

# Angiotensin-converting enzyme inhibition curbs tyrosine nitration of mitochondrial proteins in the renal cortex during the early stage of diabetes mellitus in rats

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## Abstract

Experiments were performed to evaluate the hypothesis that ACE (angiotensin-converting enzyme) inhibition (enalapril) suppresses 3-NT (3-nitrotyrosine) production in the renal cortex during the early stage of Type 1 DM (diabetes mellitus) in the rat. Enalapril was administered chronically for 2 weeks to subsets of STZ (streptozotocin)-induced DM and vehicle-treated sham rats.  $O_2^-$  (superoxide anion) and  $NO_x$  (nitrate + nitrite) levels were measured in the media bathing renal cortical slices after 90 min incubation *in vitro*. SOD (superoxide dismutase) activity and 3-NT content were measured in the renal cortex homogenate. Renal cortical nitrated protein was identified by proteomic analysis. Renal cortical production of  $O_2^-$  and 3-NT was increased in DM rats; however, enalapril suppressed these changes. DM rats also exhibited elevated renal cortical  $NO_x$  production and SOD activity, and these changes were magnified by enalapril treatment. 2-DE (two-dimensional gel electrophoresis)-based Western blotting revealed more than 20 spots with positive 3-NT immunoreactivity in the renal cortex of DM rats. Enalapril treatment blunted the DM-induced increase in tyrosine nitration of three proteins ACO2, GDH1 and MMSDH (aconitase 2, glutamate dehydrogenase 1 and methylmalonate-semialdehyde dehydrogenase), each of which resides in mitochondria. These data are consistent with enalapril preventing DM-induced tyrosine nitration of mitochondrial proteins by a mechanism involving suppression of oxidant production and enhancement of antioxidant capacity, including SOD activation.

**Key words:** diabetic nephropathy, mitochondrial proteins, nitric oxide, 3-nitrotyrosine, oxidative stress, proteomic analysis

## INTRODUCTION

The measurement of urinary albumin excretion (microalbuminuria and albuminuria) is important for diagnostics and categorization into specific stages of DN (diabetic nephropathy) [1]. In Japan, normal albumin values for morning urine samples and spot

(random) urine samples are less than 16.5 mg/l (10.8 mg of creatinine/g) and 29.3 mg/l (24.6 mg of creatinine/g) respectively [2], values that differ by approximately 2-fold. Indeed, accumulating evidence indicates that microalbuminuria is a less sensitive and less specific predictor of DN than previously reported in [3–5]. Thus it is of paramount importance to develop new diagnostic

**Abbreviations:** 3-NT, 3-nitrotyrosine; ACEI, angiotensin-converting enzyme inhibitor(s); ACO2, aconitase 2; BP, blood pressure; DM, diabetes mellitus; DN, diabetic nephropathy; GDH1, glutamate dehydrogenase 1; GFR, glomerular filtration rate; HBSS, Hank's balanced salt solution; LC, liquid chromatography; MMSDH, methylmalonate-semialdehyde dehydrogenase; MS/MS, tandem MS; NNA,  $N^{\omega}$ -nitro-L-arginine; NO, nitric oxide;  $NO_x$ , nitrate + nitrite; NBT, Nitro Blue Tetrazolium;  $O_2^-$ , superoxide anion; ONOO $^-$ , peroxynitrite; PEPCCK, phosphoenolpyruvate carboxykinase; SOD, superoxide dismutase; STZ, streptozotocin; Sham + ENAL, enalapril-treated Sham rats; STZ + ENAL, enalapril-treated STZ rats; TCA, tricarboxylic acid; 2-DE, two-dimensional gel electrophoresis.

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indices that can be employed during the early stage of DM (diabetes mellitus) in order to direct appropriate therapeutic strategies for preventing or delaying development of DN. A more complete understanding of the pathogenesis of DN may allow identification of causative and related molecules that could be useful as diagnostic markers.

ACEi (angiotensin-converting enzyme inhibitors) are widely utilized therapeutic tools for decelerating development of DN with or without hypertension [6,7]. Antioxidant effects of ACEi may contribute to the ability of these agents to prevent DN. Indeed, enalapril (an ACEi) has been reported to increase antioxidant enzyme activity in kidneys of rats with DM [6]. Our previous studies performed during the early stage of DM in the rat revealed that accelerated renal cortical  $O_2^-$  (superoxide anion) production promotes NO (nitric oxide) degradation via formation of the powerful toxic oxidant, ONOO<sup>-</sup> ( $O_2^- + NO \rightarrow ONOO^-$ ) and subsequent nitration of protein tyrosine residues [3-NT (3-nitrotyrosine)] [8]. Enalapril increases SOD (superoxide dismutase) activity and prevents accelerated  $O_2^-$  and 3-NT production in renal cortex during *in vitro* acute exposure to high glucose levels [9]. These observations suggest that in addition to its anti-hypertensive and anti-proteinuric effects, enalapril and other ACEi may suppress renal cortical 3-NT levels through a mechanism involving a reduction in AngII (angiotensin II)-stimulated NADPH oxidase activation (reducing  $O_2^-$  production) as well as enhanced  $O_2^-$  degradation resulting from SOD activation. Some beneficial effects of ACE inhibition may reflect prevention of DM-induced post-translational modification events involving nitration of tyrosine residues; however, no information is available regarding the specific renal cortical proteins undergoing enalapril-sensitive tyrosine nitration during DM. Identification of these proteins would not only reveal previously unrecognized events that may contribute to development of DN, but also could lead to establishment of new diagnostic markers that can be employed during the early stage of DM prior to development of albuminuria.

The present study was designed to test the hypothesis that ACE inhibition suppresses 3-NT production in the rat renal cortex (location of glomeruli and resistance arterioles) during the early stage of DM. An additional goal was to identify the protein(s) whose DM-induced tyrosine nitration is suppressed by ACE inhibition. Because rats studied 2 weeks after onset of STZ (streptozotocin)-induced DM exhibit renal oxidative stress without significant albuminuria [10], we chose this model for identifying ACE inhibition-sensitive post-translational protein modifications that arise early during the course of DM.

## MATERIALS AND METHODS

### Animal studies

Kitasato University Institutional Animal Care and Use Committee approved all procedures used in the present study. Male Sprague–Dawley rats (Japan SLC) were assigned to four groups ( $n = 6$  each): (i) STZ group (rats made diabetic by STZ injection); (ii) Sham group (rats receiving the STZ vehicle); (iii)

STZ + ENAL group (STZ rats receiving enalapril treatment); and (iv) Sham + ENAL group (enalapril-treated Sham rats). Injection of STZ (65 mg/kg of body weight, intravenous; Sigma–Aldrich) and implantation of a sustained-release insulin pellet (Linplant) to maintain moderate hyperglycaemia were performed as described previously [8]. All animals received penicillin G (60 000 units, intramuscular; Meiji Seika) immediately after this procedure. Beginning 1 day after STZ or vehicle injection, rats in the Sham + ENAL and STZ + ENAL groups received enalapril at 20 mg/kg of body weight per day (Sigma–Aldrich) for 2 weeks. To ensure that each rat received the same dose of enalapril, despite the polydipsia evident in STZ-treated rats, each day the amount of enalapril required for each animal was dissolved in 10–20 ml of water and this enalapril solution was provided as a substitute for drinking water. After rats finished completely the drinking of the enalapril solution, they were given *ad libitum* access to water. The total volume of enalapril solution and drinking water consumed each day was considered to represent daily water intake. The animals were allowed *ad libitum* access to food. Blood glucose concentration (GLUTEST ACE; Sanwa Kagaku Kenkyusho) and body weight were measured at 2- to 3-day intervals. Systolic BP (blood pressure) was evaluated by tail plethysmography (MK-2000; Muromachi). At 2 weeks after the injection of STZ or vehicle, the rats were housed in metabolic cages for 2 days. The first day was allowed as an acclimatization period, after which urine was collected and water intake was measured for the ensuing 24-h period. Then the animal was anaesthetized with sodium pentobarbital (50 mg/kg of body weight, intraperitoneally; Dainippon-Sumitomo Pharma), the abdominal aorta was cannulated to collect blood, and the kidneys were flushed with saline containing 20 unit/ml heparin before their rapid excision.

### NO<sub>x</sub> (nitrate + nitrite) production assay

Renal cortical slices were incubated at 37 °C for 90 min in HBSS (Hank's balanced salt solution) containing 500 unit/ml SOD (to prevent NO degradation by ONOO<sup>-</sup> formation), in either the absence or presence of 10 mmol/l NNA (*N*<sup>ω</sup>-nitro-L-arginine; Sigma–Aldrich). NO<sub>x</sub> concentration in the incubated renal cortical supernatant was measured using the Griess assay (NO<sub>x</sub> assay kit-C II, Dojindo Laboratories) [11]. NNA-sensitive NO<sub>x</sub> production is reported as nmol/mg of protein per 90 min.

### O<sub>2</sub><sup>-</sup> production assay

Renal cortical O<sub>2</sub><sup>-</sup> production was measured based on its ability to reduce ferricytochrome *c*, according to the established method [8,12]. Renal cortical slices were incubated at 37 °C for 90 min in HBSS containing 80 μmol/l cytochrome *c* and 10 mmol/l NNA (to minimize O<sub>2</sub><sup>-</sup> consumption via reaction with NO) in either the absence or presence of 500 unit/ml SOD. As a positive control, slices from each kidney were also incubated in the presence of 250 μg/ml heat-aggregated bovine IgG, which stimulates O<sub>2</sub><sup>-</sup> production by mesangial and proximal tubular cells [13]. The supernatant was removed 90 min after initiating the cytochrome *c* incubation, centrifuged (10 000 g) to remove any cellular debris and absorbance was measured at 550 nm (molar absorption coefficient of reduced cytochrome *c* =  $2.1 \times 10^4$  litres/mol per cm).

SOD-sensitive  $O_2^-$  production is expressed as reduced cytochrome *c* nmol/mg of protein per 90 min.

### SOD activity assay

Renal cortex was homogenized in ice-cold HBSS containing 10 mmol/l CHAPS, centrifuged (10 000 *g*), and the supernatant stored at  $-80^\circ\text{C}$  until assay for SOD activity by the NBT (Nitro Blue Tetrazolium) reduction method (SOD Activity Detection Kit; Wako). One unit of activity is defined as the amount of SOD required to inhibit the rate of xanthine + xanthine oxidase-induced NBT reduction by 50% at  $37^\circ\text{C}$ .

### 3-NT assay

Free 3-NT content in the renal cortex was determined using HPLC [9]. Briefly, renal cortex was homogenized and sonicated in ice-cold 10 mmol/l sodium acetate buffer (pH 6.5) containing 10 mmol/l CHAPS. After centrifugation (10 000 *g*), the supernatant was incubated with 5 mg/ml pronase (protease type XIV, EC 3.4.24.31; Sigma–Aldrich) for 20 h at  $50^\circ\text{C}$ . The resulting enzymatic digest was treated with 20% trichloroacetic acid, centrifuged (14 000 *g*) and passed through a  $0.2\ \mu\text{m}$  PVDF filter. The filtrate was assayed for 3-NT by HPLC with electrochemical detection (applied 1500 mV, EC-8020; TOHSO). The analytical column was TSK-GEL ODS-80Ts (4.6 mm  $\times$  25 cm; TOHSO). The mobile phase was 50 mmol/l acetate/50 mmol/l citrate/5% methanol, pH 3.1 (0.75 ml/min). The retention time of 3-NT is 34 min (tyrosine: 11 min). Free 3-NT concentration in renal cortex is expressed as pmol/mg of protein.

### Protein and creatinine assays

Protein concentration was determined using the Bio-Rad Protein Assay Kit. Creatinine concentration in urine and plasma was measured by the Jaffe method [14], allowing creatinine clearance to be calculated as an estimate of GFR (glomerular filtration rate).

### Agarose 2-DE (two-dimensional gel electrophoresis) for proteomic analysis of protein nitration

Frozen renal cortical tissue was disrupted in extraction solution that consisted of 7 mol/l urea, 2 mol/l thiourea, 2% CHAPS, 100 mmol/l dithiothreitol, 2.5% Pharmalyte with pH 3–10 (GE Healthcare) and 0.1 pellet/ml protease inhibitors (Complete Mini EDTA-free; Roche Diagnostics). The homogenate was centrifuged (112 000 *g*), and the clear supernatant was subjected to agarose 2-DE according to the procedure previously described [15]. Agarose 2-DE was performed at least twice from each renal cortical sample to confirm reproducibility.

### Western blot analysis for protein nitration

Proteins were separated by SDS/PAGE (10% gel) and transferred on to a PVDF membrane. Proteins in the 2-DE gel were incompletely transferred on to the PVDF membrane, leaving a portion for in-gel digestion. The blotting membranes were exposed to mouse anti-NT primary antibody (clone 1A6, 1:1000 dilution; Upstate) and anti-mouse IgG (Fc-specific) peroxidase-conjugated secondary antibody (1:1000 dilution; GE Healthcare). Spots were visualized using ECL (enhanced chemiluminescence) Plus detection reagents (GE Healthcare) and images were cap-

tured and analysed using the ATTO Light Capture system and the companion CS Analyser 3.0 software package (Atto). After blotting, 2-DE gels were stained with Coomassie Blue R-350 (GE Healthcare).

### In-gel digestion, MS and MS/MS (tandem MS)

The methods used for in-gel digestion were described previously [16]. In brief, protein spots were excised from a 2-DE gel, de-stained with 50% (v/v) acetonitrile/50 mmol/l ammonium hydrogen carbonate, dehydrated with 100% acetonitrile, and dried under vacuum conditions. Tryptic digestion was performed for 24 h at  $37^\circ\text{C}$  in a minimum volume of digestion solution containing 0.5 ng/ $\mu\text{l}$  trypsin (proteomics grade; Roche Diagnostics) and 50 mmol/l Tris/HCl buffer. Purified peptides were subjected to LC (liquid chromatography)-MS and LC-tandem MS (MS/MS), with introduction from HPLC to an LCQ-DECA system (Thermo Fisher Scientific) and an ion-trap mass spectrometer via an attached metal API2 needle (an electrospray ionization adapter). Protein identification was achieved by comparison with the Swiss-Prot protein sequence database (<ftp://ftp.ncbi.nih.gov/blast/db/FASTA/>) using the SEQUEST algorithm [17].

### Statistical analysis

Data were analysed by the unpaired Student's *t* test or ANOVA with post-hoc comparisons made using the Student–Newman–Keuls method. *P* values  $<0.05$  were considered significant. All data are reported as means  $\pm$  S.E.M.

## RESULTS

### Animal characteristics

Table 1 summarizes the salient physiological characteristics of the animals utilized in the present study, measured at the 2-weeks time point. Blood glucose levels, water intake and urine volume of STZ-treated groups (STZ and STZ + ENAL) were significantly higher than those of respective control groups (Sham and Sham + ENAL). Body weight and systolic BP did not differ among groups. Both the kidney weight and GFR were increased in the STZ group compared with the Sham group, and these changes were significantly diminished by chronic enalapril treatment (STZ + ENAL).

### Renal cortical $\text{NO}_x$ production

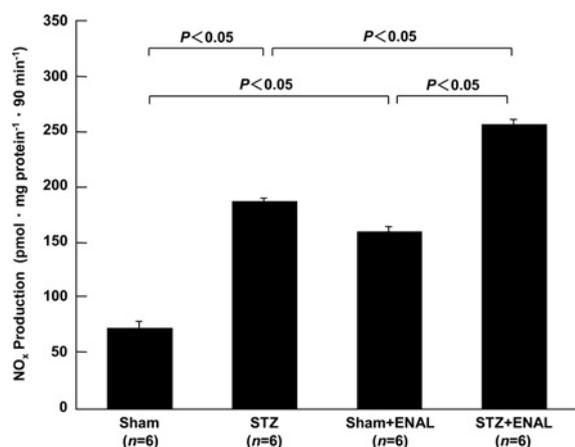
$\text{NO}_x$  production by renal cortical tissue from STZ rats was approximately three times greater than values evident in tissue from Sham rats (Figure 1).  $\text{NO}_x$  production was an additional 30% higher in the STZ + ENAL group than in the STZ group. Moreover,  $\text{NO}_x$  production in the Sham + ENAL group was significantly increased compared with untreated Sham rats. Thus enalapril treatment exaggerated the DM-induced acceleration of  $\text{NO}_x$  production by the renal cortex.

### Renal cortical $O_2^-$ production

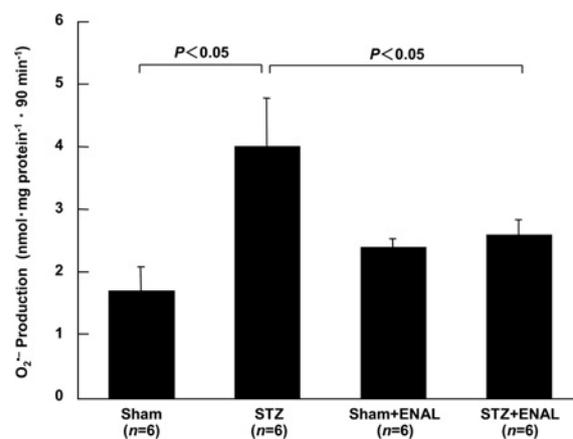
In tissue from the Sham and Sham + ENAL groups, inclusion of aggregated IgG (250  $\mu\text{g/ml}$ ) in the incubation medium yielded  $O_2^-$  production values averaging  $5.4 \pm 0.5$  and  $5.2 \pm 0.7$

**Table 1** Animal characteristicsValues are means  $\pm$  S.E.M. \* $P < 0.05$  against Sham; † $P < 0.05$  against STZ; ‡ $P < 0.05$  against Sham + ENAL.

Characteristic	Sham (n = 6)	STZ (n = 6)	Sham + ENAL (n = 6)	STZ + ENAL (n = 6)
Body weight (g)	305 $\pm$ 4	307 $\pm$ 2	316 $\pm$ 5	301 $\pm$ 5
Kidney weight (g)				
Left	1.14 $\pm$ 0.01	1.68 $\pm$ 0.05*	1.28 $\pm$ 0.07	1.40 $\pm$ 0.10*†‡
Right	1.11 $\pm$ 0.06	1.70 $\pm$ 0.05*	1.26 $\pm$ 0.06	1.40 $\pm$ 0.11*†‡
Blood glucose concentration (mmol/l)	6.0 $\pm$ 0.3	18.0 $\pm$ 4.0*	6.0 $\pm$ 0.1	18.2 $\pm$ 2.4*†
Systolic BP (mmHg)	114 $\pm$ 6	128 $\pm$ 1	113 $\pm$ 3	114 $\pm$ 3
Water intake (ml/day)	50 $\pm$ 2	190 $\pm$ 8*	59 $\pm$ 6	176 $\pm$ 12*†
Urine flow (ml/day)	17 $\pm$ 3	150 $\pm$ 2*	30 $\pm$ 4*	160 $\pm$ 7*†
GFR (ml/min)	2.9 $\pm$ 0.4	5.9 $\pm$ 0.2*	3.1 $\pm$ 0.4	5.0 $\pm$ 0.1*†‡

**Figure 1** Effect of enalapril on NO<sub>x</sub> production by renal cortical slices from normal and diabetic rats

Renal cortical slices from Sham, STZ, Sham + ENAL and STZ + ENAL groups were incubated at 37 °C for 90 min in HBSS containing 500 unit/ml SOD, in either the absence or presence of 10 mmol/l NNA. NO<sub>x</sub> concentration in the incubated renal cortical supernatant was measured using the Griess assay. Values are means  $\pm$  S.E.M.

**Figure 2** Effect of enalapril on O<sub>2</sub><sup>-</sup> production by renal cortical slices of normal and diabetic rats

Renal cortical slices from the Sham, STZ, Sham + ENAL and STZ + ENAL groups were incubated at 37 °C for 90 min in HBSS containing 80  $\mu$ mol/l cytochrome c and 10 mmol/l NNA in either the absence or presence of 500 unit/ml SOD. The absorbance of supernatant was measured at 550 nm (molecular absorbance coefficient of reduced cytochrome c = 2.1  $\times$  10<sup>4</sup> litres/mol per cm). Values are means  $\pm$  S.E.M.

nmol/mg of protein per 90 min respectively (positive control). O<sub>2</sub><sup>-</sup> production by renal cortical slices from the STZ group was approximately two times greater than that evident in the Sham group (Figure 2); however, enhanced production of O<sub>2</sub><sup>-</sup> in the STZ group was prevented by chronic enalapril treatment (STZ + ENAL). There was no significant difference between the Sham group and the Sham + ENAL group with regard to O<sub>2</sub><sup>-</sup> production.

### Renal cortical SOD activity

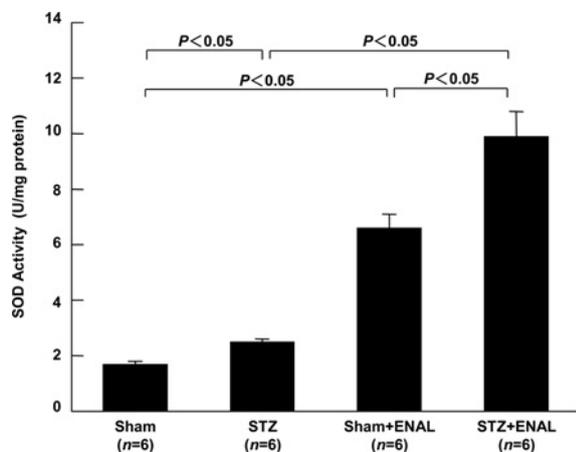
Renal cortical SOD activity (Figure 3) was elevated significantly in the STZ group compared with the Sham group. SOD activity was further accelerated nearly 4-fold by enalapril treatment of rats with DM (STZ + ENAL). In addition, SOD activity was nearly four times higher in the Sham + ENAL group than in the untreated Sham group. Thus both DM and enalapril treatments promoted enhanced renal cortical SOD activity.

### Renal cortical 3-NT content

Renal cortical 3-NT content in the STZ group was 50% higher than in the Sham group (Figure 4). Enalapril treatment did not alter 3-NT content in the Sham group (Sham + ENAL), but the effect of DM to increase renal cortical 3-NT content was completely suppressed by enalapril treatment (STZ + ENAL). Thus, despite accelerated NO production, increased 3-NT content was curbed in the renal cortex of enalapril-treated DM rats.

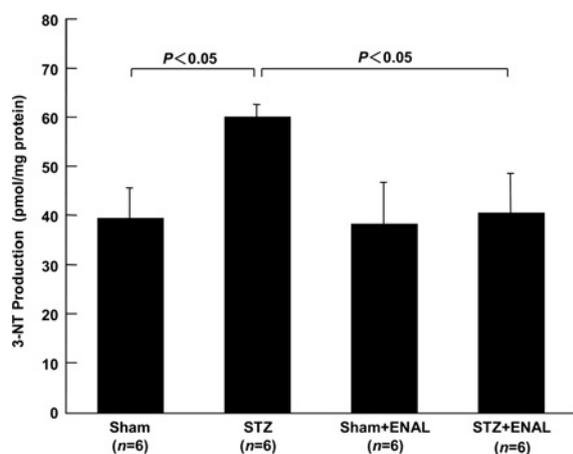
### 2-DE and Western blot analysis renal cortical extracts for protein nitration

Figure 5 shows 2-DE Western blot images of renal cortical NT-immunoreactive proteins detected on PVDF membranes (Figures 5A–5D), as well as these images superimposed with the corresponding images of Coomassie-Blue-stained proteins in the 2-DE gel after partial transfer to PVDF (Figures 5E–5H). In the STZ group, more than 20 spots in the Western blot image showed positive NT-immunoreactivity. Four of the spots exhibiting notable alterations of NT-immunoreactivity across the



**Figure 3 Effect of enalapril on SOD activity in renal cortex of normal and diabetic rats**

SOD activity in supernatant of renal cortical homogenate from the Sham, STZ, Sham + ENAL and STZ + ENAL groups was measured using the NBT reduction method. One unit of activity is defined as the amount of SOD required to inhibit the rate of xanthine + xanthine oxidase-induced NBT reduction by 50% at 37 °C. Values are means  $\pm$  S.E.M.



**Figure 4 Effect of enalapril on 3-NT concentration in renal cortex of normal and diabetic rats**

The supernatant of renal cortex homogenate from the Sham, STZ, Sham + ENAL and STZ + ENAL groups was treated with 5 mg/ml pronase for 20 h at 50 °C. The resulting enzymatic digest was treated with 20% trichloroacetic acid, centrifuged and passed through a 0.2  $\mu$ m PVDF filter. The filtrate was assayed for 3-NT by HPLC with electrochemical detection. Free 3-NT concentration in renal cortex is expressed as pmol/mg protein. Values are means  $\pm$  S.E.M.

four groups of rats are shown circled in Figure 5, with magnified images of each spot provided in Figures 6(A) and 6(B). The densitometric analysis data for spots 1–4 are shown in Figure 6(C). Spots 1, 3 and 4 were minimally evident in the Sham and Sham + ENAL groups, whereas spot 2 was somewhat more prominent in these groups. The emergence of NT-immunoreactive spot 1 in the STZ group was completely averted by enalapril treatment (STZ + ENAL). In addition, enalapril treatment of STZ rats was effective in reducing significantly the intensity of spots 2 and 3 (compare Figure 5B with 5D, and Figure 6). In contrast, NT-immunoreactive spot 4 was prominent in both the STZ group and the STZ + ENAL group. Thus the 2-DE

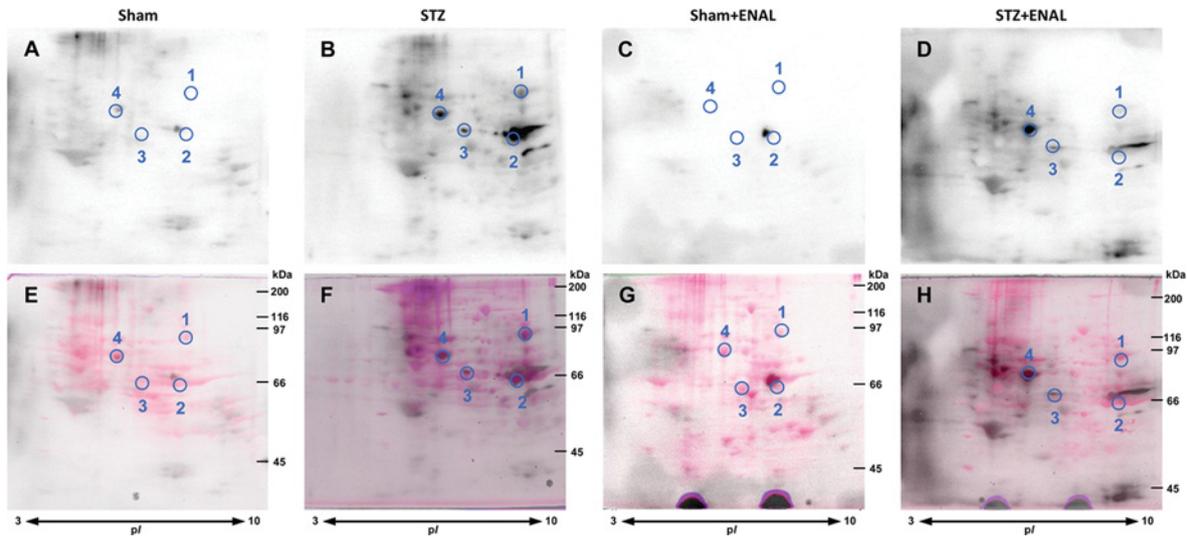
and Western blotting data revealed that the early stage of DM elicits prominent tyrosine nitration of four renal cortical proteins, and that treatment with enalapril prevents or diminishes tyrosine nitration of three of these proteins.

### Identification of tyrosine nitrated proteins from the 2-DE gels

The four prominent renal cortical DM- and enalapril-sensitive tyrosine-nitrated proteins were excised from the 2-DE gel and processed to allow their identification using LC-MS/MS, SEQUEST algorithm and Swiss-Prot protein sequence databases. The results, provided in Table 2, reveal that DM- and enalapril-sensitive spots 1–3 consisted of mitochondrial proteins: ACO2 (aconitase 2; EC 4.2.1.3), GDH1 (glutamate dehydrogenase 1; EC 1.3.1.3) and MMSDH [methylmalonate-semialdehyde dehydrogenase (acylating); EC 1.2.1.27]. Spot 4 was most similar to the cytosolic protein PEPCK (phosphoenolpyruvate carboxykinase; EC 4.1.1.32), but had a SEQUEST score below 50.

### DISCUSSION

Previous studies have utilized a proteomic approach to expose DM-induced changes in renal cortical protein expression [18,19]. In contrast, the present study utilized proteomics to identify DM-induced post-translational modifications of individual renal cortical proteins, with a focus on tyrosine nitration. Because ACEi are known to be renoprotective in DM, our approach was to determine the ability of enalapril to suppress DM-induced 3-NT production and to identify specific proteins exhibiting enalapril-sensitive tyrosine nitration during DM. Nitration of protein-bound tyrosine decreases its  $pK_a$ , makes it more hydrophobic, and may add steric restriction to the moiety [20]. As a result, tyrosine nitration can induce conformational changes that directly have an impact on protein function, with many reports of loss of enzymatic function and some reports of gain of function [20]. Tyrosine nitration probably also modulates tyrosine phosphorylation-dependent signalling events. Nevertheless, relatively little is known about the specific proteins undergoing tyrosine nitration or the contribution of these events to disease states associated with oxidative and nitrosative stress, including DM. In several regards, the present study extends our previous observations [8,9]. First, we demonstrated the ability of ACE inhibition to prevent the increase in renal cortical 3-NT production evident 2 weeks after onset of Type 1 DM in a rat model that exhibits moderate hyperglycaemia, hyperphagia, polydipsia, renal hypertrophy and little or no change in urinary albumin excretion [8,21,22]. The effect of enalapril to prevent renal cortical 3-NT production can be attributed to the concomitant suppression of  $O_2^-$  production and increase in SOD activity, both of which would suppress  $ONOO^-$  production and protein tyrosine nitration [8]. These phenomena are similar to those evident in renal cortical tissue acutely exposed to high glucose conditions [9]. Secondly, the use of a modified method of nitrated protein extraction and 2-DE revealed a large number of nitrated proteins in the renal cortex. Notably, four proteins, with molecular masses in the range of 57–86 kDa, showed prominent DM-induced tyrosine nitration. Finally, through use of a proteomics approach, the three spots displaying



**Figure 5** Effect of DM and enalapril on tyrosine nitration of proteins in the renal cortex

Proteins were separated by 2-D PAGE on the basis of differential isoelectric points (the x-axis) and molecular masses (the y-axis). (A–D) 2-DE Western blot image showing nitrotyrosine-immunopositive proteins evident in each group of rats after transferring to the PVDF membrane. (E–H) Images from (A–D) superimposed with corresponding images revealing protein levels in the SDS/PAGE gel (Coomassie Brilliant Blue, false-coloured as red to facilitate visualization). Representative image pairs are shown for the Sham group (A, E), STZ group (B, F), Sham + ENAL group (C, G) and STZ + ENAL group (D, H). Circled spots (1–4) highlight nitrotyrosine-immunopositive proteins that are prominently altered among the four groups of animals.

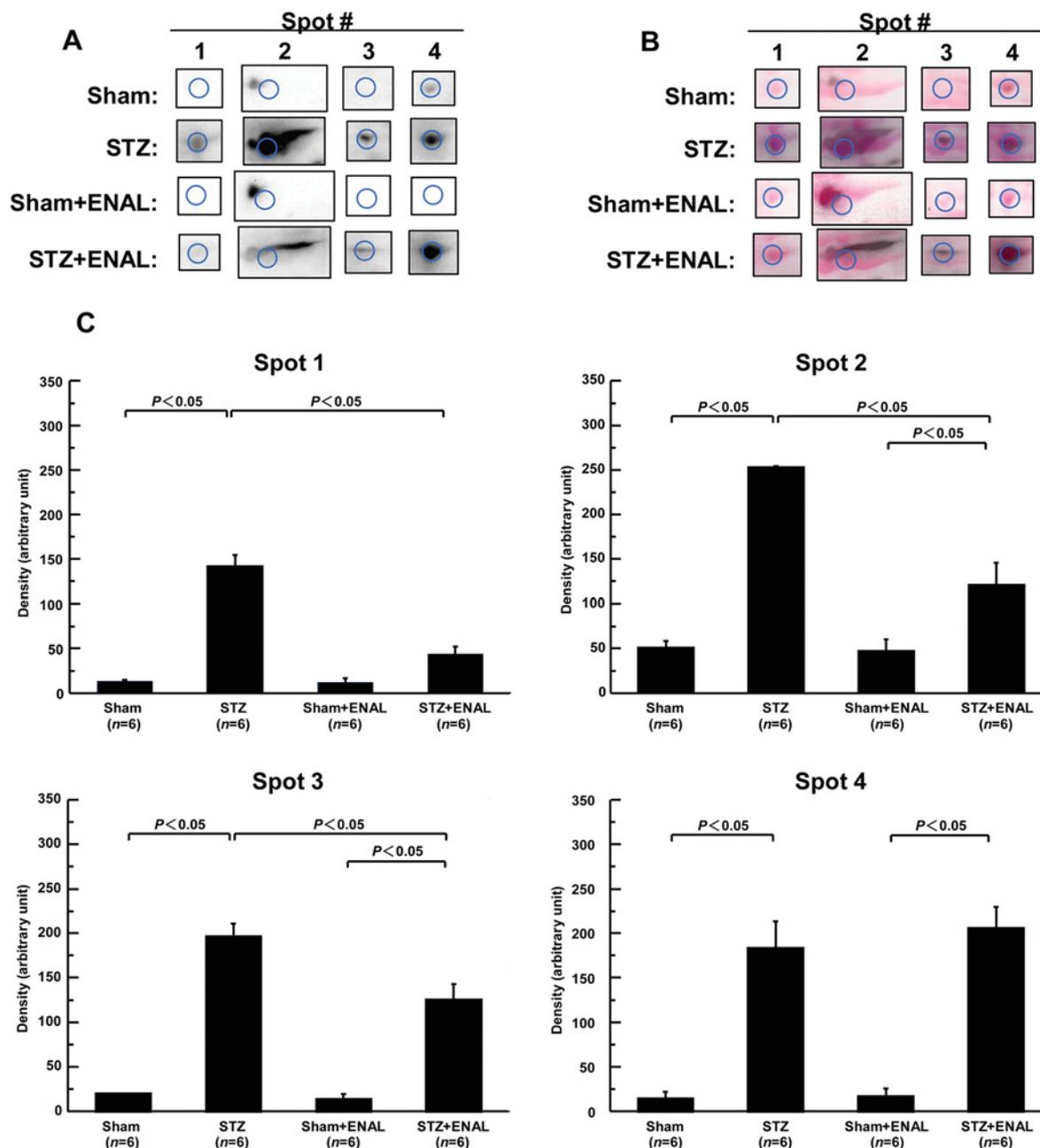
enalapril-sensitive nitration during DM were identified as ACO2, GDH1 and MMSDH.

The TCA (tricarboxylic acid) cycle enzyme ACO2 catalyses the isomerization of citrate to isocitrate. In accord with published evidence that DM increases tyrosine nitration of ACO2 in heart [23], we detected increased tyrosine-nitrated ACO2 in association with accelerated production of  $O_2^-$  and NO (ONOO<sup>-</sup> precursors) in the renal cortex during the early stage of DM. ONOO<sup>-</sup> has been shown to provoke nitration of mitochondrial ACO2 at Tyr<sup>151</sup> and Tyr<sup>472</sup>, both of which are located in close proximity to the active site of the enzyme [24]. Nitration of these tyrosine residues has been postulated to cause conformational changes that destabilize the active site, thereby contributing to the ability of ONOO<sup>-</sup> to decrease ACO2 activity [24]. These observations, together with the results of the present study, suggest that tyrosine nitration probably contributes to the decrease in renal mitochondrial ACO2 activity evident in rats and mice with Type 1 DM [25–27], and in *db/db* mice (a model of Type 2 DM) [25] despite a more than 2-fold increase in renal cortical ACO2 protein levels [18]. Interestingly, in the heart of diabetic rats, the overall activity of the TCA cycle is reduced due to ACO2 activation in the reverse mode (isocitrate → citrate) [28]. This process relies on ACO2 phosphorylation at several sites, including Ser<sup>481</sup>, which may be influenced by nitration of the adjacent Tyr<sup>472</sup> residue.

Both of the other enzymes found to undergo enalapril-sensitive tyrosine nitration in the renal cortex during DM are oxidoreductases that use NAD<sup>+</sup> or NADP<sup>+</sup> as the oxidant. The mitochondrial matrix enzyme GDH1 catalyses conversion of glutamate and NAD<sup>+</sup> to  $\alpha$ -oxo-glutarate and NADH, and is required for glutamate and NAD<sup>+</sup> to donate electrons to the electron transport system. GDH protein levels are increased more

than 2-fold in *db/db* mice (Type 2 DM), whereas renal GDH activity has been reported to be either increased [29,30] or unaltered [31,32] in rats with Type 1 DM. Bovine GDH contains 18 tyrosine residues, four of which (Tyr<sup>262</sup>, Tyr<sup>401</sup>, Tyr<sup>471</sup> and Tyr<sup>493</sup>) are known to be susceptible to nitration [33]. Despite an increase in GDH turnover resulting from tyrosine nitration [34], accumulation of tyrosine-nitrated GDH arises during conditions of sustained nitrosative stress, as evident in the liver during aging [34], in the brain during early Alzheimer's disease [35] and in the renal cortex during DM (the present study). Some evidence indicates that GDH nitration at Tyr<sup>262</sup> (located near the NAD<sup>+</sup>-binding domain) favours an increase in GDH activity, although this effect may function primarily to blunt the potent inhibitory impact of cysteine oxidation that can also result from exposure to nitrating agents [33].

The present results constitute the novel demonstration that mitochondrial MMSDH, which is more highly expressed in kidney and liver than in other organs [36], displays DM- and enalapril-sensitive tyrosine nitration. Although tyrosine nitration of MMSDH has been reported to accompany aging in rat heart [37], we are unaware of any previous reports that DM alters MMSDH nitration, expression or activity in any organ. MMSDH catalyses reactions in the distal portions of the valine and pyrimidine catabolic pathways. By catalysing the CoA- and NADP<sup>+</sup>-dependent oxidative decarboxylation of malonate-semialdehyde to acetyl-CoA, MMSDH provides a non-glycolytic means for producing the acetyl-CoA required to convert oxaloacetate to citrate in the TCA cycle. MMSDH also catalyses CoA- and NAD<sup>+</sup>-dependent oxidative decarboxylation of methylmalonate-semialdehyde to propionyl-CoA, which can subsequently be converted into the TCA cycle intermediate succinyl-CoA.



**Figure 6** Magnified 2-DE gel images of the four protein spots from Figure 5 and summary of densitometric data (A) Magnified 2-DE Western blot images showing nitrotyrosine-immunopositive proteins evident in each group of rats after transfer to the PVDF membrane. (B) Images from (A) superimposed with corresponding images revealing protein levels in the SDS/PAGE gel (Coomassie Brilliant Blue, false-coloured as red to facilitate visualization). (C) Densitometric quantification of nitrotyrosine-immunopositive protein spots (circled spots 1–4). Densities are expressed in arbitrary units. Values are means  $\pm$  S.E.M.

One of the proteins (spot 4) exhibiting prominent tyrosine nitration in the STZ rats was not influenced by enalapril treatment. Spot 4 was tentatively identified as PEPCK (SEQUEST score < 50), a cytosolic enzyme that is rate-limiting for gluconeogenesis. PEPCK protein levels are increased in the renal cortex of *db/db* mice [18] and proximal tubules of Zucker diabetic fatty rats exhibit increased gluconeogenesis in concert with increased PEPCK mRNA expression and activity [38]. PEPCK activity is

also increased in the renal cortex of rats with alloxan-induced DM [29]. It is possible that tyrosine nitration of this key enzyme contributes to enhanced intrinsic renal gluconeogenesis during hyperglycaemic states. The lack of any effect of enalapril (which reduces  $O_2^-$  production and, hence, should reduce  $ONOO^-$ -dependent protein tyrosine nitration) on spot 4 may indicate that alternative nitrating agents are primarily responsible for tyrosine nitration of this particular protein; however, further investigation

**Table 2** 3-NT-immunopositive proteins identified in the STZ rat renal cortex

Protein spots are as indicated in Figure 5. Sequence coverage shows accordance rate of peptides confirmed by MS/MS analyses in amino-acid sequences of candidate proteins. The theoretical molecular mass is from the Swiss-Prot database. The number of peptides was selected by high-quality MS/MS with an Xcorr filter.

Spot	Protein identity	Swiss-Prot accession no.	Experimental molecular mass (Da)	Theoretical molecular mass (Da)	SEQUEST score*	Peptides (n)	Coverage (%)
1	Aconitase 2, mitochondrial	Q9ER34	81 000	85 433	104.20	5	7.4
2	Glutamate dehydrogenase 1, mitochondrial	P10860	61 000	61 416	208.20	11	22.9
3	Methylmalonate semialdehyde dehydrogenase (acylating), mitochondrial	Q02253	62 000	57 808	50.21	4	9.5
4	Phosphoenolpyruvate carboxykinase 1, cytosolic	P07379	73 000	69 416	40.15	4	7.6

\*SEQUEST score of candidate proteins. (SEQUEST score >50; DeltaCn  $\geq 1.9$  for +1 charged peptides with fully tryptic ends; Xcorr  $\geq 2.2$  for +2 charged peptides with partially and fully tryptic ends; Xcorr  $\geq 3.75$  for +3 charged peptides with partially and fully tryptic ends)

is required to assess mechanisms for evoking the DM-induced tyrosine nitration of spot 4 that persists in enalapril-treated rats. For more accurate identification, 2-DE analysis using the derivative method of 3-NT to aminotyrosine by sodium dithionite and immunoprecipitation will probably be necessary, as well as analysis of the nitrated tyrosine residue site and the ratio in the nitrated protein sequence.

One somewhat surprising aspect of the present results is that the apparent quantitative effectiveness of enalapril treatment to reduce DM-induced protein nitration differed between the 3-NT assay and the 2-DE analysis. Specifically, enalapril totally blocked the DM-induced increase in total renal cortical 3-NT content (Figure 4), whereas it variably reduced the nitration status of the specific proteins highlighted in the 2-DE/proteomics aspect of the study (Figure 6C). Part of this discrepancy probably reflects the fact that we only quantified the four most prominent (out of more than 20) NT-immunoreactive spots evident after 2-DE and Western blotting. Another possibility is that the lysis and extraction methods used for the 3-NT and 2-DE assays provided different protein pools for analysis. For both assays, the zwitterionic detergent CHAPS was included in the homogenization buffer. CHAPS is a good solubilizing agent for membrane protein and disruption of non-specific protein interactions without having a denaturing effect [39]. We presume that the same cellular fractions were provided by homogenization in the presence of CHAPS for both the 3-NT assay and the 2-DE analysis. For the 3-NT assay, the CHAPS treatment was combined with sonication and the sample dissolved to the amino acid unit by enzyme digestion using pronase. Pronase acts on most peptide bonds, shows strong proteolytic action as hydrolytic degradation, and is well regarded for HPLC analysis (a key component of the 3-NT assay) [40,41]. For the 2-DE analysis, we avoided sonication and pronase treatment in order to maintain proteins intact for their identification. Rather, we combined CHAPS with chaotropic agents (urea and thiourea) that are generally employed in 2-DE analysis to dissolve more protein.

Thus the relative effectiveness of enalapril treatment to prevent protein nitration as evident in the 3-NT assay and 2-DE analysis may reflect the different protein extracting methods employed; nevertheless, the directional effects are in good agreement.

Mitochondria represent key sources of ATP and  $O_2^-$ , and participate as cell signalling organelles involved in regulating calcium homeostasis and apoptosis [31,42,43]. Hyperglycaemia in DM provokes increased glycolytic pyruvate generation and flux through the TCA cycle, generating the NADH and FADH<sub>2</sub> necessary for ATP production through oxidative phosphorylation by the electron transport chain. Electron leak from the electron transport chain results in generation of  $O_2^-$ , which is rapidly dismutated to H<sub>2</sub>O<sub>2</sub> by Mn-SOD (a mitochondrial enzyme) [43]. Insufficient antioxidant capacity, excessive  $O_2^-$  production and/or NO produced by mitochondrial NOS (nitric oxide synthase) leads to formation of ONOO<sup>-</sup> that might induce mitochondrial impairment [44]. The results of the present study suggest that this mechanism of DM-induced mitochondrial impairment may involve tyrosine nitration of specific mitochondrial proteins involved in energy metabolism. This scenario establishes mitochondria as both a source and a target of oxidative and nitrosative stress during DM. Because damaged mitochondria are degraded and removed from proximal tubular cells by autophagy [45], further studies should address the possibility that the renoprotective effect of enalapril during DM includes actions that promote autophagy to remove dysfunctional mitochondria.

Enalapril has been previously reported to blunt the increase in protein tyrosine nitration evident in the rat aorta 10 weeks after onset of STZ-induced DM [46]; however, to the best of our knowledge, our data are the first to document this effect in the kidney during the early stage of STZ-induced DM. As each of the prominent proteins exhibiting ACE-sensitive tyrosine nitration is linked to the TCA cycle, this phenomenon probably contributes to the mitochondrial dysfunction accompanying DM. Elucidating the functional impact of these proteins undergoing tyrosine nitration during DM may reveal novel mechanisms, as

well as diagnostic markers and therapeutic approaches, which can be employed during the early stage of DM to prevent or delay development of DN.

## CLINICAL PERSPECTIVES

- During the early stage of DM, renal cortical production of NO and O<sub>2</sub><sup>-</sup> is increased, leading to ONOO<sup>-</sup> formation. This oxidant enhances nitration of protein tyrosine residues to form 3-NT, a process that may contribute to development of DN. ACEis have been recommended as an initial therapy for DM to decelerate development of DN with or without hypertension.
- The results of the present study showed that ACE inhibition (enalapril) protected against DM-induced 3-NT production and protein tyrosine nitration; effects that may result from O<sub>2</sub><sup>-</sup> production and enhanced antioxidant effects. Interestingly, each of the three proteins found to exhibit enalapril-sensitive nitration during DM reside in mitochondria.
- Preventing protein nitration at tyrosine residues by ACE inhibition may be useful in developing diagnostic biomarkers and therapeutic methods that can be employed during the early stage of DM to prevent or delay development of DN.

## AUTHOR CONTRIBUTION

Naohito Ishii and Masato Katagiri contributed to the conception and design of the study, data analysis and writing of the paper. Naohito Ishii, Hiroyuki Imaizumi and Tsuyoshi Ichikawa performed the experiments. Pamela Carmines contributed to data analysis and writing of the paper. Yoshio Kodera and Masamichi Oh-ishi performed data analysis. Masanori Yokoba, Hideki Ikenagasa, Yoshikazu Aoki and Tadakazu Maeda and Tsuneo Takenaka revised the paper.

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