Renoprotective antioxidant effect of alagebrium in experimental diabetes

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Abstract

Background. Despite the beneficial effects of alagebrium (ALA), a putative advanced glycation end-product (AGE) breaker, on diabetic nephropathy, its renoprotective mechanisms are incompletely understood. Since oxidative stress exacerbates diabetic renal injury through interaction with AGE, the present study examined the antioxidative property of ALA in db/db mice, mesangial cells cultured under high glucose or H2O2 and a test tube.

Methods. ALA (2 mg/kg/day) was administered intraperitoneally for 12 weeks to 8-week-old db/db and db/db (DALLAE) mice or for 4 weeks to 16-week-old db/db mice (DALLAL). Oxidative stress markers (nitrotyrosine accumulation, expression and translocation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits, cellular DCF-DA fluorescence) together with urinary albumin excretion and histological changes including mesangial expansion were measured. The concentration of H2O2 in the presence and absence of ALA was measured by iodometric analysis in a test tube.

Results. ALA significantly reduced not only urinary albumin excretion and renal pathological changes but also accumulation of pentosidine and nitrotyrosine and expression of NADPH oxidase subunits in db/db mice regardless of treatment protocol. In mesangial cells, ALA effectively prevented not only high glucose- but also H2O2-induced membrane translocation of NADPH oxidase subunit (p47 phox, p67 phox and rac1) and protein kinase C isoform (α, βI and βII) and Nox4 messenger RNA expression concomitant with cellular reactive oxygen species. Furthermore, ALA directly decreased H2O2 in a test tube.

Conclusion. ALA has both direct and indirect antioxidant effects that may play important roles in ALA’s renoprotective effect in diabetic kidneys.

Keywords: alagebrium; diabetic nephropathy; reactive oxygen species

Introduction

Diabetic nephropathy is the leading cause of end-stage renal disease worldwide [1] and is an independent risk factor for all-cause and cardiovascular mortality [2]. Current therapies including tight control of blood glucose and blood pressure and inhibition of angiotensin II may delay but does not stop the development and progression of renal injury in diabetes, and an effective therapeutic strategy is still an unmet need.

Advanced glycation end-products (AGEs) are known to play an independent role in the development and progression of diabetic nephropathy. Inhibition of AGE has been achieved mostly by inhibitors of AGE formation and protein cross-linking [3–6]. The biological actions of AGE can also be suppressed through inhibition of the receptor for advanced glycation end-product (RAGE) [7, 8]. Given that AGE is already formed and accumulated in the kidney when the diagnosis of diabetic nephropathy is made in the clinical setting, breakage of pre-formed AGE may be an alternative approach to inhibit the actions of AGE. In this regard, ‘the putative cross-link breakers’ have emerged as a new generation of inhibitors of AGE for clinical use [9, 10].

Alagebrium [ALA or ALT-711: 3-(2-oxo-2-phenyl)-ethyl-4,5-dimethyl-thiazolium chloride] was originally introduced to break the protein cross-links formed by AGEs and was shown to reverse vascular stiffness and diastolic dysfunction in diabetic rats [11], to prevent renal tubular transforming growth factor (TGF)-beta1 expression and epithelial–mesenchymal transition in streptozotocin-induced diabetic rats [12] and to improve arterial compliance in aged humans [13]. ALA reverses diabetic renal injury in streptozotocin-induced diabetic rats [14] and db/db mice [15]. A mechanistic study showed that ALA attenuated the accumulation of extracellular matrix in diabetic kidney via a protein kinase C (PKC) α-dependent pathway [16], suggesting that ALA may have multiple actions relevant to conferring renoprotection. In this context,
thiazolium derivatives such as ALA and its hydrolysis products [17] may have chelating activity [18].

AGE activates not only PKC but also nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and induces oxidative stress [19–24]. Reactive oxygen species (ROS) and PKC together constitute a positive feedback loop in the diabetic milieu since PKC activates NADPH oxidase [25–27], which is considered the most important source of receptor-stimulated generation of ROS [28–30] and since ROS are upstream signaling molecules to PKC [31–33]. One recent study demonstrated that AGE/RAGE-mediated NADPH oxidase-derived ROS facilitated renal mitochondrial superoxide production in hyperglycemia [24]. Similarly, the observation that ROS autoinduced ROS via NADPH oxidase in non-phagocytic cells [34, 35] underscores the importance of NADPH oxidase in AGE-induced renal injury [23].

Therefore, the aim of our study was to examine the effect of ALA on oxidative stress in diabetic nephropathy. We first examined the effect of ALA on renal NADPH oxidase subunit expression accompanying renal injury in db/db mice. After we determined the antioxidant effect of ALA by glucose oxidase method of Sigma kit 510-DA; urinary creatinine was measured by a modified Jaffe method and adjusted for interference by glucose (YD Diagnostics, Yongin-Si, Kyungki-Do, Korea); and urinary albumin was measured by an Albuwell M kit (Exocell Inc., Philadelphia, PA) and normalized to urinary creatinine.

Materials and methods

Anti-fibronectin antibody was purchased from DAKO A/S (Glostrup, Denmark), anti-nitrotyrosine antibody from Calbiochem (La Jolla, CA) and anti-rac1 antibody from Upstate Biotechnology (Lake Placid, NY); antibodies against N-(carboxymethyl)lysine (CEL) and pentosidine from TransGenic Inc. (Kumamoto, Japan); antibodies against vascular endothelial growth factor (VEGF), p47phox, p67phox, NADPH oxidase 2 (Nox2, gp91phox), PKCα, PKCβI and PKCβII from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); fetal bovine serum (FBS) from GIBCO BRL (Gaithersburg, MD) and 5- and 6-chloromethyl-2-(carboxyethyl)lysine (CEL) and pentosidine from TransGenic Inc. (Kumamoto, Japan); antibodies against vascular endothelial growth factor (VEGF), p47phox, p67phox, NADPH oxidase 2 (Nox2, gp91phox), PKCα, PKCβI and PKCβII from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); fetal bovine serum (FBS) from GIBCO BRL (Gaithersburg, MD) and 5- and 6-chloromethyl-2,7'-dichlorodihydrofluorescein diacetate (CMH2DCF-DA) from Molecular Probes (Eugene, OR). Other chemicals and tissue culture plates were obtained from Sigma–Aldrich Company (St. Louis, MO) and Becton Dickinson Labware (Lincoln Park, NJ), respectively. ALA was kindly provided by Alteon Inc. (Parssippany, NJ).

Experimental animals

Male db/db mice (C57BLK1Jc1 db/db) and their age-matched non-diabetic counterparts, db/m mice (C57BL/KsJ c1 db/m), were purchased from CLEA Japan, Inc. (Tokyo, Japan). Animal experiments were conducted in accordance with institutional guidelines for animal care and use. Plasma glucose measured by the glucose oxidase method confirmed that db/db mice were hyperglycemic by 8 weeks of age.

ALA (2 mg/kg) was administered intraperitoneally (i.p.) daily for 12 weeks from 8 to 20 weeks of age to db/m mice (control-ALA) and db/db mice (diabetic-ALA-early treatment: DmgALEdE). In a separate group of db/db mice, ALA was administered for 4 weeks from 16 to 20 weeks of age (diabetic-ALA-late treatment: DLmgALAL) to evaluate the effect of delayed treatment. A group of db/m and db/db mice each received the same volume of 0.9% NaCl (p) for 12 weeks from 8 weeks of age and served as control db/m mice (C) and untreated db/db mice (D). The effective dose of ALA was decided based on previous reports [11, 12, 16] and our preliminary study.

Five each of untreated db/m and db/db mice were sacrificed at 16 weeks of age and examined for evidence of renal injury. All remaining mice were sacrificed and analyzed at 20 weeks of age. Glomeruli were obtained by a standard sieving method, homogenized in lysis buffer (20 mmol/L Tris–HCl, 137 mmol/L NaCl, 5 mmol/L ethylenediaminetetraacetic acid, 1 mmol/L ethylene glycol tetraacetic acid, 1% Triton X-100, 1 mmol/L dithiothreitol, 0.2 mg/L phenylmethylsulfonyl fluoride, 5 mg/L leupeptin, 5 mg/L aprotinin, 10% glycerol, 1 mmol/L Na3VO4, 10 mmol/L NaF, 1 mmol/L Na2PO4, 1 mmol/L [β-glycerophosphate] and stored at –70°C before use.

Mesangial cells

A murine mesangial cell line (MES-13) was obtained from American Type Culture Collection (Rockville, MD) and cultured with Dulbecco’s modified Eagle’s medium (DMEM) containing 5% FBS. Growth-arrested cells were stimulated with 5.6 mmol/L (control) or 30 mmol/L glucose (HG) or 100 mmol/L H2O2 for a given period. ALA (1–100 mg/L) was administered 1 h before the addition of HG or H2O2.

Metabolic data

Blood glucose, urinary creatinine and albumin concentrations and body weight were measured at 8, 16 and 20 weeks of age. Urine was collected over two consecutive 24-h periods with each mouse individually housed in a metabolic cage with free access to food and water. Blood glucose was measured by glucose oxidase method of Sigma kit 510-DA; urinary creatinine was measured by a modified Jaffe method and adjusted for interference by glucose (YD Diagnostics, Yongin-Si, Kyungki-Do, Korea); and urinary albumin was measured by an Albuwell M kit (Exocell Inc., Philadelphia, PA) and normalized to urinary creatinine.

Morphological study

Glomerular volume (Vg) and fractional mesangial area (FMA) were quantitated as described previously [36]. Renal tissues were fixed in 10% formalin and then embedded in paraffin. Tissue sections were cut at 4-μm thickness, dewaxed and stained with periodic acid–Schiff (PAS). Twenty randomly selected glomeruli were examined under high magnification (×400). The coded sections were read by an observer unaware of the experimental protocol. Cross-sectional glomerular area and glomerular tuft area were measured by manually tracing the outline of the Bowman’s capsule and the glomerular tuft within the Bowman’s capsule, respectively, and analyzed using Image-Pro Plus 4.5.1. (Media Cybernetics, Silver Springs, MD). Vg was calculated by the equation: Vg = B/A (glomerular cross-sectional area) where B = 1.38 is the shape coefficient for spheres and a = 1.1 is a size distribution coefficient. The mesangial matrix area was defined as the PAS-positive area within the tuft area. FMA represents the percent of mesangial matrix area occupying the tuft area.

Immunoblot analysis

Protein concentration in glomerular lysate was measured by a Bio-Rad assay reagent (Bio-Rad Laboratories, Hercules, CA). Aliquots of glomerular lysate were mixed with sample buffer containing sodium dodecyl sulfate (SDS) and β-mercaptoethanol and heated. After SDS–polyacrylamide gel electrophoresis, the proteins were transferred onto nitrocellulose membranes. The membranes were blocked and incubated with peroxidase-conjugated rabbit anti-human fibronectin (1:5000). For VEGF, CEL, pentosidine and β-actin, membranes were incubated with 1:1000 primary antibodies, washed and incubated with an appropriate secondary antibody. After washing, the membranes were incubated with ECL reagents (Amersham Life Science, Little Chalfont, UK). Positive bands were quantified, normalized to β-actin and compared to controls.

Both pentosidin and CEL are protein modifications that will be shown as multiple proteins in the immunoblot. We detected the strongest band of which molecular weight is between 35 and 50 kDa with appropriate exposure.

Immunohistochemistry

Immunoperoxidase staining was performed to determine the renal expression of NADPH oxidase subunits and nitrotyrosine using avidin–biotin–horseradish peroxidase technique (Elite Vectastain ABC kit for rabbit IgG; Vector Laboratories, Burlington, CA) as previously described [37]. Sections were dewaxed, dehydrated and incubated with 1.4% methanolic H2O2 for 30 min to inhibit the intrinsic peroxidase. After treatment with blocking serum for 30 min, the tissue sections were incubated with antibodies against nitrotyrosine (1:100) and NADPH oxidase subunits, p47phox, p67phox and Nox2 (1:100), overnight at 4°C. After rinsing in
phosphate-buffered saline (PBS), the sections were incubated with biotinylated goat anti-rabbit antibody for 1 h. Following further washes, the sections were incubated with 0.3% avidin–biotin–peroxidase complex for 1 h. After dehydrlation with increasing concentrations of ethanol, the sections were covered with glass coverslips and observed by light microscopy. The site of the antigen–antibody reaction was visualized by 3,3'-diaminobenzidine tetrahydrochloride reaction, quantitated using Image Pro Plus 4.5.1, and expressed as a % of total glomerular and tubular areas. A total of 20 different areas in the cortex of kidney tissue sections from each mouse were coded and read by three observers unaware of the experimental protocol.

Translocation of NADPH oxidase subunits and PKC isoforms in mesangial cells

Membrane and cytosol fractions were obtained as previously described [38]. Antibodies to p47 phox (1:500), p67 phox (1:500), rac1 (1:1000), PKC α (1:1000), PKC β (1:1000) and PKC θ (1:1000) were used to measure NADPH oxidase subunit and PKC isoform proteins in membrane and cytosol fractions by immunoblot analysis.

Reverse transcription and real-time quantitative polymerase chain reaction

Total RNA was extracted from cells with RNA STAT, and real-time quantitative polymerase chain reactions (PCRs) with β-actin internal control were performed using the Sybrgreen PCR master mix kit (Applied Biosystems, Foster City, CA) with an ABI 7300 real-time PCR thermal cycler (Applied Biosystems) as previously described [39]. Reaction conditions were 95°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All reactions were performed in triplicate in a final volume of 20 µL. Standard curves were generated using Applied Biosystems software. Dissociation curves were run to detect nonspecific amplification and to confirm that single products were amplified in each reaction. The quantities of the test gene and internal control β-actin messenger RNA (mRNA) were then determined from the standard curve using the Applied Biosystems software, and mRNA expression levels of test genes were normalized to β-actin levels. Primer sequences can be found in Supplementary Table S1.

Cellular ROS in mesangial cells

DCF-sensitive ROS in mesangial cells were measured by flow cytometry (Becton Dickinson, Mountain View, CA) as described previously [40]. In brief, at various time points after stimulation with HG or H2O2 in the presence or absence of ALA, cells were washed with serum-free DMEM and loaded with 5 µM/L of CM-H2DCF-DA. After incubation for 20 min in the dark, cells were washed with PBS, detached and resuspended in PBS. DCF intensity was analyzed using flow cytometry (excitation, 488 nm; emission, 515-540 nm).

Iodometric analysis

H2O2 standards were prepared by diluting ACS reagent H2O2 with LC/MS-grade water. For the standard curve, each standard was mixed with 0.1 mol/L potassium biphthalate and iodide reagents (0.4 mol/L potassium iodide, 0.06 mol/L NaOH and 0.1 mol/L ammonium molybdate) and incubated at room temperature for 3 min before measuring the absorbance at 352 nm. To measure the antioxidant capacity of ALA, H2O2 was incubated with 1, 10 and 100 mg/L of ALA at room temperature for 0.5, 1, 5 and 10 min, and iodometric reactions were performed in the same manner as for standard H2O2.

Analysis of data

All results are expressed as mean ± SE. The mean values obtained from each group were compared by analysis of variance with subsequent Fisher’s least significant difference method. Nonparametric analyses by the Kruskal–Wallis and the Mann–Whitney U-test were also used where appropriate. A P-value <0.05 was used as the criterion for a statistically significant difference.

Results

Effect of ALA on renal injury in db/db mice

We first confirmed the evidence of renal injury in 16-week old db/db mice in order to determine the effect of delayed treatment with ALA. At 16 weeks of age, db/db mice had significant albuminuria, glomerular hypertrophy, mesangial area expansion and increase in glomerular expression of fibronectin and VEGF as compared to db/m mice. Glomerular accumulation of pentosidine, a cross-linking AGE, was also increased in db/db mice compared to db/m mice at 16 weeks of age (Supplementary Figure S1).

Albumin excretion rate (AER) increased with time in untreated db/db mice and was 4-fold higher at 16 weeks (Supplementary Figure S1a) and 10-fold higher at 20 weeks of age (Figure 1A) compared to control db/m mice. ALA significantly reduced AER in both early (DALA/E) and delayed (DALL/L) treatment groups of db/db mice at 20 weeks of age without significant effect in db/m mice. V_G and FMA also increased with time in untreated db/db mice (Supplementary Figure S1b and S1c; Figure 1C–E). Untreated db/db mice exhibited significant (P < 0.05) increases in body weight, plasma creatinine and kidney weight compared to control db/m mice at 20 weeks of age (Figure 1B, Table 1). ALA did not affect kidney weight (Figure 1B), body weight, plasma glucose or creatinine concentration (Table 1) in db/m mice. Early, but not delayed, treatment with ALA significantly (P < 0.05) reduced V_G in db/db mice (Figure 1C and D). ALA significantly reduced FMA in both early and delayed treatment groups of db/db mice (Figure 1C and E).

Glomerular expression of fibronectin in untreated db/db mice at 20 weeks of age was 8-fold greater than that of db/m mice and VEGF was 1.6-fold higher (Figure 2). Both early and delayed treatment with ALA significantly reduced fibronectin expression in the glomeruli of db/db mice without significant effect in db/m mice (Figure 2A). ALA also inhibited glomerular VEGF expression in db/db mice although the difference did not reach statistical significance (Figure 2B).

Effect of ALA on glomerular accumulation of pentosidine and N-(carboxyethyl)lysine in db/db mice

Glomerular accumulation of pentosidine and CEL was 2.2-fold (Figure 3A) and 1.4-fold (Figure 3B) higher, respectively, in untreated db/db mice at 20 weeks of age than in db/m mice. Both early and delayed treatment with ALA significantly reduced pentosidine accumulation in the glomeruli of db/db mice without significant effect in db/m mice (Figure 2A). ALA also decreased glomerular CEL accumulation in db/db mice although the difference did not reach statistical significance. Glomerular CEL accumulation in both ALA-treated db/db mice was not different from that in db/m mice (Figure 3B).

Effect of ALA on renal expression of NADPH oxidase subunit in db/db mice

Since NADPH oxidase-derived ROS play an important role in AGE-induced renal injury, renal expression of the NADPH oxidase subunit along with the accumulation of nitrotyrosine was measured. In untreated db/db mice, there was a significant increase in glomerular and tubular expression of p47 phox, p67 phox and Nox2 with respect to control db/m mice (Figure 4). Expression of p47 phox, p67 phox, Nox2 and Nox4 (Renox) mRNA was also significantly increased in db/db mouse kidney (Supplementary
Nitrotyrosine, a marker of protein oxidation, was minimally detected in mouse kidney but was significantly increased in glomeruli and tubules in untreated db/db mice, suggesting that upregulation of NADPH oxidase may lead to increased oxidative stress in the kidneys of db/db mice (Figure 4A and E). Both early and delayed treatment with ALA reduced renal accumulation of nitrotyrosine as well as expression of each NADPH oxidase subunit to control level.

Effect of ALA on HG- or H$_2$O$_2$-stimulated membrane translocation of NADPH oxidase subunit and PKC isoform and cellular ROS in mesangial cells

Since AGE inhibitors including phenacylthiazolium and phenacyldimethylthiazolium have been reported to have chelating or antioxidant activity [18] and since ALA inhibited the activation of NADPH oxidase when administered from the onset of diabetes, before significant AGE accumulation.
accumulation, the effect of ALA on HG- or H2O2-treated mesangial cells was examined. HG, but not L-glucose, significantly increased NADPH oxidase subunits p47 phox, p67 phox and rac1 and PKC isoforms α, βI and βII in the membrane fraction without affecting the cytosol fraction (Figure 5). H2O2 itself also stimulated membrane translocation of all NADPH oxidase subunits and PKC isoforms in the present study (Figure 6). ALA effectively prevented not only HG- but also H2O2-induced membrane translocation of all NADPH oxidase subunits and PKC isoforms.

Nox4 is most abundantly expressed in the kidney and has been suggested to play a critical role in hyperglycemia-induced ROS in diabetic kidneys [41, 42]. Expression of Nox4 mRNA was also significantly increased in HG- or H2O2-treated mesangial cells and ALA also ameliorated HG- and H2O2-induced Nox4 mRNA (Figure 7).

Both HG and H2O2 significantly increased cellular ROS, which was effectively inhibited by the pretreatment of ALA at concentrations >1 mg/L (Figure 8).

**Effect of ALA on H2O2 concentration in a test tube**

Finally, we tested the direct effect of ALA on H2O2 in a test tube. H2O2 concentration in standard solutions remained stable for up to 10 min in the absence of ALA. ALA reduced H2O2 concentration in a dose-dependent manner by 35, 59 and 100% at 1, 10 and 100 mg/L of ALA, respectively, at 30 s after the addition (Figure 9A).

**Discussion**

The present study provides evidence that ALA, a putative AGE cross-link breaker, has both direct and indirect antioxidant effects in preventing diabetic renal injury (Figure 9B). We successfully showed that (i) ALA, when administered either early (8 weeks of age) or late (16 weeks of age), significantly decreased renal accumulation of nitrotyrosine and NADPH oxidase expression as well as features of renal injury in a model of type 2 diabetes; more importantly (ii) ALA inhibited not only HG- but also H2O2-induced membrane translocation of cellular ROS in cultured mesangial cells and (iii) ALA decreased H2O2 in a test tube.

We hypothesized that ALA may retard renal injury in diabetes, in part, through attenuation of oxidative stress in the kidney, since AGEs activate NADPH oxidase and...
induce oxidative stress [19–24]. We chose the \( \text{db/db} \) mouse as a model since type 2 diabetes is the predominant type of diabetes in humans.

ALA prevented albuminuria in \( \text{db/db} \) mice when administered early and reduced albuminuria to control level when administered late. This finding is consistent with previous studies [14, 15] in which ALA, independent of treatment period, retarded albuminuria in diabetic animals. Recent observations suggested that VEGF has an important role in the development of albuminuria in type 2 diabetes. Chronic inhibition of VEGF by neutralizing anti-VEGF antibody from 8 weeks of age ameliorated albuminuria in \( \text{db/db} \) mice [43]. Furthermore, blockade of RAGE from 8 weeks of age prevented the increase in VEGF expression in podocytes and albuminuria in \( \text{db/db} \) mice [7]. In the present study, glomerular VEGF expression was significantly increased in untreated \( \text{db/db} \) mice, and treatment with ALA, regardless of initiation of treatment, reduced VEGF to control level.

![Fig. 4. Effect of ALA on renal expression NADPH oxidase subunits p47 phox (A and B), p67 phox (A and C), and Nox2 (A and D), accumulation of nitrotyrosine (A and E). Renal immunohistochemical analysis was done as described in Materials and Methods. Data are presented as mean ± SE of 150 different glomerular and tubular areas per group. C: \( \text{db/m} \) treated with 0.9% NaCl for 12 weeks from age 8 weeks, \( \text{C}_{\text{ALA}} \): \( \text{db/m} \) treated with ALA for 12 weeks from age 8 weeks, D: \( \text{db/db} \) treated with 0.9% NaCl for 12 weeks from age 8 weeks, \( \text{D}_{\text{ALA/E}} \): \( \text{db/db} \) treated with ALA for 12 weeks from age 8 weeks, \( \text{D}_{\text{ALA/L}} \): \( \text{db/db} \) treated with ALA for 4 weeks from age 16 weeks. ALA 2 mg/kg/day was i.p. administered. Scale bar, 50 \( \mu \)m; magnification \( \times 400 \). *P < 0.05 compared to C, †P < 0.05 compared to D.

development of albuminuria in type 2 diabetes. Chronic inhibition of VEGF by neutralizing anti-VEGF antibody from 8 weeks of age ameliorated albuminuria in \( \text{db/db} \) mice [43]. Furthermore, blockade of RAGE from 8 weeks of age prevented the increase in VEGF expression in podocytes and albuminuria in \( \text{db/db} \) mice [7]. In the present study, glomerular VEGF expression was significantly increased in untreated \( \text{db/db} \) mice, and treatment with ALA, regardless of initiation of treatment, reduced VEGF to control level.
ALA has been reported to attenuate glomerular VEGF expression in streptozotocin-induced diabetic rats [16]. We also confirmed that both early and late treatment with ALA suppressed mesangial expansion and glomerular fibronectin accumulation in db/db mice. Mesangial expansion resulting from extracellular matrix protein accumulation is a characteristic finding in diabetic nephropathy and is best correlated with decreased renal function [44]. Upregulation of total renal collagen [14] and glomerular fibronectin and laminin [16] in streptozotocin-induced diabetic rats were also inhibited by ALA. Unlike mesangial expansion, glomerular hypertrophy was prevented by early treatment but was not affected by late treatment in the present study. It remains to be determined whether late intervention initiated at a time point of decreased reversibility of glomerular hypertrophy or whether a longer treatment period in the delayed intervention group, i.e. increasing drug intervention from 4 to 12 weeks, would suppress the glomerular hypertrophy.

Glomerular accumulation of pentosidine, a cross-linking AGE, in untreated db/db mice was 2.2-fold higher than that of control db/m mice at 20 weeks of age, and this increase was significantly reduced by both early and late treatment with ALA. Glomerular accumulation of CEL, a non-cross-linking product of oxidative stress, in untreated db/db mice was 1.5-fold higher than that of control db/m mice at 20 weeks of age. ALA also decreased CEL accumulation in db/db mice, although the difference was not statistically significant. We tried to quantify carboxymethyl lysine (CML) in the present study by using various antibodies against CML but could not get a specific immunoblot for some reason. ALA, regardless of the intervention period, effectively retarded serum AGE peptides, immunostaining for renal CML and renal AGE fluorescence in streptozotocin-induced diabetic rats [14] and renal CML immunoreactivity in db/db mice [15]. These results suggest that the renoprotective effects of ALA in diabetic kidney may result not only from AGE breaking activity but also from antioxidant activity since ROS promote AGE formation [32].

In this context, there was upregulated expression of NADPH oxidase subunit p47 phox, p67 phox, Nox2 and Nox4 concomitant with intense staining of nitrotyrosine proteins in untreated db/db mouse glomeruli and tubules at 20 weeks of age and these were effectively attenuated by both early and late treatment with ALA. The inhibitory effect of ALA on renal nitrotyrosine in streptozotocin-induced diabetic rats was evident only in early intervention [14]. This discrepancy may result from the initiation of intervention; we initiated at either the onset or 8 weeks after the onset of diabetes in db/db mice but Forbes et al. [14] initiated treatment at either 16 or 24 weeks after streptozotocin. Increased NADPH oxidase in the kidneys of diabetic animals has been demonstrated as summarized in recent reviews [28, 29]. Given that AGE can activate NADPH oxidase [20, 23, 24], inhibition of NADPH oxidase by ALA could be the result of decreased AGE leading to inhibiting AGE/RAGE interaction. However, our data
from cell culture suggest that ALA may directly inhibit the activation of NADPH oxidase.

In mesangial cells, ALA effectively and simultaneously prevented HG-induced membrane translocation of NADPH oxidase subunits and PKC isoforms and generation of cellular ROS. Given that PKC activates NADPH oxidase [25–27] and that ALA inhibits PKC activation [16], the inhibition of NADPH oxidase translocation by ALA may be the result of inhibition of PKC activation. However, the simultaneous inhibition of NADPH oxidase and PKC activation by ALA suggests that ALA may have a direct effect on NADPH oxidase. On the other hand, PKC is activated by ROS [31–33] and it is possible that inhibition of PKC isoforms α, βI and βII in this study was the result of the antioxidant effect of ALA. To support this, H2O2 effectively increased PKC isoform and NADPH oxidase subunit membrane translocations and these increments were abrogated by ALA in this study. Furthermore, amelioration of H2O2-induced Nox4 mRNA upregulation was effectively inhibited by ALA.

Considering the time required for AGE formation, it is possible that ALA inhibited NADPH oxidase activation independent of its action on AGE. Since NADPH oxidasederived ROS facilitate renal mitochondrial superoxide production under hyperglycemia [24] and since functional mitochondrial Nox4 is upregulated in mesangial cells cultured under high glucose and in renal cortex of diabetic rats.
ALA may also inhibit mitochondrial ROS generation. A recent study demonstrated that ALA decreased mitochondrial ROS [46].

In addition, we found that ALA at concentrations >1 mg/L (3.7 μmol/L) directly decreased H2O2 in test tube within 1 min. This observation is consistent with chelating or antioxidant activity of phenacylthiazolium and phenaclydimethylthiazolium and their hydrolysis products in vitro [17, 18]. The direct action of ALA on H2O2 shown in this study may be an alternative mechanism for its antioxidant effects. It remains to be studied whether ALA eliminates H2O2 in situ kidneys, despite recent progress in the measurement of H2O2 in biological samples [47].

In conclusion, the present study demonstrates that ALA can prevent the development of diabetic nephropathy when administered early and retard established renal injury when administered late in db/db mice, through direct and indirect antioxidant effects. It remains to be determined whether the add-on therapy of ALA to control of blood glucose and blood pressure and inhibition of angiotensin II is safe and effective in diabetic nephropathy.

Supplementary data

Supplementary data is available online at http://ndt.oxfordjournals.org.

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Conflict of interest statement. None declared.

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Quercetin reduces cisplatin nephrotoxicity in rats without compromising its anti-tumour activity

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Abstract

Background. Nephrotoxicity is the major limitation for the clinical use of cisplatin as an anti-tumoural drug. Our aim was to investigate the protective effect of quercetin on cisplatin nephrotoxicity in a rat tumour model in vivo and to examine the mechanisms of renal protection.

Methods. Breast adenocarcinoma (13762 Mat B-III) cells were inoculated subcutaneously in male Fischer rats and 7 days later, the rats were administered daily with quercetin [50 mg/kg/day, intraperitoneally (i.p.)] or vehicle. Four days after that, the rats were given a single dose of cisplatin (4 mg/kg, i.p.) or vehicle. Tumour growth and renal function were monitored throughout the experiment. Two or 6 days after cisplatin administration, the rats were killed and the kidneys and tumours were removed to examine renal function and toxicity markers in both tissues.

Results. In the kidney, cisplatin treatment induced: (i) a decrease in renal blood flow and glomerular filtration rate, (ii) tubular necrosis/apoptosis, (iii) increased lipid peroxidation and decreased endogenous antioxidant systems, (iv) increased expression of inflammation markers and (v) increased activity of the apoptosis executioner caspase-3. Cisplatin effectively reduced tumour size and weight.

Conclusions. Co-treatment with quercetin partially prevented all the renal effects of cisplatin, whereas it did not impair its anti-tumour activity. In conclusion, in a model of tumour-bearing rats, quercetin prevents the nephrotoxic effect of cisplatin without affecting its anti-tumour activity.

Keywords: acute kidney injury; cisplatin; nephrotoxicity; quercetin; renoprotection

Introduction

Cisplatin (cDDP; cis-diamminedichloroplatinum II) is a drug widely used against different types of solid tumours. However, its therapeutic utility is limited by acute and chronic nephrotoxicity. About 25% of patients receiving high-dose cisplatin undergo severe renal dysfunction. Cisplatin nephrotoxicity is chiefly characterized by tubular damage, mainly affecting the renal proximal and distal tubuli. Tubular damage may range from a mere loss of the brush border of epithelial cells to an overt tubular necrosis in severe cases [1]. Tubular damage causes impaired reabsorption, which underlies the observed proteinuria, hypomagnesemia and hypokalemia. In addition, cisplatin nephrotoxicity often progresses with reduced glomerular filtration rate (GFR) and increased serum creatinine [2, 3], which may result from the onset of the tubuloglomerular feedback mechanism and to reduced renal blood flow (RBF) resulting from renal vasoconstriction.

Although the mechanisms involved in cisplatin nephrotoxicity have been extensively studied, they are not yet fully elucidated. Recent studies suggest that cisplatin nephrotoxicity is a complex and multifaceted process in which cisplatin triggers cellular responses involving multiple pathways that culminate in renal damage and death [4, 5]. By inducing mitochondrial injury, cisplatin stimulates the production of reactive oxygen species (ROS) [6], which trigger an inflammatory response [7, 8] and several apoptotic signalling pathways mediated by caspases and mitogen-activated protein kinases [9–11].

Many different substances including antioxidants [4] have been investigated for their beneficial effects