The uraemic toxin phenylacetic acid increases the formation of reactive oxygen species in vascular smooth muscle cells

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Abstract

Background. Cardiovascular events are the most common cause of death in end-stage renal disease (ESRD) patients. Traditional risk factors do not sufficiently explain the marked increment in cardiovascular morbidity and mortality as compared with the general population. The role of uraemic toxins in the genesis of atherosclerosis remains elusive. Reactive oxygen species (ROS) play a major role in the development of atherosclerosis. In the present study, we describe the effect of the uraemic toxin phenylacetic acid (PAA) on the inducible nitric oxide synthase (iNOS) and the consecutive production of ROS.

Methods. Vascular smooth muscle cells (VSMC) were stimulated by IL-1β in the absence and presence of different concentrations of PAA (0.1–5.0 mM). Inducible NOS (iNOS) mRNA was determined by real-time PCR, iNOS protein was examined by western blotting. The NO degradation product, nitrite, was measured by Griess-assay and peroxynitrite (ONOO−) was assessed by hydroethidium, 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) fluorescence assays. To evaluate the iNOS cofactor tetrahydrobiopterin (BH4), the expression of the key enzyme GTP cyclohydrolase (GTPCH) was determined by real-time PCR.

Results. PAA enhanced the IL-1β-mediated induction of iNOS expression regarding both mRNA and protein. Nitrite was significantly increased only with high concentrations of 5 mM PAA. ONOO−, however, was enhanced in a dose-dependent manner. GTPCH, the key enzyme in BH4 synthesis, was not enhanced by PAA.

Conclusions. The uraemic toxin, PAA, leads to an induction of iNOS expression, resulting in an increase of ONOO− production. The increased production of ONOO− might be explained by a lack of GTPCH-catalysed BH4 synthesis leading to an uncoupled electron transfer. Thus, PAA might contribute to the oxidative stress in ESRD patients.

Keywords: atherosclerosis; end-stage renal disease; iNOS; peroxynitrite; phenylacetic acid; uraemic toxin

Introduction

Cardiovascular disease in consequence of advanced atherosclerosis is the most common cause of death in patients with end-stage renal disease (ESRD) [1]. Despite the identification of 90 different uraemic toxins, listed by the European Uremic Toxin Work Group (EUTox) [2,3], little is known about the role of uraemic toxins in atherosclerosis. We have recently identified phenylacetic acid (PAA) as a new uraemic toxin. We have shown that PAA inhibits inducible nitric oxide synthase (iNOS) in RAW 264.7 cells [4]. PAA is a degradation product of the phenylalanine metabolism. In young healthy patients, PAA is not detectable, whereas in ESRD patients a plasma concentration of 3.49 ± 0.33 mmol/l was measured [4]. Former research by our group showed that 69.8% of the PAA is non-protein bound [4]. Thus, the biologically active concentration is 2.44 mM.

Nitric oxide (NO) shows diverse effects in the physiology and pathophysiology of the cardiovascular system. NO has vasodilatory properties and inhibits platelet aggregation, vascular smooth muscle cells (VSMC) proliferation and leucocyte invasion [5]. On the other hand, increased expression of iNOS has been detected in atherosclerotic plaques, producing large amounts of NO. Reactive nitrogen species (RNS)—as a result of excessive NO production—and reactive oxygen species (ROS)—as a consequence of an uncoupled electron transfer by iNOS—might be involved in atherosclerotic pathogenesis [6]. The aim of this study was to elucidate the effect of PAA on iNOS expression and ROS production in VSMC.
Methods

Cell culture of vascular smooth muscle cells

VSMC were obtained from thoracic aortas of Wistar-Kyoto rats and cultured by the tissue explant method according to published procedures. Briefly, cells were incubated in Dulbecco’s modified Eagle’s medium (Biochrom, Berlin, Germany), containing 10% (vol/vol) fetal calf serum (Biochrom, Berlin, Germany), 100 U/ml penicillin G and 100 mg/ml streptomycin. Cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The medium was changed initially after 24 h and then every 2–3 days. After the first subculture, cells were subcultured every week at a seeding density of about 1.3 × 10⁴ cells/cm² and reached confluence in 8–10 days. They were harvested by adding 0.05% trypsin, and the culture was continued up to eight passages as previously described. To ascertain that cultured cells were VSMC, immunocytochemical localization of smooth muscle-specific α-actin was carried out using monoclonal antibodies ASM-1 (Progen, Heidelberg, Germany) raised against smooth muscle α-actin and labelled with a fluorescence marker. Staining of cultured VSMC with that antibody revealed that all cells in the preparation were labelled, and actin stress fibres were seen throughout the cytosol. It was confirmed that cultured VSMC were free from contamination with endothelial cells or fibroblasts by immunocytochemical staining of cells with antibodies against von Willebrand factor coupled with a fluorescence marker. Staining of cultured VSMC with a fluorescence marker. Staining of cultured VSMC with a fluorescence marker. Staining of cultured VSMC with a fluorescence marker. Cell culture of vascular smooth muscle cells

Preparation of RNA and real-time PCR

Real-time PCR primers and probes for rat iNOS, GTP cyclohydrolase (GTPCH) and for the housekeeping gene β-actin were designed using the computer program Primer Express 2.0 (Perkin Elmer Applied Biosystems, Foster City, CA, USA) (Table 1). Total RNA from VSMC was extracted using QIAGEN RNeasy Mini-Kit according to the manufacturer’s protocol (Qiagen GmbH, Hilden, Germany). Target RNA (2 μg) was reversely transcribed using 100 U Superscript II RT (Invitrogen GmbH) at 42°C for 80 min in the presence of 50 mmol/l Tris–HCl (pH 8.3), 5.7 mmol/l KCl, 3 mmol/l MgCl₂, 5 mmol/l DTT, 0.5 mmol dNTPs, 8 U RNasin (Promega Corp., Madison, WI, USA) and 5 μmol/l oligo(dT)₁₆ (Perkin Elmer Applied Biosystems). Real-time PCR was done by using the Eppendorf Mastercycler ep realplex (Eppendorf AG, Hamburg, Germany), using commercial reagents (TaqMan PCR Reagent Kit, Perkin Elmer Applied Biosystems).

Preparation of proteins from VSMC and western blot analysis

VSMC were harvested after stimulation and lysed in lysis buffer containing 50 mmol/l Tris–Cl (pH 7.4), 150 mmol/l NaCl, 100 μg/ml PMSF, 1% Nonident P-40 and 4% protease inhibitor cocktails. After centrifugation for 20 min, proteins present in the supernatant were loaded (20 μg) and separated in 10% SDS-polyacrylamide gel electrophoresis. The gels were blotted onto nitrocellulose membranes. Nitrocellulose membrane blots were locked for 1 h in Tris-buffered saline (TBS; 150 mmol/l NaCl, 100 mmol/l Tris, pH 7.5) containing 5% non-fat milk and incubated overnight at 4°C with a specific antibody against iNOS (1:500 dilution; BD Biosciences, San Jose, CA, USA) in TBS containing 0.5% non-fat milk. Membranes were washed in TBS and incubated with secondary horseradish peroxidase conjugated antibody (1:5000 dilution, BD Biosciences, San Jose, CA, USA) for 1.5 h. After further washing with TBS, blots were detected by the enhanced chemiluminescence method using an immunoblot assay kit (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Commercial markers (Bio-Rad prestained, Hercules, CA, USA) were used as molecular weight standards and β-actin was used to normalize the protein lane charge of the blot.

Measurement of nitrite formation

Measurement of nitrite production as an assay of NO release was carried out using the Griess reaction with sodium nitrite as standard. Aliquots of culture medium were mixed with

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TP, fluorogenic probe; RP, reverse primer; FP, forward primer.
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an equal volume of Griess reagent (1% sulphanilamide/0.1% N-(1-naphyl) ethylenediamine dihydrochloride in 5% phosphoric acid), the mixture was incubated at room temperature for 10 min, and the absorbance at 540 nm, measured using a photometer (iEMS reader, Labsystems, Helsinki, Finland). Standard curves were constructed using known concentrations of sodium nitrite.

**Measurement of ROS production**

VSMC were seeded on 8-well cell culture-treated chamber slides and grown to subconfluency. After starving for 24 h, cells were treated with interleukin-1β (IL-1β, 10 ng/ml) for 24 h in the absence or presence of different concentrations of PAA (0.1, 0.5, 1.0 and 5.0 mM). VSMC were stained for 10 min with 10 μM hydroethidium, 2,7-dichlorodihydro- fluorescein diacetate (H2DCFDA) at 37°C in the dark. Non-flourescent H2DCFDA permeates live cells and is deacetylated by non-specific intracellular esterases. In the presence of ROS, the reduced fluorescein compound DCFH is oxidized to DCF and emits bright green fluorescence. Cells were rinsed with Hanks buffered salt solution (HBSS) and images were obtained with an inverted microscope (Axiovert 200M, Zeiss, Oberkochen, Germany) equipped for fluorescence microscopy (excitation 485 nm, emission 535 nm). For quantification of ROS-formation, fluorescence by H2DCFDA was measured by a fluorescence image plate reader (FLIPR, Berthold Mithras LB 940) in 12-well plates. Fluorescence of IL-1β-stimulated VSMCs were set as 100%.

**Statistical methods**

Data are presented as mean ± SEM. Nitrite concentrations, mRNA levels and data from fluorescence analyses in presence of PAA were compared with mere stimulation by IL-1β by paired two-tailed t-tests. *P < 0.05 was regarded statistically significant.

**Materials**

PAA was purchased from Sigma. PAA was diluted in pre-warmed water and enhanced by sonification, in a stock concentration of 100 mM. The pH was adjusted to 7.0. Further dilutions were done with DMEM cell culture medium.

**Results**

PAA induces and enhances cytokine-induced iNOS expression in VSMC.

IL-1β (10 ng/ml) lead to an increase of rat VSMC iNOS mRNA and protein (Figure 1A and C). Nitrates as a degradation product of NO is induced in a time-dependent manner by IL-1β over a period of 48 h (10 ng/ml, Figure 2A). First, we examined the effect of PAA on the IL-1β-mediated induction of iNOS mRNA and protein. Therefore, VSMC were stimulated with IL-1β (10 ng/ml) in the presence or absence of different concentrations of PAA. After 6 h, mRNA was isolated and iNOS mRNA was detected by real-time PCR. IL-1β leads to a 345.3 ± 57.0-fold induction of iNOS mRNA compared with unstimulated cells (Figure 1A). With this value as a reference, the IL-1β (10 ng/ml)-mediated stimulation of iNOS mRNA was presented as mean ± SEM. Nitrite concentrations, mRNA levels and data from fluorescence analyses in presence of PAA were compared with mere stimulation by IL-1β by paired two-tailed t-tests. *P < 0.05 was regarded statistically significant.
was significantly enhanced in the presence of PAA (Figure 1B). PAA of 5 mM leads to a 2.9 ± 0.8-fold increase of iNOS mRNA (Figure 1B). The IL-1β-mediated increase of iNOS protein was also enhanced by PAA (Figure 1C).

To answer the question, if lower concentrations of IL-1β, comparable to those of ESRD patients, enhance iNOS in the same manner, we repeat the mRNA experiment with a reduced concentration of 1 ng/ml IL-1β. PAA enhances iNOS mRNA even in low IL-1β-stimulated VSMC (Figure 1A; 1 ng/ml IL-1β: 17.4 ± 2.3-fold iNOS mRNA expression vs w/o IL-1β; 1 ng/ml IL-1β + 1 mM PAA: 22.0 ± 2.5-fold iNOS mRNA expression vs w/o IL-1β; 1 ng/ml IL-1β + 5 mM PAA: 69.2 ± 6.1-fold iNOS mRNA expression vs w/o IL-1β). PAA itself, in absence of IL-1β, induces iNOS in a slight, but significant manner (Figure 1A; iNOS + 1 mM PAA: 2.2 ± 0.2-fold vs iNOS w/o PAA, iNOS + 5 mM PAA: 18.9 ± 4.8-fold vs iNOS w/o PAA; \(P < 0.05\)).

PAA increases ROS formation in IL-1β-stimulated VSMC

To further analyse the effects of the PAA-mediated increase of iNOS mRNA and protein, we examined the end products of iNOS activation. IL-1β-stimulated nitrite production after 24 h was set to 100%. The 24-h time point is located in the exponential phase of the time-dependent IL-1β-induced nitrite stimulation (Figure 2A). Nitrite production was significantly enhanced by 5 mM PAA, compared with IL-1β-stimulated VSMCs (IL-1β with 5 mM PAA: 193.2 ± 23.0% vs IL-1β without PAA: 100%), whereas concentrations up to 1 mM PAA did not significantly affect nitrite production (Figure 2B). The H2DCFDA fluorescence microscopy findings and fluorescence microplate reader quantification results show that PAA increases ROS concentration in IL-1β-stimulated VSMC. As displayed in Figure 3A and B, there is a dose-dependent increase in the fluorescence intensity in the presence of PAA (0.1, 0.5, 1.0, 5.0 mM) compared with IL-1β stimulation without PAA. As shown in Figure 4, IL-1β markedly increased the synthesis of GTPCH mRNA, the key enzyme for tetrahydrobioppterin (BH₄) synthesis. PAA, however, had no influence on IL-1β-mediated GTPCH expression.

Discussion

PAA is a recently identified uraemic toxin. PAA has gained some interest because of its effects on iNOS regulation and the role of NO synthases in cardiovascular disease. Cardiovascular mortality is the leading cause of death in ESRD patients. Yearly all-cause mortality ranges from 20 to 25%, half of this is caused by cardiovascular events [1]. To date, the physiological and pathophysiological properties of PAA remain largely elusive. The findings of the present study demonstrate that PAA enhances the cytokine-mediated production of ROS in VSMC.

Traditional risk factors like hypertension, smoking or hyperlipidaemia do not sufficiently explain this exceedingly high mortality risk. ESRD patients show a distinct form of vascular calcification. Several factors are involved in progression of atherosclerosis, such as hyperphosphataemia, chronic inflammation and uraemic toxins. Circulating levels of pro-inflammatory cytokines, like interleukine-6, interleukine-13, TNF-α and IL-1β, are elevated in haemodialysis patients and have been shown to be associated with an increased mortality risk [7]. IL-1β has several proatherogenic properties. Increased levels of IL-1β enhance vascular adhesion, macrophage activation, endothelial and VSMC proliferation [8].

Increased plasma levels of 3.49 ± 0.33 mmol/l PAA were measured in patients with ESRD, whereas
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69.8% ± 22.5% of the PAA is non-protein bound. Thus, the biologically active concentration is 2.44 mM. Comparable concentrations of PAA lead to a significant increase of iNOS mRNA in unstimulated cells and to an enhancement of IL-1β-induced iNOS mRNA and protein synthesis in present experiments. This finding is surprising in context with earlier studies showing that PAA inhibits iNOS expression in a macrophage cell line [2]. Apparently, the effects of PAA on iNOS expression are tissue dependent. A first hint on tissue-specific differences in iNOS regulation was given in the previous study by Pahan et al. [9] since iNOS expression in rat primary astrocytes was inhibited by PAA and lovastatin in contrast to the stimulating effects of lovastatin on iNOS in VSMC [10].

To explain the differential effects of PAA on iNOS expression, the different mechanisms regulating iNOS expression in various cell types have to be considered, given that our knowledge on iNOS regulation is still incomplete. Transcriptionally, iNOS mRNA is regulated in a complex way. The activation of the iNOS promoter is based on two different major cascades, the NF-κB activation by phosphorylation and degradation of the inhibitory complex IκB and the phosphorylation of the mitogen-activated protein kinases (MAPKs). The extracellular signal-regulated kinases (ERKs), c-jun N-terminal kinase (JNK) and p38 MAPK influence the activation of iNOS, ERK and JNK augment and p38 MAPK inhibits iNOS activation [11].

The mechanism of effects of PAA on iNOS activation is largely elusive. In LPS-stimulated rat primary astrocytes, PAA and lovastatin lead to an inhibition of NF-κB [9], which yields to an iNOS inhibition [9]. The effect of PAA on MAPKs in rat primary astrocytes was not determined. In contrast, lovastatin leads to a potentiated NF-κB activation in IL-1β-stimulated VSMC [10]. Further experiments will have to elucidate the differences in the regulation of iNOS and the properties of PAA on iNOS regulation in different cell types.

The role of iNOS in the pathogenesis of atherosclerosis still remains controversial. iNOS has been detected in atherosclerotic lesions [12,13]. Some studies support the hypothesis, that iNOS plays an antiatherogenic and vasculoprotective role by replacing NO, if the production by endothelial NOS (eNOS) is reduced in endothelial lesions. NO inhibits leucocyte adherence, LDL-oxidation, platelet aggregation, VSMC proliferation and migration [5]. This position is supported by the finding that activation of iNOS reduces the development of atherosclerosis after solid organ transplantation [14]. Other authors, however, could detect elevated iNOS in atherosclerotic lesions, suggesting iNOS as an atherosclerosis promoting factor [15–17]. Although, simply an iNOS elevation per se is no proof for a pathophysiological importance.
in atherogenesis, since it may be just a compensatory mechanism. Besides NO, iNOS can produce ONOO⁻. ONOO⁻—a member of the ROS family—was identified in high concentrations in atherosclerotic lesions. ONOO⁻ causes oxidative damage, nitration and S-nitrosylation of biomolecules, including proteins, lipids, and DNA [18,19]. Thus, the impact of iNOS on the vasculature does not only depend on the quantity of the enzyme itself, but the differential end product pattern with a predomination of either NO or ONOO⁻.

Regarding the iNOS end products in our experiment, high concentrations of PAA increase the amount of nitrate. In lower concentrations, however, nitrate production is not significantly influenced. Contrarily, ROS production is enhanced in a homogeneous dose-dependent manner, leading to an increase of ROS even in low concentrations of PAA. The pattern of the diverse iNOS products is known to depend on the presence or absence of cofactors:

Haem, flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) and BH₄ are essential cofactors for the synthesis of NO by NOS [20]. BH₄ is required for homodimerization, binding affinity of the substrate, spin state equilibrium of the haem, and for the electron transfer in NOS [20]. If BH₄ is available in sufficient amounts, NOS preferably synthesizes NO and O₂. In conditions of reduced BH₄ availability, there is an increased production of NO and O₂⁻ mediated by a haem-catalysed reduction (uncoupled electron transfer) [20,21]. The reaction of NO and O₂⁻ finally leads to the formation of ONOO⁻. The availability of BH₄ strictly depends on GTPCH, the rare-limiting enzyme in the de novo synthesis of BH₄ [22]. It is predominantly regulated on a transcriptional level and induced by cytokines, such as interferon-γ, tumour-necrosis-factor-α, IL-1β and endotoxin (lipopolysaccharide). Our studies show that PAA does not affect the IL-1β-mediated induction of GTPCH. In a condition of enhanced iNOS activity—as induced by PAA—this might result in a lack of BH₄. This phenomenon might explain the dose-dependent increase of ONOO⁻ in the depicted experiments. In contrast to PAA, statins were shown to enhance both iNOS expression and GTPCH expression in VSMC [23]. A vasoprotective effect was demonstrated by a shift of the NOS-catalysed generation of deleterious ROS towards protective NO [23]. Our experiments demonstrate that the uraemic toxin PAA shifts the iNOS-catalysed equilibrium of NO and ONOO⁻ production towards the deleterious ONOO⁻. Thus, the combination of elevated levels of IL-1β and PAA may contribute to atherosclerosis progression in ESRD patients.

In summary, the uraemic toxin PAA induces and enhances the IL-1β-mediated induction of iNOS. GTPCH, the key enzyme for the production of the essential cofactor BH₄, is not elevated. This might lead to the observed shift of the iNOS end products towards the deleterious ONOO⁻. Therefore, PAA might contribute to the rapid progression of atherosclerosis in ESRD patients.

Conflict of interest statement. None declared.

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