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N-Palmitoylethanolamide protects the kidney from hypertensive injury in spontaneously hypertensive rats via inhibition of oxidative stress

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ABSTRACT

Hypertension is an important risk factor for kidney failure and renal events in the general population. Palmitoylethanolamide (PEA) is a member of the fatty acid ethanolamine family with profound analgesic and anti-inflammatory effects, resulting from its ability to activate peroxisome proliferator activated receptor (PPAR)α. A role for this nuclear receptor has been addressed in cardiovascular system and PPARα ligands have been shown to protect against inflammatory damage especially resulting from angiotensin II hypertension.

In this study, we demonstrated that PEA significantly reduced blood pressure in spontaneously hypertensive rats (SHR) and limited kidney damage secondary to high perfusion pressure. To investigate the mechanisms involved in PEA effect, we found that PEA reduced cytochrome P450 (CYP) hydroxylase CYP4A, epoxygenase CYP2C23 and soluble epoxide hydrolase enzyme expression in the kidney, accompanied by a reduction of 20-hydroxyeicosatetraenoic acid excretion in the urine. Moreover, it markedly reduced kidney oxidative and nitrosative stress accompanied by decreased expression of renal NAD(P)H oxidase and inducible nitric oxide synthase and increased expression of Cu/Zn superoxide dismutase, in the kidney of SHR. Moreover, angiotensin II receptor (AT) evaluation revealed a decrease in AT1 receptor expression and a restoration of AT2 receptor level in the kidney from PEA-treated SHR. Consistently, angiotensin converting enzyme expression was reduced, implying a decrease in angiotensin II synthesis. These results indicate that PEA treatment lowers blood pressure and can protect against hypertensive renal injury by increasing the antioxidant defense and anti-inflammatory response and modulating renin-angiotensin system.

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1. Introduction

N-Palmitoylethanolamine (PEA) is a fully saturated, and endogenous *N*-acylethanolamine, first identified half a century ago in lipid extracts from various tissues [1]. In addition to its known role in fatty acid metabolism, PEA produces profound analgesic and anti-inflammatory effects in several animal models [2–4]. Among the molecular mechanisms of PEA effects, we have previously demonstrated that its anti-inflammatory and analgesic effects were mediated by peroxisome proliferator activated receptor (PPAR)α, since PEA failed to exert these properties in PPARα knockout mice [4–7]. A part from PPARα capability in transcriptional repression of genes encoding for key pro-inflammatory proteins, such as tumor-necrosis factor-α, inducible nitric oxide synthase and cyclooxygenase-2 [8], very recently it has been demonstrated that

Abbreviations: PEA, palmitoylethanolamide; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator response elements; Ang II, Angiotensin II; RAAS, renin-angiotensin aldosterone system; SHR, spontaneously hypertensive rats; WKY, Wistar Kyoto; SBP, systolic blood pressure; HR, heart rate; HETE, hydroxyeicosatetraenoic acid; AT, angiotensin receptor; SOD, superoxide dismutase; ROS, reactive oxygen species; RNS, reactive nitrogen species; iNOS, inducible nitric oxide synthase; GFR, glomerular filtration rate; CYP, cytochrome P-450; AA, arachidonic acid; EET, epoxyeicosatrienoic acid; sEH, soluble epoxide hydrolase; ACE, angiotensin converting enzyme.

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PPARα stimulation by PEA induces the transcription of genes encoding for steroidogenic enzymes, resulting in an up-regulation of neurosteroids that contributes to the analgesic and anti-oxidant effects of PEA [9,10].

PPARs, members of the superfamily of ligand regulated transcription factors, are expressed in the cardiovascular system and control diverse vascular functions by mediating appropriate changes of gene expression [11], through the binding to specific peroxisome proliferator response elements (PPREs) [12]. In particular, PPARα, which is highly expressed in kidney, liver, and heart [13], has been shown to be involved in the control of blood pressure, and hypertensive-related complications, such as stroke and renal damage [14,15]. The protective role for PPARα activators identified in cardiovascular diseases has been ascribed to various effects. Beyond the effect on vascular function, mainly attributed to the hypolipidemic and high-density lipoprotein-raising actions, PPARα agonists exert renoprotective effect through anti-inflammatory [16] and anti-oxidant properties via the downregulation of inflammatory cytokines and reducing with oxidative stress [17]. It has been shown that PPARα suppressed oxidative stress through the inhibition of angiotensin II (Ang II)-induced activation of NADPH oxidase and [18] and the increase in scavanging enzymes. As a matter of fact a PPRE has been identified in promoter regions of catalase and Cu/Zn SOD genes, which are key enzymes that reduce ROS production [19]. Moreover, gene expression of renin-angiotensin aldosterone system (RAAS) molecules have been shown to be affected by PPARγ and PPARα, making the ligands of these transcription factors potential blood pressure modulating drugs in RAAS-dependent hypertension [20–22].

This study was designed to investigate whether PEA could reduce blood pressure and hypetension-related renal damage in spontaneously hypertensive rats (SHR) by reducing oxidative stress and modulating RAAS system.

2. Materials and methods

2.1. Animals and treatment

Eight-week-old male SHRs and Wistar Kyoto normotensive (WKY) rats, obtained from Harlan Italy (San Pietro al Natisone, Udine, Italy), were used for this study. Animals were housed in temperature (23 ± 2 °C)- and light-controlled (12:12-h light–dark cycle) animal quarters with food and water freely available. The animals were divided into four groups: (1) WKY and (2) SHR control; (3) WKY and (4) SHR given PEA at a dose of 30 mg kg^{-1} d^{-1} subcutaneously. PEA (Tocris Cookson Ltd., UK) was dissolved in PEG400 and Tween 80 2:1 (Sigma–Aldrich), and kept over night under gentle agitation with a micro stirring bar. Before injection, sterile saline was added so that the final concentrations of PEG400 and Tween 80 were 20 and 10% v/v, respectively. Control WKY and SHR received vehicle. All animals were treated for 35 days (5 wks). All procedures with animals were carried out in accordance with the Institutional Guidelines and complied with the Italian D.L. no.116 of January 27, 1992 and associated guidelines in the European Communities Council Directive of November 24, 1986 (86/609/ECC), and approved by the Animal Ethics Committee of the University "Federico II" of Naples (Italy), using as few animals as possible. At the end of the treatment period, the animals were housed in metabolic cages for 24 h to collect urine for subsequent measurements and evaluate water intake. Before killing, animals were anesthetized by enflurane and blood was collected for creatinine evaluation. The kidneys were excised, and harvested. Portions were fixed in 10% formaldehyde and embedded in paraffin for histopathological analysis and immunohistochemical staining. Additional portions were snap frozen in liquid nitrogen for protein evaluation. Aorta, carotid, and mesenteric bed were also excised and carefully cleaned for later measurements of oxidative stress or protein expression (see supplementary data).

2.2. Measurement of arterial blood pressure and heart rate in conscious rats

Systolic blood pressure (SBP) and heart rate (HR) were measured in conscious rats with a non-invasive common indirect method using a tail-cuff device in combination with blood flow sensor and recorder (Ugo Basile, Biological Research Apparatus, 21025 Comerio, Italy) [23]. Briefly, rats were housed for 30 min in a warmed room (28–30 °C), then a tail cuff was placed about 2 cm from the base of the tail for measuring systolic blood. Care was taken in selecting an appropriate cuff size for each animal. Rats were allowed to habituate to this procedure for 2 weeks before experiments were performed. Heart rate was detected by a pulse rate counter placed distal to the tail cuff and monitored with the audio signal. SBP and HR were measured every week between 09.00 h and 12.00 h and values were recorded and were averaged from at least three consecutive readings per rat.

2.3. Histopathological analysis

Coronal sections of renal tissue (3–4 μm thick) were stained with periodic acid-Schiff (PAS) and Masson trichrome for analysis of glomerular sclerosis and tubulointerstitial fibrosis, and examined by light microscopy in a blinded fashion. A score was used to evaluate the degree of glomerulosclerosis. Twenty glomeruli in every cross section per animal were selected randomly for the score analysis. Sclerosis was defined as collapse and/or obliteration of glomerular capillary tuft accompanied by hyaline material and/or increase of matrix. Severity of sclerosis for each glomerulus was graded from 0 to 3+ as follows: 0, no lesions; 1+, mild; 2+, moderate; 3+, severe. Tubulointerstitial fibrosis was assessed semiquantitatively. Ten random high-power fields (400×) per kidney were evaluated independently by two different pathologists, checking for fibrosis (blue staining) within the area assessed and graded as follows: 0, no fibrosis, 1 mild, 2 moderate, and 3 severe.

2.4. Analysis of urine

Urine samples were assayed for creatinine, sodium, and potassium by using standard diagnostic analyzer (Dimension® RxL Max® Integrated Chemistry System). The protein concentration of the samples was determined by Bio-Rad protein assay (Bio-Rad Laboratories, Segrate, Milan, Italy), using bovine serum albumin as the standard. Urine osmolarity was calculated as follows 2 × Na (mmol/L) + glucose (mg/dL)/20 + urea (mg/dL)/6.4. The urinary 20-hydroxyeicosatetraenoic acid (HETE) levels were assessed using 20-HETE ELISA kits (Detroit R&D Inc., Detroit, MI, USA), according to the manufacturer's instructions.

2.5. Western blot analysis

Cortical and medulla from kidneys were separately homogenized on ice in lysis buffer (Tris–HCl, 20 mM pH 7.5, 10 mM NaF, 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulphonyl fluoride, 1 mM Na_3VO_4, 10 μg/ml leupeptin and trypsin inhibitor). After 1 h, tissue lysates were obtained by centrifugation at 21,000 × *g* for 15 min at 4 °C. The protein concentration of the samples was determined by Bio-Rad protein assay (Bio-Rad Laboratories, Segrate, Milan, Italy), using bovine serum albumin as the standard. For Western blot analysis, tissue lysate was dissolved in Laemmli's sample buffer, boiled for 5 min, and subjected to SDS-PAGE. The blot was performed by transferring proteins from a slab gel to

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