157 thickness of 1 mm. All the scans were read by the SIEMENS Syngo CT Workplace at
158 the same radiological department in our unit, and calcification in the distal abdominal
159 aorta above the aortic bifurcation was used for analysis. AAC Agatston score was
160 calculated by multiplying each CP area volumes by a weighted score assigned to the
161 highest density of calcification (1 for 130-199 HU, 2 for 200-299 HU, 3 for 300-399
162 HU, 4 for 400 HU and greater) within the individual CP area. According to the AAC
163 score, patients were grouped as having no detectable AAC (Agatston score = 0), mild
164 (1-100), and severe (> 100) AAC as previously described.[25] All the abdominal arterial
165 datasets were analyzed by two blinded and experienced investigators.

166 **14, 15-DHET measurements**

167 Peripheral venous blood samples were collected from each recruited patient at 7:00
168 a.m. before patients had the breakfast. After repeating the procedures of acidification,
169 extraction and saponification for three times, we pooled all the organic phase (ethyl
170 acetate) together and evaporated under argon gas. Then, we dissolved the dried
171 residue of each sample in a minimal amount of ethanol (~20uL). An enzyme-linked
172 immunosorbent assay (ELISA) was used to measure the plasma 14, 15-DHET (14,
173 15-DHET ELISA kit; Detroit R&D Inc., Detroit, MI, USA) according to the manual.[26]

174 **Laboratory testing**

175 Blood samples were drawn between 08:00 a.m. and 11:00 a.m. after at least two-hour

upright posture, usually after they had been seated for 5-15min. Aldosterone in plasma and urine were measured by radioimmunoassay using a commercial kit Diagnostic Products (DSL, Texas, USA). The intra- and inter-assay coefficients of variation for PAC were 4.5% and 9.8%, respectively, and the reference range was 38.1-313.3 ng/L. Plasma renin activity (PRA) as the generation of angiotensin I in vitro was determined as previously described. The intra- and inter-assay coefficients of variation for PRA were 5.6% and 10%, respectively, and the reference range was (2.63 ± 1.32) ng/mL/h. Biochemical parameters, potassium, calcium (Ca), phosphorus (Pi), creatinine, alkaline phosphatase (ALP), fasting plasma glucose, HbA1c, triglyceride (TG), total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), high-sensitivity C-reactive protein (Hs-CRP), urinary albumin excretion rate (UAER) were analyzed using a standardized and certified TBA-120 auto-analyzer (Toshiba Medical Systems, Japan) in the institutional central laboratory. Estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration equation with modified coefficients for the Chinese population.[27]

Statistical analysis

Continuous variables with a normal distribution were reported as mean (± SD), skewed data as median (interquartile range). Categorical variables were presented as numbers (percentages). Baseline variables between patients with PA and matched EH were compared using paired Student's t-test, Wilcoxon sign test or McNemar test according to the types of variables. Correlation analysis and linear regression analysis

was used to explore the factors affecting serum 14, 15-DHET levels and AAC severity in PA patients. Univariate and multivariate logistic analysis were used to investigate the association between serum 14, 15-DHET and AAC scores as well as in further subgroup analysis. Data were expressed as the odds ratio (OR) and 95% confidence interval (CI). Subgroup analysis was divided according to the cardiovascular risk factors affecting AAC.[28] Data were analyzed with SPSS version 20 (SPSS, Inc, Chicago, Illinois, USA), and two-side *P* values less than 0.05 were considered statistically significant.

Results

Baseline characteristics of the study population

The clinical and biochemical characteristics of 69 patients with PA caused by aldosterone-producing adenomas and 69 EH controls are shown in **Table 1**. Patients with PA had higher PAC, ARR and lower PRA, serum potassium than matched EH patients (all $P < 0.05$). Patients with PA showed lower BMI, accompanied by lower serum levels of LDL-C, HDL-C and TG (all $P < 0.05$). In contrast, UAER and urinary aldosterone were significantly higher in PA patients than that in controls (both $P < 0.05$). However, no significant difference was found in the other biochemical parameters (serum Ca, Pi, TC, creatinine, ALP, Hs-CRP, fasting plasma glucose, HbA1c and eGFR) between these two groups. Of interest, patients with PA had a higher prevalence of abdominal aortic CPs than matched EH controls [27 (39.1%) *vs.* 14 (20.3%), $P = 0.023$]. Moreover, the degree of AAC tended to be more severe in PA patients than EH patients [no AAC: mild AAC: severe AAC, (60.9%: 20.3%: 18.8%)

220 *vs.* (79.7%: 14.5%: 5.8%), $P = 0.002$] (**Table 1**).

221 **Risk factors of serum 14, 15-DHET in PA patients**

222 As shown in **Figure. 1**, serum 14, 15-DHET levels were significantly higher in
223 patients with PA than matched EH controls [(7.18 ± 4.98) *vs.* (3.50 ± 2.07) ng/mL, $P <$
224 0.001]. Notably, PA characteristic factors, including serum potassium or PRA, showed
225 no significant relationship with serum 14, 15-DHET (**Figure S1** and **Figure S2** in the
226 **online-only Data Supplement**). In order to explore whether LnPAC was an
227 independent ~~possible~~ risk factor~~s~~ influencing 14, 15-DHET in PA, multiple linear
228 regression analysis was used. We found that LnPAC was an independent risk factor of
229 increased serum 14, 15-DHET after adjustment for age, BMI, SBP, DBP and serum
230 lipid profiles ($\beta = 0.444$, $P < 0.001$) (**Table 2**).

231 **Association between serum 14, 15-DHET and AAC scores in patients with PA**

232 To evaluate the relationship between serum 14, 15-DHET and AAC scores in patients
233 with PA, we measured serum levels of 14, 15-DHET in PA patients with different
234 levels of AAC. As shown in **Figure. 2A**, serum 14, 15-DHET levels progressively
235 increased as the severity of AAC increased [Median 5.01 *vs.* 6.37 *vs.* 15.09 ng/mL, in
236 no AAC, mild AAC, severe AAC group, respectively, $P < 0.001$]. Moreover, serum 14,
237 15-DHET levels were significantly associated with AAC scores in patients with PA (r
238 $= 0.593$, $P < 0.001$; **Figure. 2B**).

239 **Identifying the PA population with high-risk of AAC**

240 As shown in **Table 3**, 14, 15-DHET was significantly associated with increased risk
241 of AAC on univariate analysis (OR = 1.329, 95% CI 1.153-1.532, $P < 0.001$). This

association remained after adjusting for age, SBP, Ca, Pi, ALP, LDL-C, Hs-CRP, and eGFR (OR = 1.371, 95% CI 1.145-1.640, $P < 0.001$). In order to explore the specific PA patients with increased risk of AAC induced by 14, 15-EET alteration, we performed subgroup analysis based on traditional risk factors. As shown in **Figure. 3**, we found that the positive association between serum 14, 15-DHET level and AAC extent was significant in PA patients with age less than 50 years (OR = 1.552, 95% CI 1.116-2.158, $P = 0.009$), mild hypertension (OR = 1.530, 95% CI 1.158-2.020, $P = 0.003$) and normal BMI (OR = 1.320, 95% CI 1.063-1.639, $P = 0.012$). However, the association between 14, 15-DHET and AAC in PA was not significant in gender subgroups (both $P > 0.05$).

Discussion

Major findings from our study demonstrate that 1) patients with PA exhibit more frequent and more severe AAC compared with matched EH controls. 2) increased serum 14, 15-DHET, which possibly associates with excess PAC, is an independent risk factor for AAC, especially in PA patients with age younger than 50 years with normal BMI and SBP lower than 160mmHg.

Target organ damage in PA patients is a major cause of cardiovascular complications worldwid.[29] Thus, early diagnosis and appropriate control of risk factors of CVD is critical for PA patients. By comparing PA patients with matched controls, our study revealed that VC might be a novel important risk factor for PA patients beyond blood pressure elevation. As a nontraditional indicator for CVD, VC has been reported to be associated with increased risk of CVD.[6] So exploring the

possible mechanisms of VC has caused great attention. Instead of passive calcium and phosphate precipitation previous thought, VC is now thought to be an actively regulated process.[30] Many factors may contribute to the development of VC, among which chronic inflammation is a key player.[31,32] In humans, focal arterial inflammation, as quantified by ^{18}F-fluorodeoxyglucose/positron emission tomography, was suggested to precede calcification within the same locations.[33] Fish oils, such as eicosapentaenoic acid, inhibited osteoblastic differentiation in vascular smooth muscle cells as well as VC through anti-inflammatory effects on nuclear factor-κB.[34] In support this, our study revealed that low anti-inflammatory 14, 15-EET, reflected by high 14, 15-DHET levels, was an independent risk factor of AAC in PA patients. Therefore, suppressing inflammation by increasing EETs may be cardiovascular protective and reduce the risk of VC in PA patients.

Produced and generated from endothelial cells, EETs possess potent vasodilatory and anti-inflammatory effects in maintaining vascular homeostasis.[35] It was reported some isoforms of EETs (such as 11, 12-EET) except for 14, 15-EET might induce vasoconstriction when cyclooxygenases/prostaglandins signaling was altered.[36] However, our study patients had no history of taking non-steroidal drugs, which is known to influence prostaglandins balance. It still warrants further investigation to identify this issue. Clinical studies have demonstrated that increasing EET levels have utility as a cardioprotective therapeutic strategy in coronary heart diseases, stroke, diabetes, *et al*.[37-39] Many factors influence EET metabolism, including obesity, age and serum lipids. In obese individuals with coronary heart disease, increased body

mass was significantly associated with low plasma EET levels and 14, 15-EET/14, 15-DHET ratio.[14] Increasing EET levels by sEH inhibitors also had anti-atherosclerotic effects, which was associated with LDL-C reduction and HDL-C elevation.[26,40] But in PA patients, we observed that excess PAC was independently associated with higher serum 14, 15-DHETs after adjustment for these traditional risk factors. In DOCA-salt mice, production of 20-hydroxyeicosatetraenoic acid was reduced.[21] Clinical studies revealed that abnormal activation of the renin-angiotensin-aldosterone system might be the main reason for altered production of EETs by increasing sEH expression.[35] Our study also showed that excess PAC was associated with decreased 14, 15-EET levels in PA patients.

Another interesting result of our study was the association between serum 14, 15-DHET levels and the extent of AAC. This was especially evident in younger PA patients with mild hypertension and normal BMI. These data suggest that in low-risk PA patients,[28] downregulation of 14, 15-EET may trigger AAC formation in the early stage. This may explain why older PA patients with severe hypertension, and abnormal nutrition, 14, 15-EET showed no effect on AAC. With a series of traditional cardiovascular risks, AAC in patients PA became advanced and severe, even increasing EETs could not bring further benefits to AAC regression. However, further investigation was still required to verify our results.

This study has several limitations that should be highlighted. Firstly, since the prevalence of PA is low in the Chinese population,[41] the number of PA patients enrolled in the study was relatively small. Therefore, further studies with larger

sample size are needed to verify our findings. Secondly, due to the cross-sectional design of the present study, causality between plasma 14, 15-DHET levels and AAC extent can not be established despite adjustment for possible factors. Thirdly, angiotensin II (Ang II) was not measured in our study, Ang II can upregulate EET production and thereby complicate interpretation.[42] However, overproduction of aldosterone is the characteristic feature of PA patients, and changes in levels of Ang II and changes in levels of angiotensin II may be inhibited because of the negative feedback, this may be difficult to assess. Further studies are needed to unravel this aspect. Fourthly, it was not possible to obtain vascular tissue for direct measurement of sEH activity, thus we measured 14, 15-DHET to reflect serum 14, 15-EET levels.[26] Finally, high-performance liquid chromatography was a standard method for quantifying cytochrome P450-derived eicosanoid metabolite concentrations.[43] In this present study, we used a simpler method, ELISA to specifically measure serum 14, 15-DHET levels. However, the results from both methods were proven to be comparable.[21]

In conclusion, we provide clinical evidence that patients with PA have significantly higher AAC compared with matched EH controls. Downregulaiton of 14, 15-EET is probably an important predictor of AAC in patients with PA, especially in younger PA patients with mild hypertension and normal BMI.

Perspectives

Our findings suggest that downregulated serum 14, 15-EET is closely with an increase of AAC in patients with PA. Therefore, measuring serum 14, 15-DHET, a

surrogate marker of 14, 15-EET, may provide a valuable additional tool for future AAC evaluation in PA.

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Disclosure

None.

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Novelty and Significance

What Is New?

- Primary aldosteronism (PA) is an important cause of-secondary hypertension with exaggerated target organ damage. We provide evidence that hyperaldosteronism is a risk factor of abdominal aortic calcification (AAC) and identify novel predictors.
- We show an association between 14, 15-epoxyeicosatrienoic acid (14, 15-EET) and AAC in PA patients.

What Is Relevant?

- Our findings define a new marker for target organ damage, specifically AAC, in PA patients.

- Decreased 14, 15-EET is significantly associated with AAC in PA patients.

Summary

We demonstrated for the first time that patients with PA had a high risk of AAC. Notably, low levels of anti-inflammatory 14, 15-EET, reflected by high 14, 15-DHET levels, was an independent risk factor of AAC in PA patients. New strategies to increase the anti-inflammatory EET may be cardiovascular protective and prevent AAC in patients with PA, especially in younger patients with mild hypertension and normal body mass index.

Figure legends

Figure 1. Comparison of serum 14, 15-DHET levels between patients with PA and EH. Each black dot referred to one patient. The middle horizontal line represented median values, 25th and 75th quartiles were shown as the lower and the upper line. 14, 15-DHET, 14, 15-dihydroxyeicosatrienoic acid; EH, essential hypertension; PA, primary aldosteronism.

Figure 2. The correlation between serum 14, 15-DHET levels and AAC scores in PA patients. A, The serum level of 14, 15-DHET in different degree of AAC groups. We found that the serum 14, 15-DHET levels were gradually elevated with AAC severity increased. Boxplots showing median values (horizontal line inside the box), quartiles (box boundaries), and the largest and smallest observed values (lines drawn from the end of the box) $^{*}P < 0.05$ *vs.* no AAC group; $^{**}P < 0.05$ *vs.* mild AAC group. **B**, Spearman correlation analysis showed that the serum levels of 14, 15-DHET were significantly associated with the AAC scores. ($r = 0.593$; $P < 0.001$). AAC, abdominal aortic calcification; 14, 15-DHET, 14, 15-dihydroxyeicosatrienoic acid; PA, primary aldosteronism.

Figure 3. Subgroup analysis of assessing the association between serum 14, 15-DHET and AAC score in PA. Multivariate logistic analysis after adjustment for Ca, Pi, ALP, LDL-C, Hs-CRP, and eGFR was perform in subgroup according to age (less than 50y or over than 50y), sex (male or female), SBP (less than 160mmHg or over than 160mmHg), BMI (20-25kg/m^2 or over than 25kg/m^2). Data were expressed as the odds ratio and 95% confidence interval. AAC, abdominal aortic calcification;

ALP, alkaline phosphatase; BMI, body mass index; Ca, calcium; 14, 15-DHET, 14, 15-dihydroxyeicosatrienoic acid; eGFR, estimated glomerular filtration rate; Hs-CRP, high-sensitivity-C-reactive protein; LDL-C, low density lipoprotein cholesterol; Pi, phosphate; SBP, systolic blood pressure; PA, primary aldosteronism.