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Inhibition of renin angiotensin system decreases renal protein oxidative damage in diabetic rats

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Abstract

Renin angiotensin system (RAS) worsens diabetic nephropathy (DN) by increasing oxidative stress. We compared the effect of three different RAS inhibitors: the angiotensin converting enzyme inhibitor Ramipril, the vasopeptidase inhibitor AVE7688 and the angiotensin receptor (AT1) antagonist Losartan on the formation of oxidative and carbonyl stress derived protein modifications in kidney from Zucker obese hyperglycemic rats (ZDFn Gm-fa/fa). Gas chromatography–mass spectrometry was used to measure representative markers of several protein oxidative pathways: direct oxidation [dinitrophenylhydrazine reactive carbonyls (DNP), glutamic (GSA), and aminoadipic (AASA) semialdehydes], mixed glyco- and lipoxidation [N^{ε}-carboxyethyl-lysine (CEL) and N^{ε}-(carboxymethyl)-lysine (CML)] and lipoxidation-[N^{ε}-(malondialdehyde)-lysine-(MDAL)], as well as renal fatty acid composition. Urinary albumin (a marker of DN), DNP, GSA, and MDAL levels, were increased in all obese rats and were dose dependently decreased by AVE7688 whereas Ramipril and Losartan were less efficient. These results show that RAS inhibition improves DN at several levels, independently of its effects on blood pressure and glycemic control, via mechanisms depending of renal oxidative stress.
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Diabetic nephropathy (DN) is a progressive and an irreversible renal disease which has become the most common single cause of renal failure [1]. The onset of DN involves several additional mechanisms such as hemodynamic dysfunctions, inflammatory reaction, growth factor and cytokine secretion and finally formation of glucose-derived toxic compounds. The knowledge and the control of these different mechanisms has become a fascinating therapeutic challenge, aimed to reduce the progression of DN. It is now generally admitted that advanced glycation end products (AGEs) play a pivotal role in the onset of detrimental side effects of diabetes including mainly diabetic neuropathy, retinopathy, and nephropathy [2–5]. Although no efficient therapy is yet available, an increasing number of reports indicate that blockade of the renin angiotensin system (RAS) is effective to delay the progression of DN and thereby to protect against end-stage renal failure. In addition to inhibitors of the RAS, a new class of pharmaceutical agents with high therapeutic potential, termed inhibitors of vasopeptidase (IVP) has emerged recently. The effect of IVP on the management of DN start being investigated in various models [6–9]. The overall effect of these pharmaceutical agents is a significant reduction of

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glomerular fibrosis progression. However, besides their well-documented efficiency, the precise mechanism and targets of these inhibitors, with special attention to oxidative stress, remain to be elucidated.

The inhibition of the RAS could affect nephropathy by different, non-excluding, mechanisms including regulation of hemodynamic factors, but also modulation of protein kinase-C activation, inhibition of TGFβ system and more recently inhibition of AGEs formation and reduction of growth factor activation [10,11]. Among those mechanisms, high-glucose induced reactive oxygen species (ROS) overproduction has recently received increasing attention [2–5]. In this line, angiotensin converting enzyme (ACE) inhibition leads to a diminished ROS production through diminished angiotensin signaling and possibly increased activation of the B2-bradykinin receptor [11,12]. It is well admitted that AT1 receptors increase oxidative stress [5,13]. This is consistent with suggestions that ACE inhibition has a marked antioxidant activity [10,14,15].

However, direct evidences of oxidative damage to proteins have not been offered in these or similar diabetic nephropathy models. Usually, protein oxidative damage is assessed by examining 2,4-dinitrophenylhydrazine (DNP) reactivity, an assay which has been criticized due to possibility of artefacts [16]. Because AGEs include a very large and heterogenous family resulting from multiple mechanisms of formation, direct in vivo measurement of structurally identified products could bring precise information regarding the preferential formation pathways of AGEs. The major established pathway results from the initial covalent bond between a reducing sugar (i.e. glucose) and an amino acid group, to produce an Amadori product with subsequent reactions leading to the formation of irreversible AGEs. In addition to glucose, a new pathway termed metal catalyzed oxidation (MCO) of lipids, or lipoxidation, has emerged as a new source of AGE formation and is being currently explored as biomarker for specific pathological processes [17,18].

In the present study we compared the effect of chronic treatment with three different classes of inhibitors i.e. Ramipril, an angiotensin converting enzyme inhibitor, Losartan, an AT1 receptor antagonist, and AVE7688, an inhibitor of vasopetidase, on the long term renal accumulation of five different species of AGEs in DN in obese hyperglycaemic Zucker diabetic fatty rats, as well as in the fatty acid composition of renal cortex due to the new potential importance of lipids in AGE formation.

Materials and methods

The animal experiments were performed in accordance with the Sanofi Aventis Laboratory Animal Science and Welfare (LASW) guidelines, the German law for the protection of animals and the National Institute of Health (NIH) Guide for Care and Use of Laboratory Animals. Male ZDF rats (Gmi ZDF fa/fa) were purchased from Charles River (Sulzfeld, Germany). The animals were housed individually in standard cages with tap water *ad libitum* and standard chow containing 0.2% sodium and 19% crude protein (Standard diet #1320, Altromin, Lage, Germany).

At age of 30 weeks, after baseline measurements, the animals were randomly assigned to one of eight groups ($n = 6$ in each group) receiving either no specific treatment (Placebo), the ACE inhibitor Ramipril (0.1 or 1 mg/kg/day), the vasopeptidase inhibitor AVE7688 (0.3, 3, and 30 mg/kg/day) or the AT1 receptor antagonist Losartan (3 and 30 mg/kg/day). Ramipril and Losartan were administered via drinking water. AVE7688 was administered orally pressed in chow [8]. The duration of treatment was 23 weeks. Heterozygous (Fa/fa) rats were used as non-diabetic control ($n = 6$). Urine and blood samples were taken to determine urinary albumin/creatinine ratio, as an index of renal function, and blood glucose, respectively. Systolic arterial blood pressure and heart rate were measured using the tail-cuff method (TSE GmbH, BP system V2.2, Bad Homburg, Germany) in all animals. Kidneys were then carefully removed and stored frozen until further measurements.

Biochemical studies

Gas chromatography-mass spectrometry (GC/MS) measurements of glutamic semialdehyde (GSA), aminoadipic semialdehyde (AASA), N^{ε}-(carboxyethyl)-lysine (CEL), N^{ε}-(carboxymethyl)-lysine (CML), and N^{ε}-(malondialdehyde)-lysine (MDAL). GSA, AASA, CML, CEL, and MDAL concentrations in total proteins from renal cortex homogenates were measured by GC/MS as routinely performed in our laboratory [18,19]. Quantification was performed by external standardization using standard curves constructed from mixtures of deuterated and non-deuterated standards. Analytes were detected by selected ion-monitoring GC/MS. The amounts of products were expressed as the ratio μmol glutamic semialdehyde, aminoadipic semialdehyde, CML, CEL, or MDAL/mol lysine.

Immunodetection of protein-bound 2,4-dinitrophenylhydrazones and the glycoxidation product CEL. Prior to SDS electrophoresis, pools of samples were derivatized with DNP as previously described and routinely performed in the laboratory [19]. Immunodetection was performed using as primary antibodies a rabbit anti-DNP antiserum (1:4.000, Dako V401, Carpenteria, CA) and a monoclonal anti-CEL antibody (1:2.000, Transgenic Inc., Kumamoto, Japan). Peroxidase-coupled secondary antibodies were used from the Tropix chemiluminescence kit (Bedford, MA). Signal quantification and recording was performed with a CCD camera-based system (Lumi-Imager) from Boehringer Mannheim, scanning bands comprised between 30 and 250 kDa.

Fatty acid analysis. Fatty acid analysis was performed as previously described [19]. Results are expressed as mol%. The following fatty acid indexes (FA) were calculated as previously described [19]: saturated fatty acids (SFA); unsaturated fatty acids (UFA); monounsaturated fatty acids (MUFA); polyunsaturated $n - 3$ fatty acids (PUFA$n - 3$); polyunsaturated $n - 6$ fatty acids (PUFA$n - 6$); average chain length (ACL); unsaturation index (UI); and peroxidizability index (PI).

Statistical analysis

All statistics calculations were performed using the SPSS software (SPSS, Chicago). Once normality of variable's distribution was checked by the Kolmogorov–Smirnoff test, differences between samples were analyzed by the ANOVA test, and by the DMS test for intra-group analyses. A $p < 0.05$ level was selected as the point of minimal statistical significance in every comparison.

Results

Blood pressure and albumin excretion

As shown in Table 1, no significant change in blood pressure and heart rate can be found between the control

and the diabetic placebo group. In contrast, a very significant increase in the albumin/creatinine ratio (an early easy measurable and reliable biomarker of DN) was found in the diabetic untreated group (Placebo vs non-diabetic control). Interestingly, all the pharmacological treatments decreased significantly the albumin/creatinine ratio to various extents. A complete normalization to the value of non-diabetic control was achieved with 30 mg/kg/day of AVE7688, whereas blood glucose of diabetic rats remained unaffected by the various treatments.

Analysis of renal protein oxidative damage markers

The Western blot analyses of renal cortex homogenates showed diabetes-induced differences in the distribution of protein oxidative damage, especially in bands between 40 and 75 kDa, as shown in Fig. 1. Treatment with Losartan,

Table 1
Effect of chronic treatments (23 weeks) with RAS inhibitors on systolic arterial pressure (SAP), heart rate (HR), albumin excretion and blood glucose in male ZDFn Gm-fa/fa rats

Group	SAP (mm Hg)	HR (b.p.m)	Alb/creat ratio (mg/mg)	Blood glucose (mmol/L)
Ctl	154.5 ± 3.3	321 ± 10.6	84.9 ± 17.7	9.1 ± 2.1
Plc	156 ± 3.5	311.1 ± 8.5	274.4 ± 39°	32.4 ± 2.8°
Rmp 0.1	155.1 + 4	331.1 ± 12.9	284.3 ± 79	29.9 ± 3.1
Rmp 1	145 ± 4.8	331.1 ± 6.5	132.1 ± 28*	28.8 ± 2.4
AVE7688 0.3	157.7 + 2.9	297.9 ± 6	281.5 + 66	30.9 ± 1.2
AVE7688 3	154.2 ± 3.4	321.1 ± 10.5	140.3 ± 28*	31.3 ± 1.7
AVE7688 30	149.4 ± 5.5	321 ± 10.5	63.7 ± 13.2**	29.2 ± 3.4
Lst 3	151 ± 2.7	317.1 ± 9.1	197 ± 18*	32.2 ± 1.8
Lst 30	141 ± 3.6*	327 ± 8.9	163.5 ± 21*	33.1 ± 1.5

Values: mean ± SEM, $n = 6$ in each group. Abbreviations of treatments are as Fig. 2. $^{\circ}p < 0.01$, diabetic placebo vs value of non-diabetic control; $^{*}p < 0.05$ and $^{**}p < 0.01$ diabetic treated vs value of diabetic placebo before treatment.

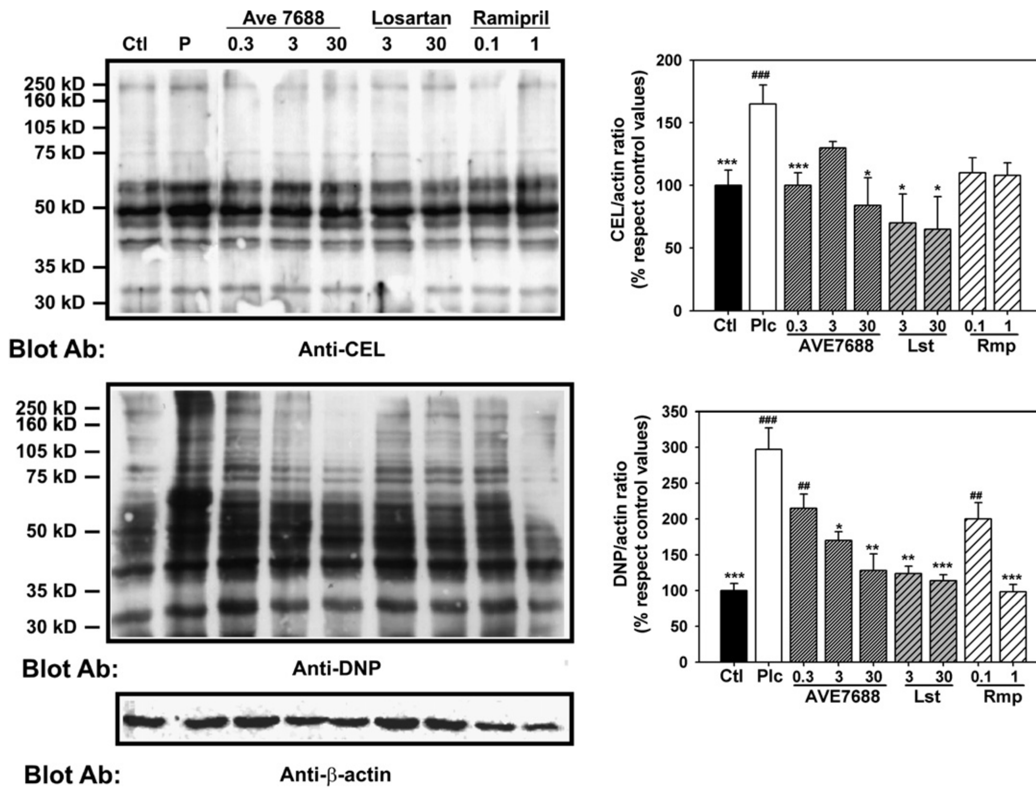

Fig. 1. Diabetes induces differences in the distribution of protein oxidative modifications in renal cortex homogenates. Western blot analyses measured the distribution of the AGE marker CEL (upper blot). Protein carbonyl groups, markers of protein oxidative damage, were derivatized with DNPH and, after their separation by SDS–PAGE, their amount was revealed by immunoblotting (middle blot). Anti-actin Western blot was done as a test for protein load. Numbers appearing at left of the blots indicate apparent molecular weight. The right panels show the quantification of these blots by densitometry, scanning bands located between 30 and 250 kDa. Blots shown are representative of at least three different measurements. Ctl: control-lean specimens, P: placebo-treated animals; doses for treatments shown are in mg/Kg/day. $^{*}p < 0.05$; $^{**}p < 0.01$, and $^{***}p < 0.001$ respect to placebo group and $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ respect to control-lean samples by DMS multiple comparison test in an ANOVA analyses.

Ramipril, and AVE7688 led to a dose-related decrease of oxidative damage, as evidenced by densitometric analyses. To identify which DNP-reactive carbonyls were related to glycoxidation/lipoxidation, we performed Western blot with an anti-CEL monoclonal antibody. CEL is a non-DNP-reactive carbonyl stress product resulting from mixed glycoxidation and lipoxidation reactions. These analyses, presented in Fig. 1, revealed only minor changes induced by diabetes, suggesting that MCO is an important determinant for DNP-reactive increased carbonyl modifications in ZDFn Gm-fa/fa rat kidneys. Therefore, to offer a more accurate quantitative measurement of protein oxidation in renal cortex proteins, we measured structurally defined MCO products by isotope-dilution GC/MS [19]. Proteins from renal cortex samples contained oxidation products resulting from MCO, glycoxidation and lipoxidation. Quantitative analyses revealed that the more abundant products were the amino acid derived oxidation products derived from MCO, AASA, and GSA (almost 95% of measured markers) (Fig. 2). GSA stood as the more MCO derived frequent modification (concentrations in the 30–35 mmol/mol lysine range), with levels being two orders of magnitude higher than of those of AASA with concentrations in the range between 250 and 290 μmol/mol lysine (Fig. 2). The mean concentrations of both GSA and AASA, presented in Fig. 2, were significantly higher ($p < 0.01$) in samples from diabetic rats than in control age-matched lean, the change being more marked for GSA. Treatment with AVE7688 abolished completely the increased GSA content, in a dose-related fashion, whereas only higher doses of Ramipril were efficient in this sense. Regarding the AASA content, only the high dose of Losartan was able to maintain oxidative modification to control levels.

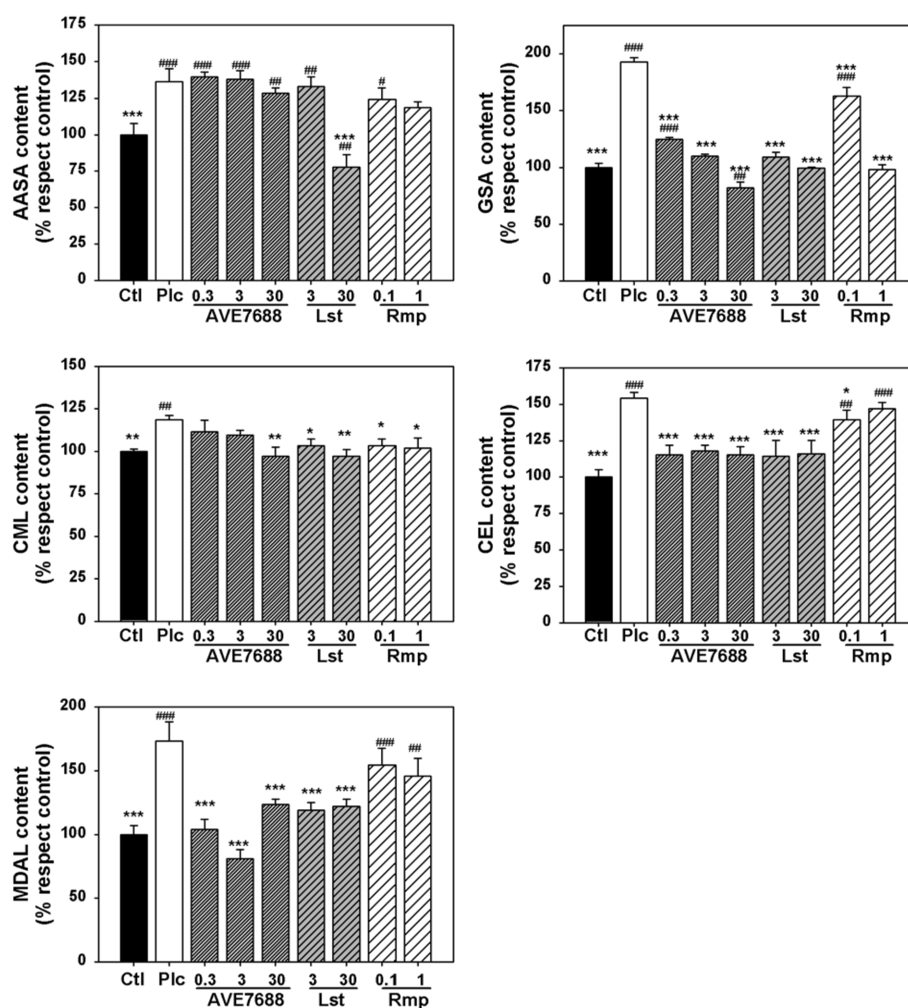

Fig. 2. Proteins from placebo-treated diabetic rats (Plc) show significant increases in the amounts of GSA and AASA, markers of MCO (upper graphs), in the concentrations of CML and CEL, arising from glycoxidation and lipoxidation (middle graphs), and in the concentration of MDAL, surrogate of lipoxidation (lower graph). Values shown are % changes of mean ± SEM over values from control-lean (Ctl) samples (GSA: 33164 ± 1353 μmol/mol lysine; AASA: 278 ± 22 μmol/mol lysine; CEL: 211 ± 11 μmol/mol lysine; CML: 589 ± 8 μmol/mol lysine; and MDAL: 127 ± 9 μmol/mol lysine). $^{*}p < 0.05$; $^{**}p < 0.01$, and $^{***}p < 0.001$ respect to placebo group and $^{\#}p < 0.05$; $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ respect to control-lean samples by DMS multiple comparison test in an ANOVA analyses. Values are also shown for AVE7688, Losartan-treated (Lst), and Ramipril-treated (Rmp) animals, doses shown in mg/Kg/day.

Table 2
Fatty acid composition (mol%) total lipids from rat kidney

	Control	Placebo	Losartan (mg/kg/day)		AVE7688 (mg/kg/day)			Ramipril (mg/kg/day)	
			3	30	0.3	3	30	0.1	1.0
14:0	0.30 ± 0.012	0.38 ± 0.05	0.23 ± 0.007*	0.33 ± 0.04	0.33 ± 0.03	0.24 ± 0.01*	0.24 ± 0.02*	0.31 ± 0.02	0.36 ± 0.03
16:0	22.38 ± 0.26	21.95 ± 0.50	20.91 ± 0.17	22.39 ± 0.53	21.75 ± 0.27	21.84 ± 0.37	21.79 ± 0.70	21.61 ± 0.51	22.72 ± 0.31
16:1	0.89 ± 0.04$^{\circ\circ}$	0.37 ± 0.012	0.10 ± 0.01†	0.13 ± 0.01†	0.10 ± 0.007†	0.09 ± 0.003†	0.09 ± 0.007†	0.44 ± 0.08	0.58 ± 0.16*
18:0	18.10 ± 0.20	18.92 ± 0.45	18.40 ± 0.44	16.85 ± 0.66†	18.76 ± 0.65	17.21 ± 0.47	17.61 ± 0.30*	18.07 ± 0.31	17.91 ± 0.75
18:1	8.45 ± 0.23°	10.13 ± 0.33	9.59 ± 0.26	10.69 ± 0.80	10.87 ± 0.70	10.49 ± 0.42	9.55 ± 0.17	11.33 ± 0.32	10.43 ± 0.56
18:2$n-6$	13.97 ± 0.21$^{\circ\circ}$	19.48 ± 0.15	19.78 ± 0.36	21.23 ± 0.29*	20.29 ± 0.54	18.47 ± 0.92	19.79 ± 0.28	21.19 ± 0.72*	19.47 ± 0.73
18:3$n-3$	0.11 ± 0.007	0.18 ± 0.01	0.20 ± 0.01	0.28 ± 0.05	0.22 ± 0.04	0.20 ± 0.02	0.26 ± 0.07	0.28 ± 0.03	0.21 ± 0.01
20:3$n-6$	0.47 ± 0.02°	0.73 ± 0.03	0.76 ± 0.03	0.64 ± 0.05	0.63 ± 0.14	0.85 ± 0.03	0.77 ± 0.04	0.68 ± 0.07	0.74 ± 0.06
20:4$n-6$	28.58 ± 0.41$^{\circ\circ}$	22.10 ± 0.57	23.97 ± 0.54	21.62 ± 0.38	21.41 ± 0.57	24.39 ± 0.69	23.48 ± 0.46	20.66 ± 0.48	21.81 ± 0.91
20:5$n-3$	0.18 ± 0.01	0.18 ± 0.01	0.17 ± 0.006	0.18 ± 0.003	0.18 ± 0.01	0.19 ± 0.01	0.21 ± 0.01	0.16 ± 0.01	0.18 ± 0.02
22:4$n-6$	0.28 ± 0.002$^{\circ\circ}$	0.46 ± 0.04	0.34 ± 0.01*	0.32 ± 0.02*	0.32 ± 0.007*	0.39 ± 0.01*	0.34 ± 0.01*	0.41 ± 0.01	0.38 ± 0.02
22:5$n-6$	0.17 ± 0.01$^{\circ\circ}$	0.08 ± 0.004	0.08 ± 0.008	0.15 ± 0.01†	0.07 ± 0.003	0.08 ± 0.005	0.07 ± 0.007	0.07 ± 0.002	0.08 ± 0.005
22:5$n-3$	0.57 ± 0.03	0.47 ± 0.04	0.50 ± 0.07	0.47 ± 0.12	0.36 ± 0.04	0.51 ± 0.09	0.35 ± 0.06	0.41 ± 0.01	0.33 ± 0.02
22:6$n-3$	5.47 ± 0.12$^{\circ\circ}$	4.49 ± 0.06	4.90 ± 0.10	4.65 ± 0.21	4.63 ± 0.13	4.99 ± 0.19*	5.38 ± 0.08†	4.30 ± 0.15	4.72 ± 0.25
ACL	18.36 ± 0.01	18.21 ± 0.02	18.30 ± 0.01	18.20 ± 0.02	18.21 ± 0.01	18.29 ± 0.01	18.28 ± 0.02	18.18 ± 0.02	18.19 ± 0.03
SFA	40.79 ± 0.06	41.25 ± 0.46	39.56 ± 0.47*	39.58 ± 0.34*	40.86 ± 0.61	39.30 ± 0.29*	39.64 ± 0.40*	40.00 ± 0.74	41.00 ± 0.98
UFA	59.20 ± 0.06	58.74 ± 0.46	60.43 ± 0.47*	60.41 ± 0.34*	59.13 ± 0.61	60.69 ± 0.29*	60.35 ± 0.40*	59.99 ± 0.74	58.99 ± 0.98
MUFA	9.35 ± 0.26	10.51 ± 0.33	9.69 ± 0.26	10.38 ± 0.79	10.97 ± 0.70	10.58 ± 0.42	9.65 ± 0.17	11.77 ± 0.34	11.02 ± 0.68
PUFA	49.84 ± 0.31	48.23 ± 0.58	50.74 ± 0.43	49.58 ± 0.50	48.15 ± 0.71	50.10 ± 0.14	50.70 ± 0.51	48.21 ± 0.73	47.97 ± 1.45
PUFA$n-6$	43.49 ± 0.23	42.88 ± 0.50	44.95 ± 0.37	43.98 ± 0.41	42.74 ± 0.61	44.20 ± 0.37	44.47 ± 0.49	43.04 ± 0.81	42.51 ± 1.26
PUFA$n-3$	6.34 ± 0.10$^{\circ\circ\circ}$	5.34 ± 0.08	5.78 ± 0.17	5.60 ± 0.21	5.41 ± 0.11	5.90 ± 0.28*	6.22 ± 0.04†	5.17 ± 0.15	5.45 ± 0.29
DBI	192.08 ± 1.43$^{\circ\circ}$	173.27 ± 2.50	182.70 ± 2.25*	175.89 ± 1.94	172.04 ± 2.22	183.77 ± 2.0*	183.24 ± 1.88*	170.51 ± 2.06	173.04 ± 5.01
PI	180.28 ± 2.03$^{\circ\circ}$	152.35 ± 2.89	163.07 ± 2.84*	153.29 ± 3.09	150.07 ± 2.99	164.69 ± 2.9*	164.41 ± 2.06*	146.12 ± 2.50	151.90 ± 5.67

Values: mean ± SEM, $n = 6$ in each group. Comparisons: $^{\circ}p < 0.05$, $^{\circ\circ}p < 0.01$, $^{\circ\circ\circ}p < 0.001$ non-diabetic control vs value of diabetic placebo; $^{*}p < 0.05$; and $^{\dagger}p < 0.01$ diabetic treated vs value of diabetic placebo before treatment. ACL, average chain length; SFA, saturated fatty acids; UFA, unsaturated fatty acids; PUFA$n-6/n-3$, polyunsaturated fatty acids $n-6$ or $n-3$ series; MUFA, monounsaturated fatty acids; DBI, double bond index; PI, peroxidizability index.

The mean concentrations of CEL and CML were also significantly higher ($p < 0.01$) in diabetic rats than in controls, as shown in Fig. 2. Treatment with AVE7688 normalized CEL content, as Losartan, but not Ramipril. All treatments prevented the increase of CML induced by diabetes (Fig. 2). The concentration of MDAL, a lipoxidation product, presented in Fig. 2, was also significantly increased in diabetic samples ($p < 0.01$). Notably, AVE7688 and Losartan treatments were able to normalize increased values of MDAL, but not Ramipril, which led to significant decreases with respect to placebo.

Since CML and CEL could arise from lipoxidation, and due to the importance of MDAL as lipoxidation marker, we measured the fatty acid composition of renal cortex, to ascertain whether increased availability of peroxidizable substrate (i.e. increased amount of polyunsaturated fatty acids) could explain increases in carbonyl stress markers. In addition, indexes of fatty acid composition would also reflect increased lipoxidative consumption of polyunsaturated fatty acids. The analyses of fatty acids revealed significant differences associated with diabetes in renal cortex samples, both in individual fatty acids and in global indexes (Table 2). Significant increases were noted for $18{:}2n-6$, and decreased levels of $20{:}4n-6$ and $22{:}5n-6$. The highly peroxidizable docosahexaenoic acid (DHA, $22{:}6n-3$) also showed a significant decrease. With reference to the derived indexes, diabetes led to significant decreases for DBI and PI indexes. In contrast with changes in protein oxidative modification, no major changes induced by treatment were evident, with the exception of significant normalization of PI and DBI indexes induced by Losartan at lower doses and by AVE7688 in a dose-dependent fashion.

Discussion

The present work brings new information regarding the pathways of MCO, AGE, and lipoxidation-derived protein modifications in the Zucker fatty rats and extends the knowledge of vasopeptidase inhibitor treatment on the accumulation of products derived from carbonyl and oxidative stress. This is the first report demonstrating the presence of increased MCO in a diabetic nephropathy model. GSA derives from the MCO of proline and arginine while AASA results from lysine oxidation [17]. These products are among the main carbonyl products of MCO of proteins, thus represent specific probes of oxidation of amino acids in protein. However, their presence and the factors affecting their concentrations in renal tissues were unknown up to date. Nevertheless, the chemical pathways linking increased free radical efflux and protein structural modification also involve third-party molecules, which may give rise to increased DNP-reactive carbonyls in proteins [16,17,19]. Particularly, carbohydrates, when reacting with free radicals, generate highly reactive dicarbonyl compounds, such as glyoxal and methylglyoxal. In the cellular context, these are mainly derived from glycolysis and triose phosphate metabolism, lipid peroxidation, and hypochlorite mediated reactions being also potential sources. These compounds generate stable adducts reacting with lysine, arginine, and cysteine residues in proteins. CEL and CML are two of these adducts, first described as AGE, later named glycoxidation products and now recognized as mixed AGEs-advanced lipoxidation products. Despite CML has been thoroughly detected in the DN lesions by immunohistochemical analyses [2], no chemical evidences have been reported for this product in renal tissues until very recently. Polyunsaturated fatty acids are other third-party molecules very susceptible to the oxidative action of free radicals [20]. Free radical attack of those lipids generates specific reactive aldehydes, such as malondialdehyde or 4-hydroxynonenal, which in turn could react with proteins, generating also DNP-reactive moieties. However, there were no chemical evidences for lipid peroxidative-induced damage of proteins in DN. Using GC/MS, our results demonstrate the presence of lipid peroxidation-derived markers in renal tissues, and evidence increased modification in a type II diabetes mellitus model. Globally, the present work, evaluating the effects of different RAS inhibitors (AVE7688, Losartan, and Ramipril) on the renal accumulation of different non-enzymatic protein modifications points out several differences reflecting that mechanisms of action of these three compounds could have specific effects on each oxidative pathway. Moreover, these data support the importance of the vasopeptidase system (inhibited by AVE7688) in diabetic nephropathy.

Two previous works from our group described the beneficial effect of AVE7688 on diabetic nephropathy in the diabetic and hypertensive models [6–8]. We found that AVE7688 ameliorates proteinuria without affecting glycemia as sustained by steady level of Hb1Ac [6]. Reduction in proteinuria was also associated with reduction in the severity of glomerular and tubulo-interstitial kidney damage, these effects being partly mediated by B2 kinin receptor activation [9]. In addition, these nephroprotective effects, in the Zucker fatty rat model, were not dependent of a blood pressure lowering effect. This suggests that development of DN in this model may be dependent on peculiar mechanisms and especially on an exaggerated concentration of lipids resulting in an increased formation of AGEs and lipoxidation products.

Noteworthy, changes in fatty acid composition related to diabetes were not corrected by any treatment essayed, despite AVE7688 (and Losartan, in a lower extent) showed a trend to normalize both PI and DBI, mainly by reversing the effects of diabetic status in the content of PUFA$n-3$. This suggests that RAS inhibition acts on free radical generation [13], more than actuating on tissue susceptibility (i.e. fatty acid profile) to oxidative damage. In this sense, AVE7688 also normalized the

level of lipoxidation and MCO products namely GSA and MDAL, whereas it demonstrated a weaker effect on CEL, CML, and AASA. However, it is interesting to underline that AVE7688 is more efficient than the two other inhibitor on the products more sensible to hyperglycemia. With respect to the Zucker diabetic fatty rat model, this observation could be also of great interest, as the diabetes-induced increases in GSA and MDAL concentrations were much higher when compared to the three other measured products, which showed more moderate but significant increase. These findings could reveal a major pathway—relying on MCO and lipoxidation—responsible for renal AGEs accumulation.

When compared to the effects of the ACE inhibitor Ramipril and the AT1 receptor antagonist Losartan, AVE7688 was more effective to reduce the concentration of GSA and MDAL. This reduction has to be associated with normalization of the albumin/creatinine ratio, only achieved with AVE7688 treatment. It has been previously reported that both ACE inhibitors and AT1 receptor blockers reduced AGEs formation [10,11,14,15]. However the relationship linking inhibition of the RAS, AGE formation, and normalization of DN index has not been extensively investigated. These previous results demonstrate that AVE7688 has potent chelating activity, and that by inhibiting metal-catalysed formation of AGE compounds, it decreases CML formation. The present results therefore suggest that inhibition of vasopeptidases seems more efficient than blockade of RAS alone to reduce renal AGEs formation in type II diabetes, an effect significantly associated with reduction of DN.

In summary, our work offers chemical evidences for differentially increased protein oxidative and carbonyl stress in renal cortex of this model of DN, suggesting the important role of lipid peroxidation and MCO. Nevertheless, RAS activity in diabetes underlies these changes, as different inhibitors, and specially IVP, are able to normalize the concentration and distribution of oxidized proteins.

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