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Effects of valsartan therapy on protein glycoxidation

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Abstract

Several lines of evidence suggest that both advanced glycation end products (AGEs) and oxidation processes play key roles in the physiology of aging and age-related pathologies, leading to irreversible proteins modifications in both tissues and the extracellular matrix. Such an accelerated accumulation of these modifications has been reported to be present in several age-related chronic diseases, such as atherosclerosis, diabetes, arthritis, and neurodegenerative diseases. The current literature reveals that the specific inhibition of AGEs may constitute an innovative therapeutic goal. In experimental animals, the use of sartans significantly reduces blood pressure and kidney pentosidine content, improving both histologic renal damage and proteinuria. In this study, 12 subjects who were affected by diabetes mellitus and hypertension were subjected to oral antihypertensive therapy with valsartan (class of sartans) with timed sampling of plasma and urine pentosidine, N^{ε}-(carboxymethyl)lysine (CML), malondialdehyde, and isoprostanes levels, respectively, at baseline and after both 3 and 6 months, with parallel ongoing evaluation of glycemic control and blood pressure levels. Valsartan elicited a good antihypertensive effect with a 30% decrease in plasma pentosidine levels ($P < .05$) after 3 months of therapy, followed by a slight increase after 6 months. Urinary pentosidine concentrations exhibited a 40% decrease after 3 months (215 ± 19 vs 129 ± 23 nmol/24 h) and a further significant reduction after 6 months of therapy (105 ± 24 nmol/24 h). Plasma CML levels showed a progressive decrease after 3 months (23.15 ± 3.215 vs 19.88 ± 1.684 μmol/mL) and achieved a further slight reduction after 6 months of therapy (19.48 ± 1.339 μmol/mL); for urinary CML, a statistically significant reduction was gained after the sixth month of therapy (48.51 ± 5.70 vs 30.30 ± 2.77 μmol/24 h after 3 months and 27.02 ± 4.13 μmol/24 h after 6 months; $F = 7.62$, $P < .005$). Plasma and urinary concentrations of malondialdehyde were slightly modified by valsartan treatment; the mean levels after both 3 and 6 months did not significantly differ from baseline. Urinary 15-F2t-isoprostanes (2.96 ± 0.45 ng/24 h) levels displayed a progressive decrease after both 3 (2.27 ± 0.31 ng/24 h) and 6 months (1.70 ± 0.23 ng/24 h) with statistical significance achieved only at the end of the study ($P < .05$). The present data suggest interesting in vivo antiglycation and antioxidation effects of this angiotensin II receptor antagonist with reductions in plasma and urinary pentosidine, plasma CML, and urinary isoprostanes levels. The present study supports an antagonistic role of valsartan in the production of AGEs precursors through the chelation of transition metals and an antioxidant activity that scavenges reactive oxygen species. This property of valsartan may broaden the scope of newly developed pharmacologic inhibitors of advanced glycoxidation.

1. Introduction

Advanced glycation and oxidation processes occur over time and contribute to age-related damage through a variety of effects, from irreversible modifications of tissue proteins to cellular and tissue injury [1].

More than a dozen advanced glycation end products (AGEs) have been identified in vivo and almost half are known to accumulate with age in collagen, thus affecting the biochemical and physical properties of both tissue proteins and the extracellular matrix.

Advanced glycation end products accumulate to high levels in tissues in age-related chronic diseases, such as atherosclerosis [2,3], diabetes [4,5], arthritis, neurodegenerative diseases, and uremic complications [6,7], playing an important role in the onset and/or maintenance of these pathologies.

The direct implication of AGEs in the progression of these chronic diseases has stimulated a large field of research on their inhibition, which may constitute an interesting and new therapeutic goal.

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Inhibition of AGEs may limit oxidative, inflammatory responses, and tissue damage; retard the progression of physiopathology and the onset of complications; and subsequently improve the quality of life during the aging process.

Aminoguanidine, the first AGEs inhibitor, was discovered in 1986 [8]. Both aminoguanidine and the compound called OPB-9195 are hydrazine derivatives that are able to inhibit the formation of AGEs, such as pentosidine [8] and N^{ε}-(carboxymethyl)lysine (CML) [9], in vitro from a series of precursors (ie, ascorbic acid and ribose), as well as from advanced lipoxidation end products. These molecules have useful biologic effects, but are, at the same time, heavily hampered by detrimental side effects, such as the sequestration of pyridoxal activity and clinical B_6 deficiency [10].

However, more recently, other drugs that antagonize the formation of AGEs have been discovered and are clinically more favorable [11].

The aim of the present study was to evaluate the long-term effectiveness of AGEs inhibition in vivo by a well-tolerated and well-recognized antihypertensive drug of the angiotensin II type I receptor (AIIR) antagonist family.

This pharmacologic class, composed of a series of chemical compounds that are characterized by their pleiotropic effects, shares the capacity to block the renin-angiotensin system, leading to a progressive lowering of blood pressure and a decreased risk of long-term micro/macrovascular diabetic complications and overall cardiovascular risk. Moreover, both the general tolerability and safety profile are more than acceptable for this drug class [12-15].

Recently, it was reported that this class of molecules was also able to inhibit the formation of 2 AGEs in vitro and to subsequently reduce kidney pentosidine and CML levels, proteinuria, and histologic renal damage in spontaneously hypertensive diabetic rats [11].

Accordingly, we tested the ability of valsartan to reduce AGEs levels at an antihypertensive dosage, contributing to our knowledge about the use of AGEs-inhibiting compounds in humans.

2. Materials and methods

Twelve subjects (11 men and 1 woman; age range, 55-80 years; mean, 67.83 $\pm$ 2.65 years) who were affected by type 2 diabetes participated in the study. Informed consent was obtained from all participants.

The subjects had persistent microalbuminuria (median urinary albumin excretion rate of 3 consecutive 24-hour urine collections in the range of 30-300 mg/d) and mild to moderate hypertension (grade 1-2 according to World Health Organization guidelines) [16] that was previously treated unsatisfactorily or not treated at all. Glycemic control was stable (glycated hemoglobin $<8\%$) within the last 6 months.

At baseline, valsartan, a molecule belonging to the class of AIIR inhibitors, was administered to all patients as an antihypertensive therapy; the mean dosage varied between 80 and 160 mg/d.

Six subjects were treated with oral hypoglycemic therapy with sulfonylurea plus metformin, 2 were treated with sulfonylurea only, and 4 subjects were treated with other hypoglycemic agents. No dropout was observed during the time of follow-up.

Systolic and diastolic blood pressures (SP and DP) were measured at each visit in the sitting position according to the European Society of Hypertension guidelines [17].

Fasting blood and 24-hour urine (of the previous day) samples were obtained at 3 different times (time 0, at baseline; time 1, after 3 months; time 2, after 6 months). Plasma and urine samples were stored at -80°C until the determination of pentosidine (plasma and 24-hour urine), CML (plasma and 24-hour urine), malondialdehyde (plasma and 24-hour urine), and 15-F2t-isoprostane (urine) levels was performed.

Pentosidine levels in both plasma and urine were analyzed by a high-performance liquid chromatography (HPLC) method as previously described [12], with a modification in the preparative step. Briefly, an aliquot of either plasma (containing 10 mg of protein) or urine (containing 3 mg of protein) underwent a solid-phase extraction using an OASIS MAX extraction cartridge (Waters, Milford, MA) with 125 mmol/L sodium acetate, pH 7.0, and 2% methanol as the mobile phase. After extraction, each sample was lyophilized and hydrolyzed with 6 mol/L HCl for 20 hours at 110 °C in borosilicate screw–capped tubes that had been flushed with nitrogen. The acid was evaporated in a SpeedVac Concentrator System (SPD111V concentrator, RVT400 refrigerated vapor trap, Savant Instruments, Holbrook, NY), and each sample was reconstituted in HPLC-grade water containing 0.01mol/L heptafluorobutyric acid (Pierce, Prodotti Gianni, Milan, Italy). A volume equivalent to 3 mg of protein was injected into the HPLC system (Waters, Milford, MA) after filtration through a 0.45-μm filter (Ultrafree MC, Millipore, Milan, Italy).

Separations were performed on a C_{18} column (X-Terra MS 5 μm-4.6 $\times$ 250 mm, Waters, Milan, Italy) with a curvilinear gradient program of 20% to 40% methanol from 0 to 30 minutes, containing HPLC-grade water and heptafluorobutyric acid as the counter-ion. The effluent was scanned by a Waters 470 fluorescent detector; the excitation and the emission wavelengths were 335 and 385 nm, respectively. A pentosidine synthetic standard was injected at the beginning of every run to quantitate the pentosidine levels in the sample by peak area comparison.

Plasma CML levels were measured by competitive enzyme-linked immunosorbent assay (ELISA). Sodium cyanoborohydride, glyoxylic acid, bovine serum albumin - fraction V (BSA), horseradish peroxidase–conjugated antimouse immunoglobulin G, and 3,31,5,51-tetramethylbenzidine were purchased from Sigma (Milan, Italy). Plasma CML levels were measured in duplicate by a competitive ELISA using a mouse anti-CML monoclonal antibody (6D12, clone NF-1G, TransGenic, Kumamoto, Japan). Carboxymethylated BSA (CML-BSA) was prepared according to Ikeda et al [18]. Briefly, 50 mg/mL BSA was incubated

at 37°C for 24 hours with 45 mmol/L glyoxylic acid and 150 mmol/L sodium cyanoborohydride in 10 mL of 0.2 mol/L phosphate-buffered saline (PBS, pH 7.4) and then extensively dialyzed against PBS. As a negative control, BSA was incubated in parallel without glyoxylic acid and sodium cyanoborohydride. The CML content of the modified BSA was measured by amino acid analysis after hydrolysis of the protein in 6 mol/L HCl and 0.2% phenol for 24 hours at 110°C.

Each well of a 96-well microtiter plate (Nunc-Immuno Plate, Nunc, Denmark) was coated overnight with 100 μL of a 1 μg/mL CML-BSA solution in 50 mmol/L sodium carbonate buffer (pH 9.6), washed with PBS/0.05% (vol/vol) Tween 20, and blocked for 2 hours at room temperature (RT) with 6% (wt/vol) nonfat dry milk in PBS. Wells were then washed and 50 μL of potentially competing antigen were added (either serum samples or serial dilution of CML-BSA), followed by 50 μL of monoclonal antibody clone 6D12. The plate was incubated for 2 hours at RT, washed, and incubated for 2 hours at RT with 50 μL horseradish peroxidase–conjugated antimouse immunoglobulin G.

After the incubation, wells were developed with 100 μL per well of 3,31,5,51-tetramethylbenzidine solution and incubated for 30 minutes at RT on a shaker platform. Color reactions were stopped by adding an equal volume of 0.5 mol/L H_2SO_4, and absorbance was read at 450 nm in an ELISA plate reader (Microplate Manager 5.0 PC, Bio-Rad, Milan, Italy).

The concentration of malondialdehyde as a thiobarbituric-reactive substance (malondialdehyde [MDA]) was used as an index of lipid peroxidation and was determined following the method described by Young and Trimble [19], based on the thiobarbituric (TBA) reaction. The TBA reaction was carried out by mixing 750 μL of 0.44 mol/L phosphoric acid, 450 μL HPLC-grade water, 50 μL of sample/standard, and 250 μL of 42 mmol/L TBA. Tightly capped tubes were incubated in a boiling water bath for 60 minutes then rapidly cooled and kept in an ice-water bath until HPLC analyses were performed. Ten minutes before injection onto the HPLC column, all samples were neutralized with 0.5 mL of a methanol-NaOH solution (4.5 mL of 1 mol/L NaOH in 50 mL HPLC-grade methanol) and centrifuged (9500*g*, 5 minutes) for protein precipitation.

Fifty microliters of clear supernatant was injected onto a 3.9 × 300-mm C_{18} μBondapak (Waters, Milan, Italy). The mobile phase contained 50% methanol and 50% of 25 mmol/L phosphate buffer at pH 6.5. A flow rate of 0.8 mL/min was used.

The detection system (Waters 470 scanning fluorimetric detection) was set at 532 and 553 nm for excitation and emission wavelengths, respectively. The levels of malondialdehyde (in nmol/mL) were calculated relative to the MDA standard calibration curve obtained from the hydrolysis of 1,1,3,3-*tetra*-ethoxy-propane. Quantification was performed by peak area integration using 1,1,3,3-*tetra*-ethoxy-propane as a standard.

The 15-F2t-isoprostane levels in urine samples were measured, without prior extraction, by a competitive ELISA (Oxford Biomedical Research, Oxford, UK). The assay quantitatively measures 15-F2t-isoprostane, the best characterized isoprostanes, a true marker of oxidative stress [20].

Glycated hemoglobin levels were measured by a standard HPLC method [21] (reference range, 4.6%-6.1%).

Data were reported as the mean ± SE; the measurements of monitored variables were compared by repeated measures one-way analysis of variance (rANOVA) and the differences between columns were assessed by the Dunnett multiple comparison post test. Differences between controls and diabetic subjects were assessed using the unpaired *t* test. The relationships between 2-parameter series were examined by linear regression analysis and the strength of the correlation was calculated by correlation coefficients. A *P* value of less than .05 was considered significant.

3. Results

During the period of observation, blood glucose control did not show significant variation (mean glycated hemoglobin: baseline, 6.65% ± 0.38%; 3 months, 7.13% ± 0.33%; 6 months, 6.44% ± 0.37%, *P* = not significant [NS]), suggesting steady metabolic control.

Valsartan treatment elicited, as expected, a significant decrease in SP, mean arterial pressure (MAP), and DP

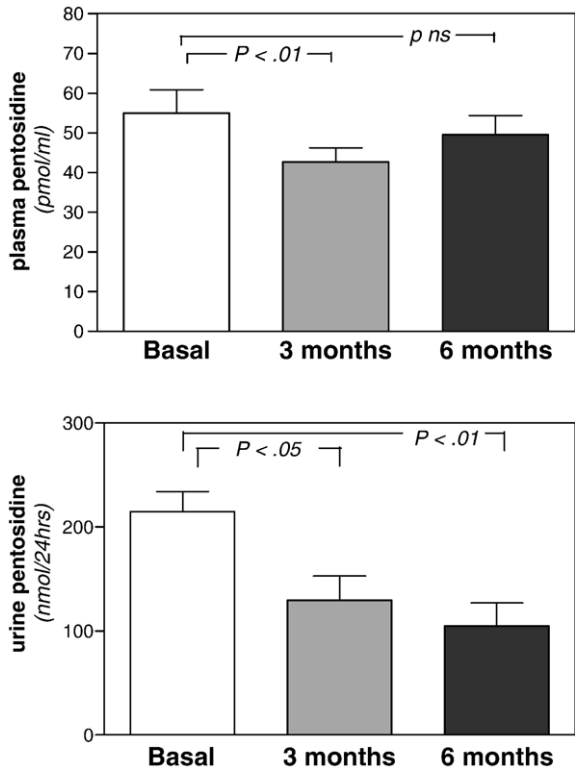

Fig. 1. Plasma and urinary (24-hour) pentosidine levels (at 0, 3, and 6 months) in diabetic subjects (n = 12) after antihypertensive therapy with valsartan.

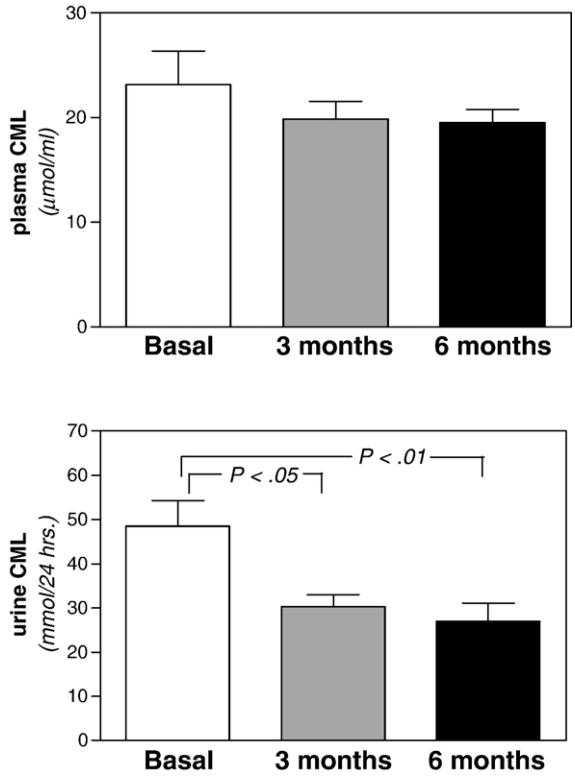

Fig. 2. Plasma and urinary (24-hour) CML levels (at 0, 3, and 6 months) in diabetic subjects (n = 12) after antihypertensive therapy with valsartan.

(baseline SP, 152 ± 3; MAP, 111 ± 1; DP, 91 ± 1 mm Hg before treatment; SP, 142 ± 2; MAP, 103 ± 1; DP, 83 ± 1 mm Hg after treatment; $P < .01$ for all parameters).

The treatment was well tolerated and no significant side effects were reported during the trial.

3.1. Effects on pentosidine levels

After 3 months of antihypertensive therapy with valsartan, plasma pentosidine levels showed a decrease of 30%; after 6 months of therapy, the levels of plasma pentosidine were still lower from baseline with no statistical significance (baseline, 55.02 ± 5.84; 3 months, 42.65 ± 3.57; 6 months, 49.52 ± 4.86 pmol/mL; F = 9.97; baseline vs 3 months, $P < .05$).

Urinary pentosidine levels demonstrated a 40% decrease after 3 months of treatment (215 ± 19 vs 129 ± 23 nmol/24 h) compared with the corresponding starting value. A further significant reduction was observed after 6 months of therapy (105 ± 24 nmol/24 h) (rANOVA: F = 11.33, $P < .001$; baseline vs 3 months and vs 6 months, $P < .01$) (Fig. 1).

3.2. Effects on CML levels

After 3 months of valsartan therapy, plasma CML levels showed a progressive decrease, declining 15% after 6 months of therapy (23.16 ± 3.22 vs 19.88 ± 1.68 vs 19.48 ± 1.34 μmol/mL). Statistical analysis, however, did not achieve significance (rANOVA: F = 1.62, P = NS). On the contrary, urinary CML levels displayed a significant progressive decline after both 3 and 6 months of therapy (48.51 ± 5.70 vs 30.30 ± 2.77 μmol/24 h at 3 months and 27.02 ± 4.13 μmol/24 h at 6 months; rANOVA: F = 7.62, $P < .005$; baseline vs 3 months, $P < .05$; baseline vs 6 months, $P < .01$) (Fig. 2).

3.3. Effects on MDA and 15-F2t-isoprostane levels

Plasma levels of MDA were slightly modified by valsartan treatment; the mean levels after both 3 (2.70 ± 0.35 nmol/mL) and 6 months (2.27 ± 0.27 nmol/mL) of therapy were not significantly different from baseline (2.94 ± 0.57 nmol/mL) (rANOVA: F = 6.21; baseline vs 3 months and vs 6 months, P = NS).

Urinary elimination of MDA (baseline 2.15 ± 0.29 μmol/24 h) showed a slight and not significant decrease after 3 months (1.72 ± 0.25 μmol/24 h) and a trend toward baseline at 6 months (2.01 ± 0.34 μmol/24 h) (rANOVA: F = 0.54, P = NS).

Urinary 15-F2t-isoprostane levels (2.96 ± 0.45 ng/24 h) displayed a progressive decrease after 3 months (2.27 ± 0.31 ng/24 h) and 6 months (1.70 ± 0.23 ng/24 h) of therapy, attaining statistical significance by the end of the study (rANOVA: F = 3.62, $P < .05$; baseline vs 3 months, P = NS; baseline vs 6 months, $P < .05$). For glycated hemoglobin levels, a significant correlation with plasma pentosidine levels (n = 36, $y = 16.16 + 4.85x$, $r = +0.410$, $P = .013$) was observed. As expected, plasma pentosidine levels correlated with urinary pentosidine levels (n = 36, $y = 71.69 + 1.61x$, $r = +0.36$, $P = .034$), and plasma MDA levels displayed a significant correlation with urinary isoprostanes levels (n = 36, $y = 1.51 + 0.29x$, $r = +0.37$, $P = .027$). Surprisingly, no correlation was found between urinary MDA and isoprostanes levels, whereas a significant correlation was present between amounts of urinary MDA and urinary pentosidine (n = 36, $y = 46.41 + 52.59x$, $r = +0.61$, $P < .001$) (Fig. 3).

Plasma CML levels demonstrated a significant correlation with plasma pentosidine (n = 36, $y = 28.38 + 1.24x$, $r = +0.37$, $P < .05$), plasma MDA (n = 36, $y = 0.91 + 0.09x$, $r = +0.37$, $P < .05$), and urinary isoprostanes levels

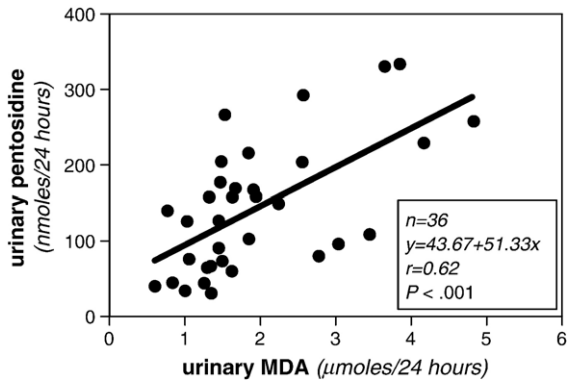

Fig. 3. Linear regression and correlation coefficients between pentosidine and MDA urinary (24-hour) levels (at 0, 3, and 6 months) in diabetic subjects (n = 12) after antihypertensive therapy with valsartan.

(n = 36, $y = 0.87 + 0.08x$, $r = +0.39$, $P < .05$). Urinary CML levels correlated with amounts of both plasma CML (n = 36, $y = 14.89 + 0.18x$, $r = +0.40$, $P < .05$) and urinary isoprostanes (n = 36, $y = 1.61 + 0.02x$, $r = +0.37$, $P < .05$).

4. Discussion

The present results focus on interesting antiglycation and antioxidation effects of a widely used antihypertensive drug in vivo. The initial finding of the antiglycation effects of this AIIR antagonist was from a Slovakian group that reported an improvement in the plasma levels of AGEs in nephrectomized rats treated with losartan; however, they attributed the lower concentrations of AGEs to the improvement of renal function and better subsequent AGEs removal [22].

In recent years, other studies have obtained very interesting data both in vitro, with a series of sartans (ie, olmesartan, candesartan, irbesartan, losartan, telmisartan, and valsartan), and in rats, with olmesartan [20], elucidating the most likely mechanism of action of these sartans.

The likely biochemical mechanism appears to hamper the production of AGEs precursors through the chelation of transition metals. The molecule also shows an antioxidant activity that scavenges reactive oxygen species [23].

Angiotensin II type 1 receptor antagonists are biphenyltetrazole derivatives; it is conceivable that their common core structure is necessary for their inhibitory effectiveness, suggesting a unique property of this specific class of molecules among the entire class of antihypertensive drugs.

Studies in animal models show that long-term administration of olmesartan and candesartan reduce the renal content of AGEs, implying an in vivo lowering effect at an antihypertensive dosage.

Because the accumulation of AGEs in the kidney is harmful and the pathogenetic role of AGEs in the development and progression of diabetic nephropathy is widely accepted, it is obvious that a pivotal effect of glycation inhibition is the prevention of diabetic nephropathy.

In the present study, we demonstrated that long-term antihypertensive therapy with valsartan in type 2 diabetes mellitus is able, as expected, to significantly decrease blood pressure and achieve, in most of subjects, ideal target values. At the same time, a lowering of plasma and urinary pentosidine levels as well as plasma CML levels was observed in the presence of steady blood glucose control. This finding is in agreement with the observation of Forbes et al [23] who reported a very slight reduction of pentosidine levels in mice kidney and skin after 6 months of valsartan treatment, notwithstanding poorer blood glucose control compared with our subjects.

According to the findings of the present study and of the current literature, it is reasonable to postulate an inhibition of the glycoxidative cascade, which leads to AGEs as a prevention therapy. The formation of both pentosidine and CML is closely related to the presence of transition metal ions and to the release of reactive oxygen species [14,15]. The ability of valsartan to inhibit the formation of AGEs can be considered an antioxidant property of this family of molecules [23,24]; this property can also be thought of as a chelating activity against transition metal ions resulting in less reactive oxygen species and fewer carbonyl compounds, which are known precursors of AGEs.

On one hand, the levels of plasma pentosidine are, at any rate, intertwined with glucose control, as confirmed by the linking of pentosidine with glycated hemoglobin. On the other hand, CML may be considered a by-product of both glycation and oxidation [25]; in fact, there was no relationship between CML and glucose control. Moreover, the results of plasma CML levels, which decrease in parallel with ongoing valsartan treatment, corroborate the above-mentioned results.

The reduction observed at 3 months reveals a significant even if slight decline of plasma protein glycation, whereas the value at 6 months is lower than the initial one without achieving a statistical significance. Plasma pentosidine concentration represents the sum of the glycation of plasma proteins (dependent on blood glucose concentration) and of the glycated peptides removed from the peripheral tissues: a value with a modest inner variability also in diabetic subjects. As the glycation process is inhibited, then this parameter reveals lower sensibility. Urinary pentosidine concentration, instead, can be considered an integrated value of the 24 hours, displaying the overall elimination of pentosidine in relation to the kidney excretion rate witnessing a more reliable estimation of tissue AGEs mobilization. Therefore, after valsartan therapy, the reduced removal rate from tissues may be ascribed to a diminished synthesis of AGEs (dependent on the inhibiting therapeutic effectiveness of valsartan). In addition, the results of plasma CML, almost superimposable, substantiate our interpretation of effectiveness of sartans to inhibit glycation cascade.

Urinary CML levels showed a positive correlation with urinary isoprostanes levels, whereas amounts of urinary pentosidine significantly correlated with urinary MDA levels, reinforcing the interrelationships between AGEs, oxidative stress, markers of lipoperoxidation, and glycometabolic control in diabetic subjects.

The results of oxidative markers were less significant; plasma MDA levels showed a decreasing trend, whereas urinary MDA values did not validate the data. However, the most specific determination of isoprostanes levels produced more convincing results, although a high degree of variability was still present. The last results agree with the findings of both Miyata and Forbes: in diabetic rats that were treated with 2 different sartans, a positive staining for the 4-hydroxynonenal protein adducts disappeared and a significant reduction in CML levels was reported [23,26,27].

Our study corroborates, therefore, the scavenging activity of valsartan through a significant decrease in the amounts of isoprostanes and the decreasing trend in MDA levels.

We are aware that the observed results of this study require validation with a larger number of subjects and a greater observational period (>1 year observation). However, because of the multiple effects of sartans (antihypertensive, antiglycative, antioxidant, and renoprotective), it is worth considering this class of molecules for first-line treatment of diabetic subjects with hypertension.

Acknowledgments

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