Adrenomedullin protects against oxidative stress-induced podocyte injury as an endogenous antioxidant

Shigeyoshi Oba, Masayo Hino and Toshiro Fujita

Department of Nephrology and Endocrinology, University of Tokyo Graduate School of Medicine, Hongo, Bunkyo-ku, Tokyo 113-8655, Japan

Abstract

Background. We previously reported that puromycin aminonucleoside (PAN) increased adrenomedullin (AM) secretion and AM mRNA expression in podocytes, through overproduction of oxidative stress. To clarify the cytoprotective role of AM as anti-oxidative and antiapoptotic substance in podocytes, we investigated the effect of exogenous AM and AM antagonist on PAN-induced apoptosis in conditionally immortalized murine podocytes.

Methods. The expression of AM, RAMP 2 and RAMP 3 was investigated using real-time PCR, western blotting analysis and immunofluorescence microscopy. Reactive oxygen species (ROS) production was measured by CM-H$_2$DCFDA fluorescence intensity method. The percentage of apoptotic cells was measured by Hoechst 33342 staining.

Results. PAN (100 μg/ml) significantly (P < 0.01) increased ROS production, associated with an increase in apoptosis; the percentage of apoptotic cells is 5.3% ± 0.05% (P < 0.01) with 36 h treatment of PAN compared to 0.24 ± 0.16% with no treatment. Several antioxidants could markedly reduce PAN-induced apoptosis in cultured podocytes, suggesting that PAN-induced apoptosis might be attributable to the overproduction of ROS. Accordingly, the administration of exogenous AM (10$^{-6}$ M) could significantly reduce not only ROS production via a PKA-dependent pathway, but also the resultant apoptosis induced by PAN. AM antagonists, CGRP8-37, augmented PAN-induced apoptosis, associated with increased ROS production, 2.2- and 2.3-Fold, respectively. RAMP 2 and RAMP 3 could be detected in podocytes and glomeruli.

Conclusions. This suggests that ROS-induced up-regulation of AM with PAN could counteract ROS-induced apoptosis, by the suppression of ROS production. Therefore, AM might have the endogenous antioxidant potential to protect against ROS-induced podocyte injury.

Keywords: adrenomedullin; aminonucleoside; antioxidants; apoptosis; oxidative stress; podocyte; proteinuria; puromycin; reactive oxygen species

Introduction

Glomerular visceral epithelial cells, which are also called podocytes, function as critical size and charge barriers of protein excretion from glomerulus, therefore, podocyte injury induced marked proteinuria [1,2]. There is a growing body of evidence suggesting that podocyte injury plays an important role in not only proteinuria but also the progression to end-stage renal disease in diabetic nephropathy [3] and hypertensive glomerulosclerosis [4]. Since podocytes lack the ability of proliferation, they do not recover from their disappearance induced by podocyte injury, resulting in the decrease of podocytes from the glomerulus, podocytopenia, which is one of the important processes of glomerulosclerosis. Apoptosis is known to be the major course of podocytopenia [5]. Although there are several factors influencing apoptosis in podocytes, oxidative stress is one of the important causative factors. Accordingly, oxidative stress, in vitro, induces podocyte injury, associated with apoptosis of podocytes. Moreover, we recently demonstrated that antioxidants could not only improve podocyte injury but also inhibit proteinuria in salt-loaded Dahl salt-sensitive rats and aldosterone/salt-induced rats [6].

Adrenomedullin (AM) is known to be a potent vasoactive substance produced abundantly in vascular endothelial and smooth muscle cells [7,8]. Moreover, AM is also an antioxidant, since endogenous AM has the potential to protect from oxidative stress-induced vascular damages in angiotensin II/salt-treated rats and mice [9,10]. ROS up-regulates AM gene expression [11,12], and, in turn, AM inhibits ROS production.

Correspondence to: Toshiro Fujita, PhD, MD Department of Nephrology and Endocrinology, University of Tokyo Graduate School of Medicine, Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Email: fujita-dis@h.u-tokyo.ac.jp

© The Author [2007]. Published by Oxford University Press on behalf of ERA-EDTA. All rights reserved.
For Permissions, please email: journals.permissions@oxfordjournals.org
Adrenomedullin protects against podocyte injury

induced by angiotensin II/salt [13]. Thus, vascular AM counteracts angiotensin II/salt as an endogenous antioxidant. Moreover, AM is reported to be expressed in podocytes as well as in the glomerular mesangial cells and tubular epithelial cells [14–16].

We previously reported that puromycin aminonucleoside (PAN) induced podocyte injury, associated with increased AM secretion and AM mRNA expression in podocytes, possibly through overproduction of ROS [17]. In order to clarify the cytoprotective role of AM up-regulated by PAN, in the present study, we investigated the effect of exogenous AM and AM antagonist on PAN-induced apoptosis, a marker of podocyte injury, in conditionally immortalized murine podocytes [18].

Materials and methods

Reagents

Interferon-γ was purchased from Toyobo (Tokyo, Japan). PAN, rotenone, antimycin A, diphenyleneiodonium chloride (DPI) and N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide hydrochloride (H-89) were purchased from Sigma Aldrich (St Louis, MO, USA). The 4-hydroxy-3-methoxyacetophenone (apocynin) was purchased from Tokyo Kasei (Tokyo, Japan). Human CGRP8-37 was purchased from Peptide Institute (Osaka, Japan).

Cell culture

We used mouse podocyte cell lines established by Mundel et al. [18] in 1997. Cells were cultured in RPMI1640 with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin in the presence of 10 U/ml recombinant murine interferon-γ at 33°C in 5% CO₂/95% air (permissive condition). To differentiate, podocytes were plated on type I collagen at a density of 1 x 10⁶ cells/cm² and cultured with 1% FBS in the absence of interferon-γ at 37°C (non-permissive condition). Two or three days after subculture, the concentration of FBS was reduced to 0.5%. Podocytes maintained under non-permissive condition for 10–14 days were used for experiments [17].

Stimulation of podocytes

Podocytes maintained under non-permissive condition for 10–14 days were exposed to PAN 1, 10 and 100 μg/ml. For ROS experiments, podocytes were treated with H₂O₂ 10 μM for 24 h. For antioxidant experiments, podocytes were pretreated with rotenone 100 μM, antimycin A 1 μM, DPI 5 μM or apocynin 60 μM 40 min before stimulation of PAN. To examine the effects of the addition of AM receptor antagonists, pretreatment of human CGRP8-37 at 10⁻⁸, 10⁻⁷ and 10⁻⁶ M was done preceded by the addition of PAN for 40 min. To examine the effects of the addition of a PKA inhibitor, podocytes were treated with H-89 at 10⁻⁷ M 30 min before stimulation of AM. Detection of apoptosis was analysed by harvesting cells at 36 h, and other experiments were analysed at 24 h.

Real-time quantitative reverse transcription-polymerase chain reaction (real-time quantitative RT-PCR)

Total RNA was extracted using an RNeasy kit (Qiagen K.K., Tokyo) and treated with DNase I (Qiagen) to remove contaminating genomic DNA. The cDNA was synthesized from 1 μg of total RNA with random primers (Promega Corporation, Madison, WI) and Superscript II reverse transcriptase (Invitrogen Corp. Carisbad, CA). Gene expression was quantitatively analysed by real-time RT-PCR using an ABI PRISM 7000 (Applied Biosystems, Foster City, CA). TaqMan chemistry and assay by design primers and probe sets were used for mouse AM, RAMP2, RAMP3 and β-actin. PCR was carried out on ABI PRISM 7000 with 7.5 x 10⁴ cells incubated on a 100 mm collagen I coated dish with 7 ml medium for 24 h with or without the agents. The supernatant was used for the 8-OHdG assay. We used the ELISA kit, New 8-OHdG Check (Japan Institute for the Control of Aging, Nikken SEIL Corp., Shizuoka), according to the protocol [19]. That is, the sample was applied to 8-OHdG-coated microplates then anti-8-OHdG antibody was added and incubated at 37°C for one hour. After washing, the enzyme-labelled antibody was added and incubated at 37°C an hour. After colour fixing under light interception, absorbance at 450 nm was measured. The concentration of 8-OHdG was calculated in comparison to the standard curve of absorbance, using standard samples included in the kit.

Western blotting

Western blot analysis was performed to measure the protein levels of adrenomedullin [6]. Adherent cells were treated with lysis buffer [composition: 50 mM Tris–HCl (pH 7.4), 10 mM EDTA, 1% Triton X-100, protease inhibitor], then the proteins (20 μg) were electrophoresised on 12.5% SDS-polyacrylamide gel, and the gels were blotted to the nitrocellulose membrane. Rabbit anti-adrenomedullin antibody (Santa Cruz Biotechnology, Inc., CA) diluted to 1:1000 was incubated at 4°C overnight after blocking with 5% non-fat dry milk powder solved in TTBS buffer (Tris-buffered saline with tween-20) for an hour. After washing with TTBS buffer, the membrane was incubated with an anti-rabbit IgG antibody-conjugated horseradish peroxidase (Amersham Biosciences) diluted to 1:4000 for an hour at room temperature. The resultant bands were detected with chemiluminescence Western blotting reagents (Amersham Biosciences) and exposed to hyperfilm ECL (Amersham Biosciences).
Measurement of CM-H₂DCFDA fluorescence intensity

Podocytes differentiated in 6-well dishes were stimulated by the agents 36 h after being cultured with serum-free medium for more than 12 h. After disruption of the cells with trypsin, the number of harvested cells was counted. Then the cells were incubated with 5- (and 6-) chloromethyl-2’,7’-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) (Invitrogen), 50 μg/ml, at 37° C for 60 min, and the fluorescence intensity of 100 μl of the sample was measured at 480 nm excitation and 535 nm emission by micrplate reader (WALLAC 1420 ARVO MX/Light, Perkin Elmer) [20,21]. Fluorescence intensities were compensated by their numbers of the cells.

Detection of caspase-3 activity

The samples were exposed to stimuli for 36 h. The caspTag™ Caspase-3 In Situ Assay Kit, sulforhodamine (Chemicon), was applied using the fluorochrome inhibitors of caspase (FLICA) method [22]. After incubation at 37° C for one hour, cells were added to Hoechst solution at a final concentration of 0.5% for 5 min. After washing three times, the fixation buffer included in the kit was added and observed by microscope for caspase-3 activity at 550 nm excitation and 580 nm emission, and for Hoechst staining, at 351 nm excitation and 460 nm emission with a 420 nm filter. We confirmed that Hoechst-positive apoptotic cells also produced fluorescence of caspase-3 activity by observation of the sample simultaneously with caspase-3 labelling and Hoechst staining.

Hoechst 33342

The sample cells were exposed to stimuli for 36 h after being cultured with serum-free medium >12 h and were incubated with 10 μM Hoechst 33342 solution (Dojindo) at 37° C for 10 min, and fixed by 4% paraformaldehyde, then observed by microscope at 351 nm excitation and 460 nm emission with a 420 nm filter [23,24]. We judged an apoptotic cell by nucleus fragmentation and aggregation characteristic of apoptosis. Two pathologists examined more than 400 cells in a blinded manner, and calculated the percentages of apoptotic cells. The results by two pathologists were almost same.

Measurement of cell viability

To investigate the cytotoxicity of PAN, AM and CGRP8-37 of the podocytes, MTT assay for cell viability was performed. We used the [3-(4,5-dimethythiazol-2-yl) -2,5-diphenyl tetrazolium bromide] MTT Cell Proliferation Kit I (Roche Diagnostics, IN, USA) to investigate the viability of podocytes. Cells were seeded into 12-well culture plates and incubated with PAN, AM, CGRP8-37 for 24 h. The 100 μl MTT reagent was added to each well and incubated for 4 h in humidified atmosphere (37° C, 5% CO₂). The total 500 μl of the solubilization solution was added to each well and 16 h later the colorimetric reaction was measured at 580 nm. Results were expressed as percentage of controls.

Immunofluorescence microscopy

An immunofluorescence study was performed according to a previously described method [25]. Cryostat sections were fixed with acetone for one minute and incubated with rabbit anti-RAMP 2, and anti-RAMP 3 (Santa Cruz Biotechnology, Santa Cruz, CA), stained with FITC-conjugated anti-rabbit IgG (Dako, Glostrup, Denmark), and observed with raser microscopy.

Statistical processing

Data were expressed as means ± SD. Statistical analyses were performed by unpaired t-test or analysis of variance and subsequent Tukey’s simultaneous multiple comparison. A P value < 0.05 was considered to be statistically significant [6]. All experiments were repeated three times.

Results

PAN induced AM expression on podocytes (Figure 1)

PAN (100 μg/ml) increased the gene expression of AM mRNA in cultured podocytes (AM/β-actin 15.99 ± 0.77 vs 1.79 ± 0.13 (P < 0.01), which is consistent with the results of the previous study [17]. Western blotting analysis revealed that H₂O₂ and PAN increased the expression of AM at the protein level (AM/β-actin 1.00 ± 0.09 vs 1.72 ± 0.19 and 2.21 ± 0.41 (P < 0.05) and that AM upregulation by PAN was almost same.
Adrenomedullin protects against podocyte injury

PAN-induced apoptosis and the effect of antioxidants (Figure 2)

To evaluate the effect of PAN on apoptotic cell death of the podocytes, we measured apoptosis morphologically by nucleus fragmentation or aggregation by staining Hoechst 33342. The percentage of apoptotic cells was significantly increased at the podocyte with 36 h treatment of PAN from 0.24±0.09% without treatment to 4.45±0.51% with.

Antioxidants could decrease apoptosis of podocytes induced by PAN significantly: rotenone: 1.21±0.15% (P < 0.01), antimycin A: 0.22±0.01% (P < 0.01), DPI: 0.08±0.10% (P < 0.01) and apocynin: 1.83±0.13% (P < 0.01). (B) The signal of activated caspase-3 was positive in cells with nucleus fragmentation and aggregation (insert of Figure 2).

PAN-induced ROS and the effect of exogenous AM and CGRP8-37 (Figure 3–6)

To confirm PAN-induced overproduction of ROS, in this experiment we measured three markers of oxidative stress. First, we measured the concentration of 8-OhdG in the medium of PAN-treated podocytes. The concentration of 8-OhdG was significantly (P < 0.05) increased, from 0.18±0.02 to 0.56±0.12 ng/ml, with the administration of PAN. Secondly, we examined the expression of nitrotyrosine by western blotting which was predominantly enhanced by PAN (data not shown). Thirdly, we measured fluorescence intensity of podocytes incubated with CM-H2DCFDA for 36 h,
the role of endogenous AM up-regulated by PAN, we added AM receptor antagonist, CGRP8-37 (10⁻⁸, 10⁻⁷ and 10⁻⁶M), on PAN-induced podocytes. CGRP8-37 could increase PAN-induced ROS production dose-dependently and CGRP8-37 10⁻⁶M could significantly increase ROS by 2.3 times; 3.57 ± 0.50 vs 1.55 ± 0.05 (P < 0.01), suggesting that endogenous AM up-regulated by PAN might inhibit overproduction of ROS with PAN (Figure 3 and 6). To understand the role of AM at the physiological state, we investigated the effects of AM and CGRP8-37 on ROS production of the cells without PAN treatment. Administration of CGRP8-37 and AM could not influence the ROS production in the cells without PAN treatment.

**AM inhibits PAN-induced ROS generation via the PKA-dependent pathway (Figure 7)**

To determine whether the antioxidant effect of AM to PAN-induced ROS is mediated via the PKA-dependent pathway, we tested the effects of the PKA inhibitor, H-89. The inhibitory effect of AM (10⁻⁶M) on ROS generation by PAN (10 μg/ml) was predominantly abolished by H-89 (10⁻⁵M): 1.10 ± 0.14 vs 1.47 ± 0.31 (P < 0.05).

**The effect of exogenous AM and CGRP8-37 on PAN-induced apoptosis (Figure 8)**

To evaluate cytoprotective role of endogenous AM, we studied the effect of CGRP8-37 on apoptosis of the podocytes by Hoechst 33342 stain. According to the changes in ROS production, CGRP8-37 increased PAN-induced apoptosis by 2.2 times; 4.50 ± 0.09% vs 2.02 ± 0.29% (P < 0.01). Moreover, the addition of...
exogenous AM could reduce PAN-induced apoptosis significantly; 1.01 ± 0.08% vs 2.02 ± 0.29% (P < 0.01).

**Discussion**

In the present study, we demonstrated that the administration of antioxidants such as rotenone, antimycin A, DPI and apocynin could markedly reduce PAN-induced apoptosis measured by Hoechst 33342 staining in conditionally immortalized murine podocytes, which is consistent with the results of previous studies indicating that PAN-induced apoptosis of podocytes might be attributable mainly to overproduction of oxidative stress. Correspondingly, the addition of exogenous AM significantly reduced PAN-induced apoptosis, associated with the decreased PAN-induced overproduction of ROS measured by CM-H2DCFDA fluorescence. Moreover, the administration of the AM receptor antagonist CGRP8-37 could augment not only ROS production but also apoptosis induced by PAN. This suggests that AM might protect against ROS-induced apoptosis with PAN, through the inhibition of ROS production.

There is a growing body of evidence that oxidative stress plays a critical role in the progression to end-stage renal failure as well as the development of cardiovascular disease. Hypertension, dyslipidaemia, diabetes, obesity and smoking cause the incidence of chronic kidney disease and renal failure, through overproduction of oxidative stress. There are several factors producing oxidative stress. Vasoactive substances such as angiotensin II and aldosterone also produce oxidative stress, resulting in atherosclerosis and renal damage. Indeed, angiotensin II and aldosterone caused podocyte injury [26], associated with proteinuria, and the administration of tempol, a membrane-permeable SOD mimetic, could correct not only podocyte injury but also proteinuria [27]. In the present study, we used puromycin aminonucleoside (PAN), since the podocyte is the main target of the injury by PAN. Many studies focused on the mechanism of PAN-induced nephropathy. Among them, some studies showed that podocyte injury induced by PAN was solely mediated by oxidative stress [28,29]. In the present study, therefore, in order to clarify the mechanism for ROS-induced podocyte injury with PAN, we firstly studied the effects of antioxidants...
on PAN-induced apoptosis and podocyte injury. In this report, we showed that exogenous AM inhibited PAN-induced apoptosis by about 50%, although AM completely inhibited ROS production increased by PAN. One of the reasons may be that induction of apoptotic cell death needs a certain amount of ROS, and so AM could inhibit apoptosis by decreasing ROS to less than the level which could induce apoptosis. According to the results of previous studies, the present experiment showed that PAN induced apoptosis, a marker of podocyte injury. It should be noted that podocytes lack the ability of proliferation because of the dominance of CDK-inhibitors as compared to cyclin-dependent kinases [30]. As a result, the podocytes that disapper can never be replaced, leading to podocytopenia, the loss of podocytes from glomerulus, which is one of the important processes of glomerulosclerosis. Apoptosis is well known to be the major course of podocytopenia leading to podocyte injury. Several studies revealed the involvement of oxidative stress in apoptosis of podocytes. Suzuki showed that PAN induced overproduction of ROS and apoptosis of podocytes, in vitro, using podocyte sieving from glomeruli [31]. Moreover, Sanwal et al. [32] demonstrated that the administration of ROS scavengers such as SOD and DMTU could suppress apoptosis induced by PAN. Consistently, in the present study the administration of antioxidants could ameliorate PAN-induced apoptosis.

AM is reported to be produced in not only vascular endothelial and smooth muscle cells but also in podocytes, mesangial cells and tubular cells. We demonstrated that AM is synthesized and secreted in the glomerular podocyte cell line established by Mundel et al. AM may play a regulatory role in the podocytes in an autocrine manner. Indeed, cAMP, one of the main second messengers of the AM signalling cascade, was indicated to regulate podocyte function [33]. Alternatively, AM secreted from the podocytes may regulate glomerular function by acting on glomerular endothelial or mesangial cells in a paracrine manner [34].

Several investigators demonstrated, in vitro, that oxidative stress induced AM secretion and AM gene expression in mesangial cells and vascular smooth muscle cells [35]. Moreover, we previously found that AM mRNA expression was enhanced by ROS generators such as H$_2$O$_2$, TNF-α, human serum albumin and PAN [17]. In addition, AM mRNA up-regulation by PAN was normalized by antioxidants. The result of western blotting analysis in this report was consistent with the previous study. Then, it can be assumed that PAN generates ROS, which activates injurious signalling, resulting in apoptosis, on one hand, and stimulates AM secretion to counteract the podocyte injury at the same time.

AM acts by binding the calcitonin receptor-like receptor (CRLR), whose interaction with the subtypes 2 and 3 of a family of receptor activity-modifying proteins (RAMP) gives rise to two distinct AM receptors, CRLR/RAMP 2 and CRLR/RAMP 3 receptors [36]. AM may prevent PAN-induced apoptosis by up-regulation of its functional receptor. We examined the expression of RAMP2 and RAMP3 in podocytes and glomeruli of vehicle and PAN-treated podocytes and glomeruli. We could detect the expression of RAMP2 and RAMP3 in podocytes and glomeruli, however, PAN cannot increase the expression of RAMP2 and RAMP3. In this report, we showed that the PKA inhibitor, H-89, could abolish the inhibitory effect of AM (10^{-7} M) on ROS generation by PAN, which is consistent with the results of previous studies that AM inhibited angiotensin II-induced oxidative stress in rat endothelial cells via a PKA-dependent pathway. [37].

It is well known that AM is not only a vasoactive substance but also an antioxidant [38–40]. AM has been reported to counteract the deleterious effects of angiotensin II [10,33]. Moreover, we previously reported that endogenous AM exerts protective effects against angiotensin II- or cuff-induced vascular injury through the inhibition of oxidative stress using AM knockout mice [10,41,42]. Accordingly, our recent study has demonstrated that renal damage by unilateral urethral occlusion, UUO, was enhanced in AM knockout mice through overproduction of ROS (manuscript in preparation), suggesting that AM may protect against ROS-induced renal damage with UUO. Moreover, several investigators demonstrated that AM has an anti-apoptotic effect in endothelial cells [43]. Consistently, in the present study, the administration of exogenous AM ameliorated not only PAN-induced ROS overproduction but also the resultant apoptosis of podocytes. Moreover, AM receptor antagonist CGRP8-37 augmented PAN-induced apoptosis in cultured podocytes, associated with the enhanced ROS production. Therefore, it may be plausible to hypothesize that upregulated AM in the podocytes with PAN protects against ROS-induced apoptosis, through the inhibition of ROS.

Acknowledgments. The authors thank Dr Peter Mundel (Mount Sinai School of Medicine, One Gustavo L. Levy Place, New York, USA) for the podocyte cell line.

Conflict of interest statement. None declared.

References

Adrenomedullin protects against podocyte injury

10. Shimosawa T, Shibagaki Y, Ishibashi K et al.