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Advanced glycation end products (AGEs) accumulate earlier and faster in long-term diabetes than in aging (1). In the glomerulus, AGE are likely to constitute the main mediator of the untoward consequences of hyperglycemia; the increase in oxidative stress, likely caused by hyperglycemia, is an additional source of damage. Both AGE accumulation and oxidative stress enhance the synthesis of extracellular matrix and the release of toxic cytokines (2,3). Mesangial cells can be damaged by peroxidation of their membrane lipids and by glycation and oxidation of intracellular proteins, as a result of the hyperglycemia-driven upregulation of glucose uptake via GLUT-1 and GLUT-2 transporters (4,5). Because AGE accumulation occurs in all tissues, the concentration of a single end product in the subcutaneous tissue is believed to constitute a handy marker of AGE-related glomerular damage (6); a case in point is the detection of pentosidine, an AGE derived from the glycoxidation reaction. The accumulation of fluorescent adducts of proteins with malondialdehyde (MDA) or 4-hydroxynonenal (HNE) in tissues can indirectly evidence oxidative damage; these two reactive carbonyl compounds are formed by lipid peroxidation, another nonenzymatic cascade involved in the pathogenesis of diabetic complications (7). Prevention of synthesis and tissue accumulation of AGE- or oxidative-derived end products could constitute a major advance in the treatment of diabetes complications. Several compounds with putative properties against AGE accumulation have been investigated in both clinical and experimental settings (8,9). In most instances, the results have been only partially satisfactory, possibly because the drugs being tested inhibited either glycation or oxidation but not both processes. The present study is a long-term comparative trial of N-acetyl-cysteine (NAC), oxerutin (OXE), and taurine (TAU) in experimental diabetes.

Not only does NAC exhibit antioxidant properties (10), but it may also counteract the glycation cascade through the inhibition of oxidation. NAC can also reduce the apoptosis elicited by reactive oxygen species (ROS) (11). These beneficial effects have been documented in endothelial dysfunction, in diabetic neuropathy, and in other instances of cell and tissue damage (12,13). OXE is a flavonoid found in staple foods and in several over-the-counter drugs and belongs to a large family of polyphenolic compounds with antioxidant properties (14). It exhibits superoxide scavenging activity and protects against lipid peroxidation (15). The beneficial effects of OXE have been documented in several conditions involving free radical damage, such as cataractogenesis, endothelial cell damage, and thrombosis (16,17). Flavonoids can also hinder the glycation cascade by inhibiting oxida-
tive stress and aldose reductase activity (16,18). The antioxidant properties of TAU, an amino acid abundant in human tissues (19), are ascribed to its scavenging activity on HOCIC, a highly toxic molecule. The product, taurine chloramine, exhibits lower oxidative activity than HOCIC. TAU might also act as an antiglycative compound, providing free amino groups that may compete for the reducing sugars (19,20).

In this study, the effects of the above-mentioned substances on the AGE accumulation and oxidative damage that accompany long-term diabetes were assessed in the subcutaneous tissue by evaluating pentosidine accumulation, quantified by liquid chromatography, and specific fluorescence of AGE and protein adducts with MDA and HNE. In addition, the glomerular accumulation of Nε-(carboxymethyl)Lysine (CML), the main AGE found at this site (21) and a known marker of glycoxidation (22), was demonstrated immunohistochemically. The mean glomerular volume (MGV), glomerular apoptosis rate, and glomerular cell density were also measured. MGV is a reliable parameter used to evaluate glomerular damage in human glomerulosclerosis and in several experimental models of this lesion. There is considerable morphometric evidence of glomerular hypertrophy in diabetes; in addition, the development of glomerulosclerosis, the increase in mean size of the glomeruli, and the total mesangial volume depend on disease duration (23). In contrast, information on glomerular cell turnover in diabetes is still fragmentary (24). A recent study by our group has shown significant rates of apoptosis and a parallel decrease in cellularity in the glomeruli of rats after 6 months of diabetes (25).

**RESEARCH DESIGN AND METHODS**

**Animals.** Male Wistar rats (n = 52) purchased from Harlan Italy Srl (Milan, Italy) were randomly housed in pairs and fed a standard diet (Piccioni SpA, Milan, Italy) ad libitum. A group of control rats (CTR; n = 8) were left untreated. At 12 weeks of age, the remaining rats were rendered diabetic by means of a single intraperitoneal injection of streptozotocin (60 mg/kg), freshly dissolved in sterile citrate buffer (pH 4.5). The glucose concentration in blood taken from a tail vein was determined by means of reactive strips and read with a reflectometer (both from Glucotrend 2, Boehringer Mannheim Italia SpA, Milan, Italy). Stable glucose levels >200 mg/dl were considered to indicate diabetes onset.

The diabetic rats were randomly assigned to the following five groups: (1) untreated diabetic rats (UD group); (2) rats treated with NAC (300 mg · rat−1 · day−1; NAC group); (3) rats treated with TAU (2.5 g · rat−1 · day−1; TAU group); (4) rats treated with both NAC and TAU (same dosages as groups 2 and 3; N + T group); and (5) rats treated with OXE (2.5 g · rat−1 · day−1; Roche Italia SpA; OXE group). All rats were maintained at 22–24°C with 12-h dark/light cycles, in accordance with common procedures for good animal care. Blood glucose levels were measured once a week. GHB levels were measured by affinity chromatography (Glycafin; Isolab, Akron, OH). The rats were killed at 40 weeks of age (~6 months after the onset of diabetes) by bleeding under anesthesia with intraarterial injection of sodium pentobarbital (65 mg/kg).

**Sample preparation.** On killing, blood was withdrawn from the inferior vena cava and aliquots were used to measure glucose and GHB. Samples of subcutaneous connective tissue were collected through a midline incision in the abdominal skin. The samples were washed thoroughly in normotonic saline solution and stored at ~80°C until measurements were taken. A small amount of tissue was finely minced and homogenized in ice-cold 0.1 mol/l PBS (pH 7.4) with a Polytron homogenizer (Kinematica, Lucerne, Switzerland), set at level 3, for 3–3 min. Lipids were extracted with chloroform/methanol (2:1) by mild shaking overnight. The pellets were washed with methanol and water, stored overnight at 4°C in 0.05 mol/l Triton-HCl buffer (pH 7.4) with 1 mol/l NaCl, and freed from soluble protein by washing with high-performance liquid chromatography (HPLC)-grade water. The remaining tissue was digested for 24 h (at 37°C with mild shaking) with purified collagenase (type VII, Sigma, St. Louis, MO) in HEPES buffer (0.02 mol/l, pH 7.5) containing 0.01 mol/l CaCl₂ (26). The supernatant was used to determine hydroxyproline content and fluorescence. The undigested collagen amounted to <20% of the total.

**Fluorescence determination.** Fluorescence intensity was determined in the digested tissue preparations with an LS55 PerkinElmer spectrophotofluorometer (at (1) 370-nm excitation (EX)/440-nm emission (EM) for Maillard products (27), (2) 335-nm EX/385-nm EM for pentosidine-like products (28), and (3) 390-nm EX/460-nm EM and 356-nm EX/460-nm EM for MDA and HNE protein adducts, respectively (29). A hydroxyproline assay was performed according to the method of Stegemann and Stalder (30) to assess collagen content. Fluorescence intensity was expressed in arbitrary units of fluorescence per milligram of hydroxyproline.

**Pentosidine quantification.** Subcutaneous collagen was used for pentosidine quantification. After homogenization and lipid extraction, 20 mg of collagen pellets were washed twice with phosphate buffer (0.1 mol/l, pH 7.4) and hydrolyzed in 2 ml of HCl 6N, under N₂ atmosphere for 24 h at 110°C in borosilicate tubes with screw caps. After hydrolysis, HCl was evaporated with Modulysylylizer (Edwards Alto Vuoto SpA, Milan, Italy); each sample was reconstituted in water containing 0.01 mol/l heparin/hydroboric acid (Pierce, Prodotti Gianni, Milan, Italy) and filtered through a 0.45-μm pore diameter filter (Ultrafree MC; Millipore, Milan, Italy). Pentosidine was determined in HPLC following the method of Odetti et al. (31).

**Morphology.** Specimens of renal tissue were fixed in buffered 10% formaldehyde and embedded either in 2-hydroxyethyl-methacrylate (Technovit 7100; Kulzer, Wehrheim, Germany) for morphometric determinations or in Paraplast to identify apoptotic nuclei by means of the transferase-mediated dUTP nick-end labeling (TUNEL) reaction. A custom-made, C-language macro was written to measure the area of glomerular tuft profiles and to count the number of cells within the glomerular areas within the glomerular tuft profiles of all animals. Image analysis was computed by means of the Labview image analysis software. The number of cells per unit of glomerular tuft area (Nₐ = 1/μm²) was used as an indirect measure of cell population density. The areas of at least 100 glomerular tuft profiles per sample were measured; by this method, MGV was estimated from the harmonic mean of the profile areas according to the DeHoff and Rhines formula, as previously described (32).

**Apoptotic nuclei on tissue sections were demonstrated by using the POD in situ cell death detection kit (Boehringer Mannheim, Monza, Italy) for the detection of single- and double-stranded DNA breaks by means of the end-labeling TUNEL reaction. Streptavidin-conjugated peroxidase was pre-
paralleled by the total GHB concentration at the time the rats were killed, with the UD group showing significantly higher mean GHB values than those of all of the treated groups (Fig. 2B).

**Subcutaneous tissue.** In the UD group, fluorescence at the various wavelengths tested was significantly more intense than in CTR rats (Fig. 3). All treatments except TAU provided partial protection against fluorescent product accumulation, with N + T treatment giving the best results (Fig. 3). Pentosidine accumulation in the skin, as determined by HPLC, showed a similar pattern, being significantly higher in UD rats than in CTR rats. OXE treatment led to a marked abatement of pentosidine content, which decreased to values not significantly different from those of CTR rats; NAC and N + T treatments provided partial protection, whereas in the TAU group, the value was higher than in the UD group (Fig. 4).

**Structural changes.** The kidneys of the diabetic rats showed severe glycogenosis of the proximal tubules, thickening of the glomerular basement membrane, and sparse periodic acid Schiff–positive deposits, which were observed mainly in the glomerulus and occasionally within the wall of the afferent arteriole. Mesangial expansion was also detected.

Immunohistochemistry for CML (Fig. 5) showed no staining in the glomeruli of CTR rats, with irregular, low-level positivity in the tubulointerstitial compartment. In UD rats, CML immunostaining was seen in both the glomerular and the tubulointerstitial compartment. In the glomeruli, there was intense, diffuse staining in the mesangium, basement membrane, and Bowman’s capsule. The tubular epithelium and basement membrane were also intensely positive. In the interstitial arteries, the intima and the luminal erythrocytes also reacted for CML. Diabetic rats treated with NAC or OXE showed marked reduction of staining for CML in the glomeruli; staining of glomerular basement membrane and Bowman’s capsule was particularly intense. On the contrary, TAU prevented CML immunostaining of erythrocytes of diabetic rats to the same degree of NAC and OXE. Diabetic rats treated with the N + T association showed reduction of CML positivity in the glomeruli similar to that observed with OXE or NAC alone.

Quantitative measurements revealed that MGV was significantly increased in UD rats in comparison with CTR rats. Diabetic rats treated with NAC or OXE showed a significant reduction in MGV in comparison with UD rats; in these groups, MGV values were not significantly different from those of the CTR group. Not only did treatment with TAU fail to prevent the diabetes-associated increase in MGV, but it was also associated with an additional increase in MGV. Treatment with N + T completely prevented the above TAU-related effect and induced a reduction in MGV comparable with that of NAC or OXE treatment (Fig. 6).

The UD group showed significant glomerular cell loss in comparison with the CTR group (data not shown). The combined effect of net glomerular cell loss and glomerular size increase determined a sharp reduction in cell density, i.e., mean number of cells per unit area, in the UD group. Treatment with NAC or OXE partially prevented the reduction in glomerular cell density found in UD rats. TAU treatment was associated with a marked reduction in glomerular cell density, an effect that exceeded that of UD rats. Again, treatment with N + T abated this negative effect of TAU-only treatment and provided protection against the decrease in glomerular cell density associated with diabetest to a similar extent to that of OXE treatment. Glomerular cell density in diabetic rats treated with OXE or N + T did not differ significantly from that of CTR animals (Fig. 7A).

Apoptosis rates showed a reverse pattern. The rate of glomerular apoptosis in UD rats was approximately four times that of CTR. OXE and, to a lesser degree, NAC abated the diabetes-associated rise in apoptosis. In particular, the low apoptosis rate in the OXE group did not differ significantly from that of the CTR group. Diabetic rats treated with TAU showed aggravation of diabetes-related
apoptosis in the glomeruli. Again, this effect was abated in the N+T group, which showed mean values comparable with that of the NAC group (Fig. 7B).

Pentosidine concentration in the skin correlated significantly with mean GHb values \((r = 0.440; P < 0.002)\), MGV \((r = 0.577; P < 0.002)\), and rate of apoptotic cells \((r = 0.564; P < 0.002)\). It correlated negatively with cell density \((r = -0.635; P = 0.0005)\; \text{Fig. 8}).

**DISCUSSION**

NAC, OXE, and TAU, the three drugs being tested in this study, significantly affected the biochemical and morphological parameters that we assessed as biomarkers of damage in diabetic rats. All of the treatments tested produced a significant slight reduction in hyperglycemia and GHb levels in comparison with the UD group. However, because all of the treated diabetic rats were still markedly hyperglycemic, we do not believe that this reduction could have affected the tissue parameters assessed. Moreover, because all antioxidant treatments were started after the development of diabetes, i.e., after virtual completion of β-cell damage, any protection of pancreatic β-cells against streptozotocin by the antioxidants is to be considered improbable.

NAC and OXE provided definite protection against the effects of long-term diabetes on the glomerulus: they drastically reduced CML accumulation, tuft enlargement,
cell loss, and rate of apoptosis. In the skin, OXE prevented partially the accumulation of fluorescent protein adducts and of pentosidine, whereas NAC induced only slight, not significant, reductions of these parameters.

Our in vivo observations are consistent with the hypothesis that ROS play a pivotal role in apoptosis and that thiol reductants, such as NAC, can block or delay this process (33). Indeed, NAC has been shown to inhibit ROS-induced mesangial apoptosis (34) and to be able to protect cells from glucose-induced inhibition of proliferation (35). Moreover, NAC has been seen to inhibit transforming growth factor-\(\beta\) (TGF-\(\beta\))-related pathways involved in collagen deposition (36). More recently, NAC proved capable of counteracting the high-glucose–dependent activation of mitogen-activated protein kinase pathways, which are probably involved in the development of microvascular complications of diabetes (37).

The beneficial effects of NAC on long-term diabetes observed in this study, together with the literature data, indicate a role of NAC-mediated mechanisms in protecting the glomeruli from diabetes-induced disorders. These data indirectly support the observation that glomerular damage in diabetes is associated with the local generation of ROS and AGE deposition.

A novel observation of our study is that administration of a flavonoid, such as OXE, could offer a promising approach to reducing glycoxidation-related damage in diabetes. Indeed, decrease in skin pentosidine concentration and in glomerular CML accumulation, maintenance of MGV, and preservation of glomerular cell number through apoptosis abatement were greatest in OXE-treated diabetic animals.

OXE, which has been studied extensively as a protector in venous hypertension (38), has also aroused attention because of its antioxidant properties: it is able to protect against ROS (39), and, in combination with other antioxidants, has shown the ability to protect against some risk factors for atherosclerosis (40).

Although we are unaware of any studies on the effects of OXE on diabetic complications, inhibition of cataractogenesis has been observed in vitro (41); however, several published reports support beneficial effects of flavonoids against diabetic complications (42,43).

In our study, the effects of TAU administration on AGE accumulation in skin and in kidney and on the renal structural changes associated with long-term diabetes were either negligible or negative. This is at variance with some studies, which have shown TAU-dependent partial prevention of diabetic glomerulopathy (44,45). In one of those studies, however, Trachtman et al. (44) failed to demonstrate in vitro inhibition of albumin glycation by TAU. Moreover, although diet supplementation with TAU reduced the morphologic damage to the kidney in rats with 12 months of diabetes, it failed to restrain the accumulation of fructoselysine, pentosidine, and carboxymethyllysine in the kidney (45), which is consistent with our immunohistochemical data.

A major point of contrast between our data and those of Trachtman’s group is the variation in glomerular size associated with TAU treatment of diabetic rats. Whereas Trachtman et al. (45) reported a reduction in the glomerular planar area in rats with 6 months of diabetes treated with TAU, we found an additional increase in MGV in this
group in comparison with UD rats. However, tissue processing and size calculation protocols were considerably different in these two studies. On the one hand, methacrylate embedding, which is used in our laboratory, minimizes tissue distortion, whereas paraffin embedding, which was used by Trachtman, is associated with considerable size artifacts. On the other hand, glomerular volume estimated from the harmonic mean of glomerular profile areas is a more reliable indicator of size than a simple mean of planar area (32).

We have no conclusive interpretation of the mechanisms implicated in the MGV increase associated with TAU treatment of diabetic rats. One hypothesis is based on the role of this amino acid as an organic osmolyte. Within the physiological pH range, TAU is a zwitterion that penetrates cell membranes (46); in the kidney, TAU works as an osmolyte (47). Although its concentration is normally regulated by proximal tubule reabsorption, TAU concentration increases in the kidney in response to diet supplementation or in pathological conditions such as diabetes (19, 45). A rise in glomerular TAU concentration could alter cell volume regulation and induce cell swelling. The subsequent increase in lysosomal pH may account for the inhibition of the proteolysis of growth factors, such as TGF-β (48): this may lead to the increased formation of extracellular matrix (49). Another hypothesis is that the effect of an increased concentration of TAU could have negative effects because the antioxidant activity of TAU is not complete and its chloramine derivative is still a weak oxidant (50).

The negative effects of TAU administration were suppressed by the combined N+T treatment protocol. Indeed, N+T provided significant protection against several of the parameter assessed in the skin and in the kidney. We postulate that NAC inhibits the residual oxidative activity of taurine chloride. Accordingly, TAU might exert a positive effect only in association with a chaperon molecule such as NAC.

The concomitant protection against glomerular CML accumulation and glomerular apoptosis exhibited by NAC, OXE, and N+T suggests the possibility that glycoxidation and cell regulation in the glomerulus are related. A corollary observation was that all treatments, including TAU only, markedly reduced CML accumulation in erythrocytes. This discrepancy between kidney and erythrocytes for the effects of TAU treatment on CML accumulation could be related to the affinity of the drug for the renal tissue (45) and to the different cell and matrix turnover of the relevant structures. A pertinent consideration is that TAU treatment reduced GHB levels in diabetic rats. Finally, it is worth noting that the level of fluorescent products in the skin correlated with the structural changes in the kidney; skin pentosidine, a recognized marker of AGE accumulation, also turned out to be a reliable index of kidney morphological damage.

In conclusion, we believe that our study provides valuable information that supports the possibility of preventing—or at least attenuating—glomerular damage in diabetes by diet supplementation with specific antioxidants; diabetic nephropathy might be delayed or slowed down by such treatments. The slight decrease in mean glycemic levels that we observed in the treated diabetic rats in comparison with the UD group, although significant, in our opinion is sufficient neither to account for the reduction of fluorescent and pentosidine accumulation in the skin nor to provide the protection against the glomerular damage observed in all treated groups except TAU; moreover, if the reduction of hyperglycemia had been the only cause of such protection, then even TAU treatment would have proved useful, which was not the case. We suggest that the antioxidant-mediated protection against diabetes-dependent damage is the consequence of global beneficial effects of the antioxidant treatments on the metabolic balance (which is testified to by the reduced hyperglycemia) and on the tissue glycoxidative modifications (which is testified to by the restricted accumulation of fluorescence and pentosidine in the skin). It is conceivable that TAU was not able to provide beneficial effects because of either residual oxidant properties of TAU derivatives (50) or unfavorable osmotic side effects in the kidney. The observation that N+T led to some of the best results suggests that associating antioxidants may be a useful procedure in the trials for protection against diabetes-dependent nephropathy. Further investigation is needed to discover the best associations and the best ratios between the dosages of the different antioxidants.

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