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Nitric oxide inhibits the formation of advanced glycation end products[1]

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Nitric oxide inhibits the formation of advanced glycation end products.

Background. Advanced glycation end products (AGEs) are elevated in renal failure and have been implicated in the pathogenesis of several uremic complications. Their formation is closely associated with oxidative stress. The recent observation that nitric oxide (NO) has an antioxidant effect led us to examine the possible role of NO in the generation of AGEs.

Methods. We examined the effect of NO donors, 2,2′-(hydroxynitrosohydrazono)bis-ethanamine (NOC18) and *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP), on the in vitro formation of pentosidine, which was used as a surrogate marker for AGEs. Bovine serum albumin was incubated under air at 37°C in a medium containing either several AGE precursors or uremic plasma. To elucidate further the mechanism of the NO effect on AGE formation, we examined the generation of free radicals and carbonyls in pentose-driven pentosidine formation.

Results. NO donors significantly inhibit the formation of pentosidine in a dose-dependent manner. The effect is abolished by the addition of a NO scavenging agent, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (carboxy-PTIO). The inhibitory effect results from NO but not from the NO donor molecule. It is best explained by the ability of NO to scavenge carbon-centered radicals, hydroxyl radical, and carbonyl compounds.

Conclusions. NO inhibits pentosidine formation by scavenging free radicals and by inhibiting carbonyl compound formation. NO might be implicated in the atherogenic and inflammatory effects of AGEs: Reduced NO production and increased oxidative stress associated with atherosclerotic lesions may accelerate AGE formation and, thus, exacerbate endothelial dysfunction and accelerate the development of atherosclerosis in uremia.

[1] **See Editorial by Devuyst and van Ypersele de Strihou, p. 1814**

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Advanced glycation end products (AGEs) are elevated in plasma and tissue proteins of uremic patients [1–3]. Their accumulation has been ascribed to the retention of various carbonyl intermediates derived from carbohydrates and lipids (carbonyl stress) [4]. The levels of pentosidine, a well-studied AGE structure, are highly correlated with those of precursor carbonyl compounds [5].

Advanced glycation end products have been implicated in the pathogenesis of vascular complications in uremia, a major cause of morbidity and mortality. Levels of AGEs in arterial tissues are higher in dialysis patients than in normal subjects [6]. Recent in vitro and in vivo studies suggest that they play an active role in atherogenesis. AGE-modified proteins initiate a range of inflammatory responses, including stimulation of monocyte chemotaxis [7, 8], secretion of inflammatory cytokines from macrophages [8, 9], proliferation of vascular smooth muscle cells [10], stimulation of aggregation of platelets [11], and stimulation of vascular endothelial growth factor expression [12]. These biological activities may account, at least in part, for the development of atherosclerosis. Furthermore, oral administration of the inhibitor of carbonyl amine reaction 2-isopropylidenehydrazono-4-oxo-thiazolidin-5-ylacetanilide (OPB-9195) to rats after balloon injury of their carotid arteries effectively reduces neointima proliferation in arterial walls [13].

The mechanism regulating AGE formation in vascular lesions is of particular interest. AGE formation is closely related to oxidative stress (glycoxidation) [14]. There is a significant correlation between the levels of AGEs and antioxidant enzymes, such as glutathione peroxidase and Cu/Zn-superoxide dismutase, in uremic plasma [15].

Nitric oxide (NO) and NO donors are known to inhibit lipid peroxidation in low-density lipoprotein [16, 17] by directly scavenging peroxyl radicals [18]. They might prove to be powerful anti-oxidants. We therefore evaluated the effect of NO on AGE formation.

We took advantage of an in vitro model of pentosidine

generation, used as a surrogate marker for AGEs, and demonstrate that indeed two NO donors, 2,2′-(hydroxynitrosohydrazono)bis-ethanamine (NOC18) and *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP), effectively inhibit the formation of AGEs. This NO-mediated inhibition of AGE formation appears mediated by the scavenging of free radicals and carbonyl compounds.

METHODS

In vitro incubation experiments

Sixty milligrams of essentially fatty acid-free grade bovine serum albumin (BSA; Sigma, St. Louis, MO, USA) were incubated with air for three days at 37°C in dark with either 10 mmol/L ribose, ascorbic acid, glucose, or arabinose in 2.0 mL of 0.2 mol/L sodium phosphate buffer (pH 7.4). One and 10.0 mmol/L NOC18 (Dojindo Laboratories, Kumamoto, Japan) and SNAP were used as NO donors [19]. The half-lives of these NO donors were determined by monitoring the decay of their characteristic absorptions at 254 and 590 nm for NOC18 and SNAP, respectively. Half-lives of NOC18 and SNAP under these conditions were 21 and 27 hours, respectively. They were thus expected to release NO continuously during the three-day incubation. The oxygen tension in the reaction medium did not decrease significantly during the procedure despite the consumption of O_2 by NO donors (52.5 ± 0.86 mm Hg at the initiation of incubation and 51.3 ± 1.29 mm Hg at the end). The effect of degradation products of NOC18 and SNAP was also examined to determine whether the observed effect by NO donors depends on liberated NO. Degradation product of NOC18, diethylenetriamine, was purchased from Aldrich (Milwaukee, WI, USA). A degradation product of SNAP was prepared by irradiating 100 mmol/L SNAP in 0.2 mol/L sodium phosphate buffer (pH 7.4) with a 75 W xenon lamp for one hour at room temperature. The degradation was confirmed by the absence of absorption above 400 nm. In some experiments, the sample incubation was performed in the presence of 2-(4-carboxyphenyl)-4,4,5,5,-tetramethyl-imidazoline-1-oxyl 3-oxide (carboxy-PTIO), which is a NO-specific scavenging agent [20].

Fresh heparinized predialysis plasma samples were obtained with informed consent from five nondiabetic hemodialysis patients (3 males and 2 females, age range of 41 to 62 years old, hemodialysis duration range of 6 to 13 years). Hemodialysis was performed with a cuprophane dialyzer and dialysate containing 30 mmol/L of bicarbonate and 8 mmol/L of acetate. All plasma samples were pooled. Two milliliters of pooled plasma were incubated with air at 37°C for seven days in the presence of 1.0 and 10.0 mmol/L SNAP or its degradation product. The experiments were performed four times. The effect of carboxy-PTIO was also examined.

Pentosidine measurement by high-performance liquid chromatography

Bovine serum albumin samples incubated with reagents (50 μL) were mixed with an equal volume of 10% trichloroacetic acid and were centrifuged at 5000 × *g* for five minutes. The supernatant was discarded, and the pellet was washed with 300 μL of 5% trichloroacetic acid. The pellet was dried under vacuum, lyophilized, and hydrolyzed by 100 μL of 6 N HCl for 16 hours at 110°C with nitrogen. Plasma samples (50 μL) were directly hydrolyzed by 50 μL of 12 N HCl for 16 hours at 110°C with nitrogen. The acid hydrolysates were subsequently neutralized with 100 μL of 5 N NaOH and 200 μL of 0.5 mol/L phosphate buffer (pH 7.4), filtered through a 0.5 μm pore filter, and diluted with phosphate-buffered saline.

The pentosidine content was analyzed in duplicate on a reverse-phase high-performance liquid chromatography (HPLC) according to our previous methods [3, 21]. In brief, a 50 μL solution of acid hydrolysate diluted by PBS was injected into an HPLC system and separated on a C18 reverse-phase column (Waters, Tokyo, Japan). The effluent was monitored using a fluorescence detector (RF-10A; Shimadzu, Kyoto, Japan) and an excitation-emission wavelength of 335/385 nm. Synthetic pentosidine was used as a standard [3]. The detection limit was 0.05 pmol of pentosidine per mg of proteins. All samples assayed in this study were analyzed in single batches to exclude interassay variation. Intra-assay coefficients of variation of the assays ranged from 5 to 12%.

Measurement of free radicals and dicarbonyl sugar formation

The free radical formation associated with ribose autoxidation was measured with spin-trapping technique. Twenty mmol/L of α-phenyl-tert-butylnitrone (PBN; Wako, Tokyo, Japan) was added to 0.2 mol/L phosphate buffer containing 10 mmol/L ribose and/or 30 mg/mL BSA, which was incubated for three days at 37°C under air. The resulting solution was sucked into a flat quartz cell (180 mL; JEOL, Tokyo, Japan), and the nitroxide radical was measured on an electron-spin resonance spectrometer (JES-FE2XG; JEOL) under the following conditions: microwave frequency, 9.42 GHz; magnetic field, 334.5 ± 5 mT; time constant, 0.3 seconds; microwave power 8 mW; field modulation width 0.2 mT; amplitude 1.6 × 1000; sweep time 2 min/10 mT. The amount of nitroxide radical was determined as the relative signal height to the third signal of external instrumental standard (Mn^{2+} in MgO).

Hydroxyl radical and dicarbonyl sugar formed during three-day incubation at 37°C were measured in 0.2 mol/L phosphate buffer (pH 7.4) containing 10 mmol/L ribose with or without NO donors and/or their degradation

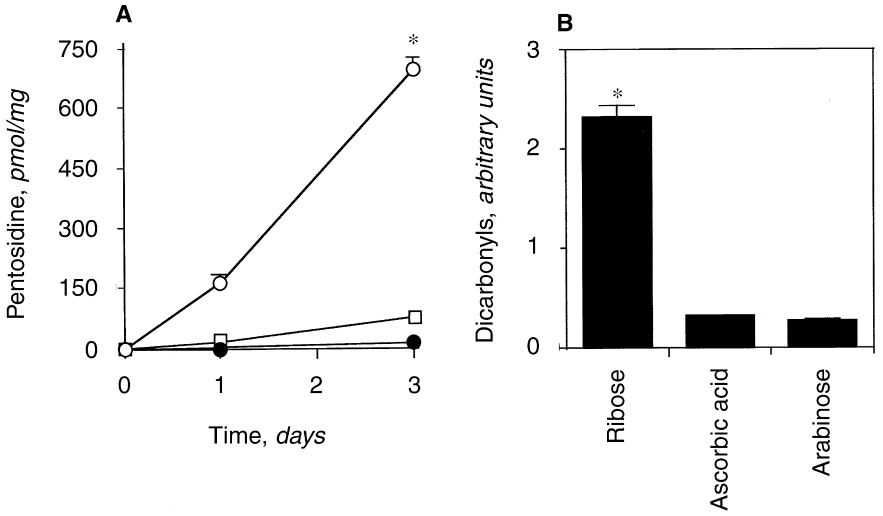

Fig. 1. Production of pentosidine (*A*) and dicarbonyls (*B*) during the glycoxidation of ribose, arabinose, and ascorbic acid (10 mmol/L each). The reaction was conducted in the presence of BSA (30 mg/mL) for one or three days at 37°C. Symbols are: (○) ribose; (□) arabinose; (●) ascorbic acid. Dicarbonyls were measured by Girard T reagent with absorbance at 295 nm. *$P < 0.0001$ vs. arabinose or ascorbic acid.

products. Hydroxyl radical formation was estimated with the hydroxylation of 1 mmol/L sodium benzoate measured by fluorospectrometer (RF-1500; Shimadzu, Kyoto, Japan) with excitation at 308 nm and emission at 410 nm [22]. The known amount of sodium salicylate (0.1 to 50.0 μmol/L) was used as an assay standard. Dicarbonyl sugar was measured using Girard T reagent [23]. The assay was performed at pH 2.9 as reported elsewhere [24], and the reaction product between dicarbonyls and the reagent was measured by spectrophotometer (BioSpec-1600; Shimadzu) with absorbance at 295 nm.

Statistical analysis

Data are expressed as means ± SD. One-way analysis of variance (ANOVA) was used for a statistical evaluation of significant difference between the groups.

RESULTS

Pentosidine and dicarbonyl formation

Ribose, ascorbic acid, and glucose (and its autoxidation product arabinose) are efficient precursors for pentosidine through the formation of intermediate carbonyl compounds [25–27]. The efficacy of these precursors on pentosidine and dicarbonyl formations was examined in incubation experiments with BSA. Pentosidine yields after three days with 10 mmol/L ribose, arabinose, or ascorbic acid averaged 697.3 ± 34.1, 77.8 ± 3.36, or 16.9 ± 3.19 pmol/mg albumin, respectively (Fig. 1A). By contrast, the pentosidine yield with 10 mmol/L glucose was below the detection limit. For dicarbonyls, the experiments were performed without BSA for three days at 37°C. As shown in Figure 1B, ribose produced approximately 7.15 or 8.39 times more dicarbonyls than ascorbic acid or arabinose (2.317 ± 0.116 vs. 0.324 ± 0.001 or 0.276 ± 0.008 arbitrary unit, respectively). Subsequently, we used only ribose as a source of pentosidine and dicarbonyls.

Inhibitory effect of NO on pentosidine formation

SNAP and its degradation product (10 mmol/L each) were incubated with ribose (10 mmol/L) and BSA (30 mg/mL) at 37°C for three days. SNAP decreased pentosidine formation by 30.0%, whereas denatured SNAP had no effect (Fig. 2). Carboxy-PTIO, a scavenger of NO, effectively quenched the inhibitory effect of SNAP on pentosidine generation. Carboxy-PTIO per se did not affect pentosidine generation during the incubation with BSA. The NO donor thus inhibits pentosidine formation on BSA from ribose. This inhibitory effect derives from NO but not from the NO donor molecule or its degradation product.

Another NO donor, NOC18, was also highly inhibitory on the pentosidine formation: In the presence of 10 mmol/L NOC18, the pentosidine generation was decreased by 58.5% on BSA incubated with 10 mmol/L ribose at 37°C for three days. However, diethylenetriamine, the degradation product of NOC18, is a potent inhibitor for pentosidine formation as it has two primary amino groups, which react with any carbonyls to form Schiff base products. NOC18 was thus inappropriate to estimate the NO effect on pentosidine formation.

Effect of NO on free radical formation during ribose glycoxidation

Free radicals are formed during the glycoxidation reactions [14, 28]. As NO acts as a free radical scavenger in lipid peroxidation [18], its inhibitory effect on pentosidine formation might result from its ability to scavenge free radicals generated during the glycoxidation. To test this hypothesis, free radical formation during the oxidation of ribose in the presence or absence of BSA was

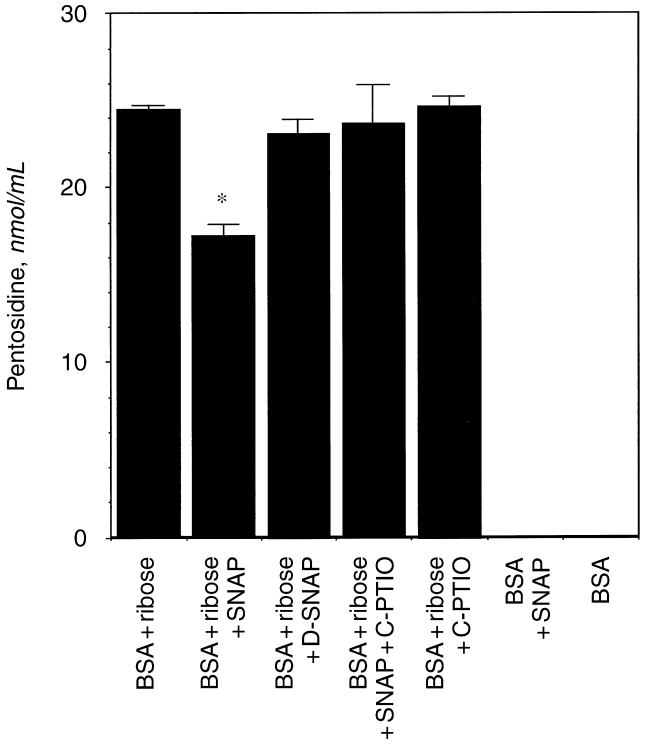

Fig. 2. Effect of SNAP on pentosidine formation during three-day incubation of BSA with 10 mmol/L ribose at 37°C. The effect of denatured SNAP (D-SNAP) and carboxy PTIO (C-PTIO) were also examined. *$P < 0.0001$ vs. ribose + BSA.

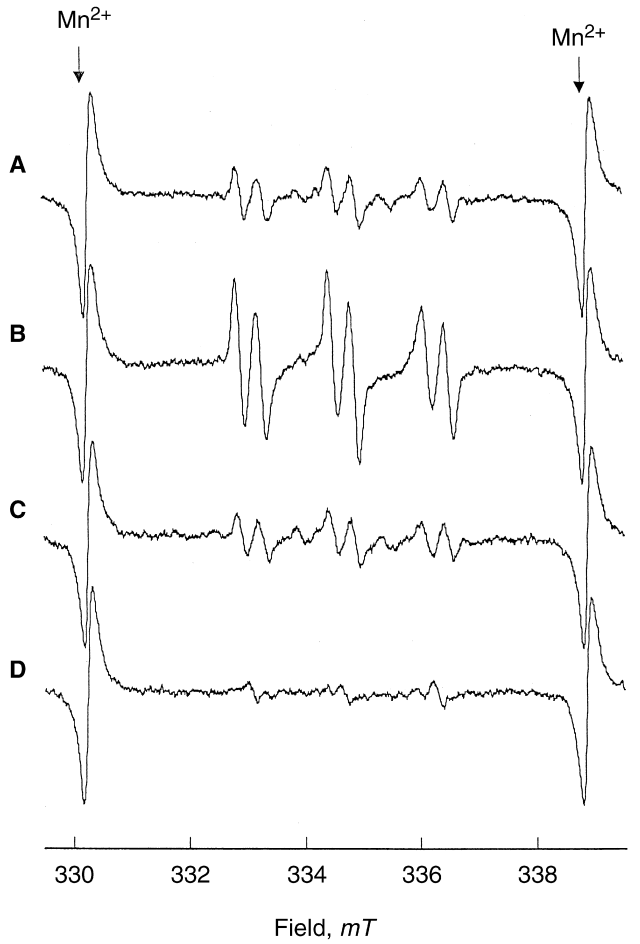

Fig. 3. Electron paramagnetic resonance (EPR) spectra for α-phenyl-tert-butylnitrone (PBN)-radical adduct generated by incubation of BSA with ribose. The signals designated with an arrow are due to Mn^{2+} in MgO used as an internal standard for signal intensity and field. (*A*) PBN + ribose + BSA. (*B*) PBN + ribose + BSA + diethylenetriamine. (*C*) PBN + ribose + BSA + NOC18. (*D*) PBN.

measured by PBN spin adduct formation with electron paramagnetic resonance (EPR). In this experiment, we had to use NOC18 because SNAP itself generates sulfur-centered radicals (S-radical), which interfere with the free radical detection. Although several radical species are formed in this system, including carbon-centered radicals (C-radical) and oxygen-centered radical (O-radical), such as hydroxyl radical, superoxide, and alchoxyl radicals via ribose autoxidation, only C-radicals are detectable because PBN spin adducts of O-radicals are short lived and cannot be detected in this time span.

Figure 3 shows typical EPR spectra obtained by the reaction of ribose, BSA, and PBN. The spectra observed in Figure 3 A and C have a *g* value of 2.0057 and hyperfine splittings of $a_N = 1.58$ mT and $a_H = 0.31$ mT, which indicates a typical PBN spin adduct of C-radicals [29]. The fact that the signal peak height did not change for at least six hours further confirms that these signals are due to C-radicals. The spectrum obtained from three days of incubation with PBN only did not show significant signals (Fig. 3D).

Figure 4 summarizes the relative peak height of the EPR signals attributed to PBN spin adducts of C-radicals. Note that the C-radical yield increased threefold in the presence of diethylenetriamine, the degradation product of NOC18, even though it decreased pentosidine formation. The reactions behind this increase can be explained by equations 1 to 3. A short-lived O-radical (R-O•) such as hydroxyl radical first forms during glycoxidation (equation 1). R-O• is trapped by PBN (equation 2a) to form PBN-O-R adduct or to abstract hydrogen from diethylenetriamine (DT), and resulting C-radicals (DT•; equation 2b), which, in turn, are trapped by PBN (equation 3):

Autoxidation

$$\text{Ribose} \rightarrow \text{R-O}\bullet + \text{Other product} \quad \text{(Eq. 1)}$$

$$\text{R-O}\bullet + \text{PBN} \rightarrow \text{PBN-O-R} \quad \text{(Eq. 2a)}$$

$$\text{R-O}\bullet + \text{DT} \rightarrow \text{R-OH} + \text{DT}\bullet \quad \text{(Eq. 2b)}$$

$$\text{DT}\bullet + \text{PBN} \rightarrow \text{PBN-DT} \quad \text{(Eq. 3)}$$

PBN-O-R is invisible because the adduct is short lived

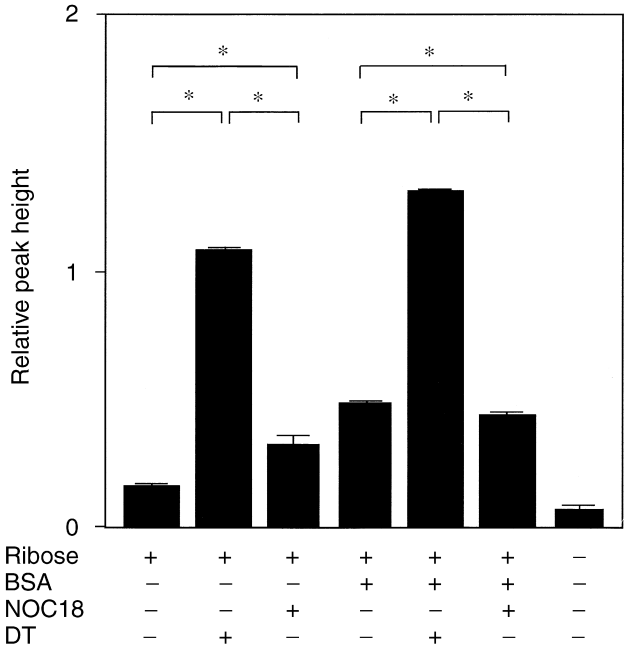

Fig. 4. Effect of NOC18 and its degradation product, diethylenetriamine (DT), on relative radical yield by incubation of BSA with ribose. *$P < 0.001$.

and cannot accumulate. DT therefore works as a converter of invisible O-radical to visible C-radical. In the presence of NOC18, the C-radical yield decreased compared with that with DT. Because NOC18 works as DT plus 2 NO molecules attached to one of nitrogens of DT, the effect of NO generated by NOC18 on free radical generation should be compared with that of DT rather than with that of ribose alone or ribose plus BSA. It may be concluded that NO scavenges C-radicals. As reported by previous authors [16–18], the most plausible mechanism of the NO-induced decrease in C-radicals is the quenching by NO of both R-O• and DT•, as NO can couple with any free radical by radical-radical reaction generated in this glycoxidation (equations 5 and 6):

$$\text{R-O}\bullet + \bullet\text{NO} \rightarrow \text{R-O-NO} \qquad \text{(Eq. 5)}$$

$$\text{DT}\bullet + \bullet\text{NO} \rightarrow \text{DT-NO} \qquad \text{(Eq. 6)}$$

Increased production of C-radicals by DT was also completely suppressed by NOC18 in the absence of BSA. The radical yield was lower in the absence than in the presence of BSA because BSA may be also attacked by O-radicals and produce fragmented C-radicals [22]. Free radicals are thus produced during ribose glycoxidation, and NO can scavenge these free radicals.

Effect of NO on hydroxyl radical and dicarbonyl formation

We next examined the effects of NO donors on the formation of hydroxyl radicals and dicarbonyls. Ribose (10 mmol/L) in 0.2 mol/L PB was incubated with or without NO donors and/or their degradation products at 37°C for three days. Ribose produces high levels of hydroxybenzoate, a product of the reaction between benzoate and hydroxyl radical (Fig. 5A). Hydroxyl radicals are so reactive that any molecule can be an effective scavenger. Diethylenetriamine and denatured SNAP effectively inhibit hydroxybenzoate formation. NOC18 suppresses the formation more than DT and SNAP completely abolishes hydroxybenzoate formation, an effect absent with denatured SNAP, indicating that NO scavenges residual free hydroxyl radical. Figure 5B shows the effects of SNAP on dicarbonyl formation. SNAP completely suppresses dicarbonyl formation. The effect of NOC18 on dicarbonyl formation was not estimated because NOC18 and DT directly react with carbonyls, as mentioned previously in this article.

Effect of NO on pentosidine formation in uremic plasma

To test the inhibitory effect of NO on pentosidine formation under physiological conditions, plasma obtained from uremic patients was incubated under air at 37°C for three to seven days in the presence or absence of SNAP. The pooled plasma from five uremic patients has a higher basal level of pentosidine residues on proteins. Incubation increases pentosidine generation from accumulated pentosidine precursor carbonyl compounds (Fig. 6). The addition of SNAP significantly inhibits pentosidine formation in plasma samples in a dose-dependent manner. Ten millimolar SNAP inhibits pentosidine formation by 25.1 and 27.6% after three and seven days of incubation, respectively. Denatured SNAP has no inhibitory effect on pentosidine formation. Carboxy-PTIO abolishes the inhibitory effect of SNAP. These findings indicate that NO inhibits the pentosidine formation in uremic plasma.

DISCUSSION

We demonstrate that NO inhibits the formation of pentosidine, a surrogate marker for AGEs. This inhibitory effect is abolished by the NO scavenger carboxy-PTIO. Further experiments with denatured product of NO donors demonstrate that the inhibitory effect of NO donors is indeed attributable to NO itself and not to a NO donor molecule or its degradation product.

We have further examined the effects of NO donors on the formation of unidentified free radicals by the PBN spin-trapping method, on hydroxyl radicals by hydroxybenzoate formation and on dicarbonyls by Girard-T reaction in order to elucidate the mechanism by which NO inhibits pentosidine formation (Fig. 7). During autoxidation of reducing sugars to dicarbonyls, dissolved oxygen receives an electron to form superoxide, the source of

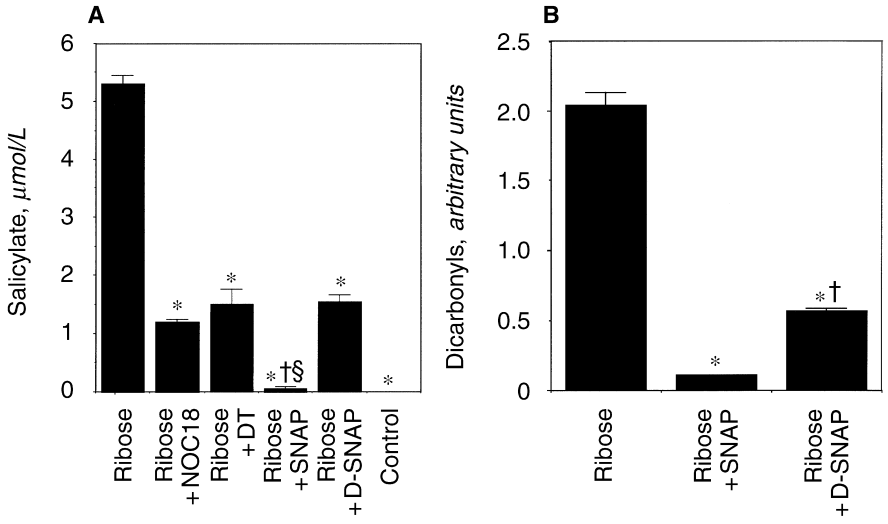

Fig. 5. Effects of NO donors on the yield of hydroxyl radical and dicarbonyls during ribose glycoxidation. (*A*) Hydroxybenzoate formation associated with the generation of hydroxyl radical, measured by the fluorescence (excitation at 308 nm and emission at 410 nm). The concentration of hydroxybenzoate was assayed as that of salicylate by comparing fluorescence intensity with the standard curve made with salicylate. (*B*) Dicarbonyls measured with Girard T reagent (absorbance at 295 nm). DT, diethylenetriamine; D-SNAP, denatured SNAP. $*P < 0.0001$ vs. ribose; $\dagger P < 0.0001$ vs. ribose + NOC18; $\S P < 0.0001$ vs. ribose + D-SNAP.

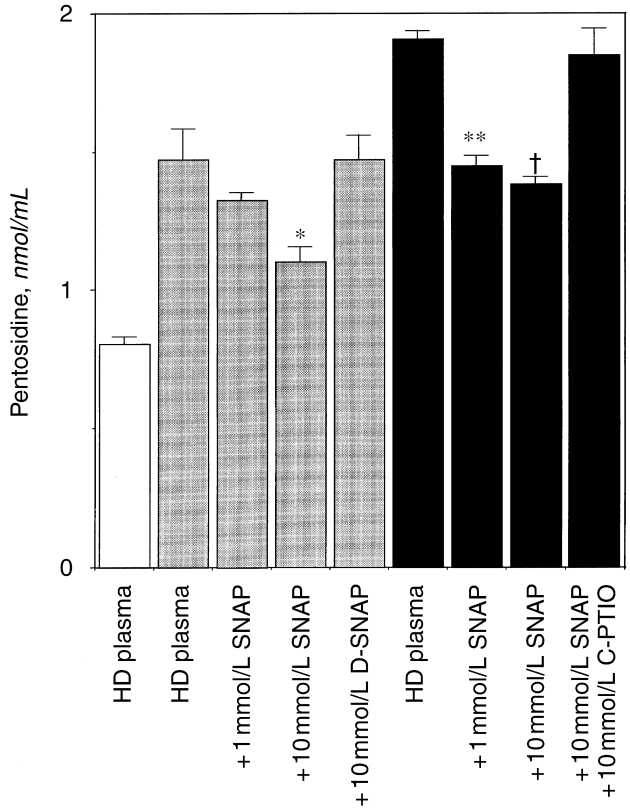

Fig. 6. Effect of SNAP on pentosidine formation by incubation of plasma of uremic patients at 37°C. Symbols are: (□) pentosidine level before incubation; (▒) three days of incubation; (■) seven days of incubation. $*P < 0.01$ vs. HD plasma (3 days incubation); $**P < 0.001$ vs. HD plasma (7 days incubation); $\dagger P < 0.0001$ vs. HD plasma (7 days incubation).

hydroxyl radicals. Hydroxyl radicals can abstract hydrogen from any molecule to promote the oxidation of sugars and form of C-centered and O-centered free radicals. NO acts as a scavenger of all free radicals and consequently suppresses dicarbonyl formation. At the very end, it is this reduction of dicarbonyl levels that results in a diminished pentosidine formation. NO couples with any free radical generated by autoxidation and breaks the chain reactions that they are able to promote.

Our observations cast a new light on the intricate relationships between AGEs and NO and their ability to generate pathological consequences. In arterial tissues, AGE levels are higher in hemodialysis patients than in normal subjects [5]. On immunohistochemical examination, AGEs, such as pentosidine and N^{ε}-(carboxymethyl) lysine, are present in the fatty streak and thickened neointima of arterial walls of uremic patients [12]. The staining pattern of AGEs corresponds with that of protein carbonyls, a biomarker of oxidative protein damage [30]. In atherosclerosis, protein expression of endothelial NO synthase (eNOS), which is responsible for NO synthesis in endothelial cells and NO release, is markedly reduced [31]. Reduced NO production together with increased oxidative stress in atherosclerotic lesions [32] might therefore accelerate the local formation of AGEs, endothelial dysfunction, and thus the progression of atherosclerosis. Interestingly, NO release and expression of eNOS protein are markedly reduced after exposure of cultured endothelial cells to AGE-modified proteins [33]. NO production and AGE formation appear therefore inextricably linked in atherosclerotic lesions.

In circulating plasma, the AGE contents of plasma proteins are also significantly higher in uremic than in control subjects [3, 5]. Interestingly, some researchers reported that the plasma levels of NO and its metabolites are significantly lower in uremic than in control subjects [34–36]. This is partially ascribed to the presence of inhibitor of NO synthase, N^{G}, N^{G}-dimethylarginine in uremic plasma [37] and to reduced arginine (substrate) availability [35]. To what extent the reduction of plasma NO contributes to increased carbonyl stress and AGE forma-

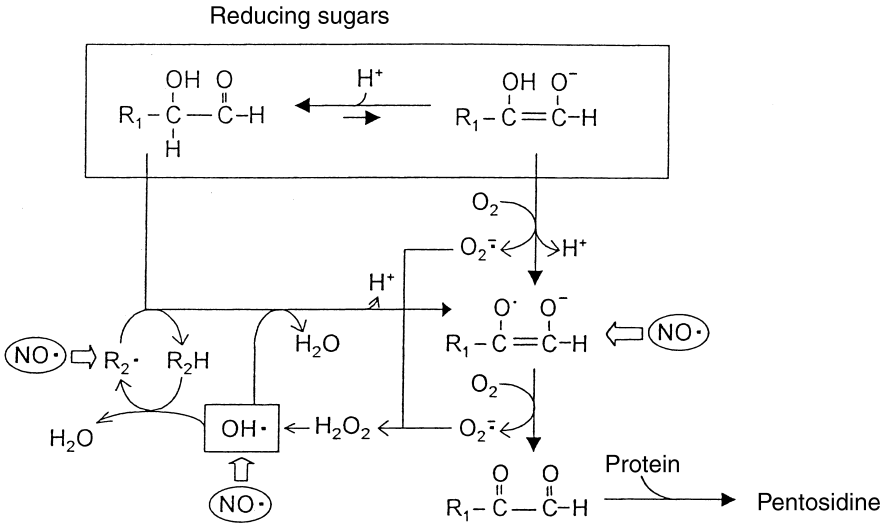

Fig. 7. Proposed mechanism for the inhibition of the pentosidine formation by NO. Reducing sugars are autoxidized to their dicarbonyls and reduce molecular oxygen to form superoxide/hydroxyl radicals. These radicals promote the chain reaction of sugar oxidation. NO traps these radicals and sugar radicals to inhibit whole sugar-autoxidation process. Consequently, NO suppresses dicarbonyl and pentosidine formation.

tion in plasma proteins of uremic patients remains to be established.

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APPENDIX

Abbreviations used in this article are: AGE, advanced glycation end product; BSA, bovine serum albumin; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide; eNOS, endothelial nitric oxide synthase; EPR, electron paramagnetic resonance; HPLC, high-performance liquid chromatography; NO, nitric oxide; NOC18, 2,2′-(hydroxynitrosohydrazono)bis-ethanamine; PBN, α-phenyl-tert-butylnitrone; and SNAP, *S*-nitroso-*N*-acetyl-DL-penicillamine.

REFERENCES

1. Odetti P, Fogarty J, Sell DR, Monnier VM: Chromatographic quantitation of plasma and erythrocyte pentosidine in diabetic and uremic subjects. *Diabetes* 41:153–159, 1992
2. Miyata T, Ueda Y, Shinzato T, Iida Y, Tanaka S, Kurokawa K, van Ypersele de Strihou C, Maeda K: Accumulation of albumin-linked and free-form pentosidine in the circulation of uremic patients with end-stage renal failure: Renal implications in the pathophysiology of pentosidine. *J Am Soc Nephrol* 7:1198–1206, 1996
3. Miyata T, Fu MX, Kurokawa K, van Ypersele de Strihou C, Thorpe SR, Baynes JW: Autoxidation products of both carbohydrates and lipids are increased in uremic plasma: Is there oxidative stress in uremia? *Kidney Int* 54:1290–1295, 1998
4. Miyata T, van Ypersele de Strihou C, Kurokawa K, Baynes JW: Alterations in nonenzymatic biochemistry in uremia: Origin and significance of "carbonyl stress" in long-term uremic complications. *Kidney Int* 55:389–399, 1999
5. Miyata T, Ueda Y, Yamada Y, Izuhara Y, Wada T, Jadoul M, Saito A, Kurokawa K, van Ypersele de Strihou C: Accumulation of carbonyls accelerates the formation of pentosidine, an advanced glycation end product: Carbonyl stress in uremia. *J Am Soc Nephrol* 9:2349–2356, 1998
6. Makita Z, Radoff S, Rayfield EJ, Yang Z, Skolnik E, Delaney V, Friedman EA, Cerami A, Vlassara H: Advanced glycosylation end products in patients with diabetic nephropathy. *N Engl J Med* 325:836–842, 1991
7. Kirstein M, Brett J, Radoff S, Ogawa S, Stern D, Vlassara H: Advanced protein glycosylation induces transendothelial human monocyte chemotaxis and secretion of platelet-derived growth factor: Role in vascular disease of diabetes and aging. *Proc Natl Acad Sci USA* 87:9010–9014, 1990
8. Miyata T, Inagi R, Iida Y, Sato M, Yamada N, Oda O, Maeda K, Seo H: Involvement of β2-microglobulin modified with advanced glycation end products in the pathogenesis of hemodialysis-associated amyloidosis: Induction of human monocyte chemotaxis and macrophage secretion of tumor necrosis factor-alpha and interleukin-1. *J Clin Invest* 93:521–528, 1994
9. Vlassara H, Brownlee M, Manogue KR, Dinarello CA, Pasagian A: Cachectin/TNF and IL-1 induced by glucose-modified proteins: Role in normal tissue remodeling. *Science* 240:1546–1548, 1988
10. Satoh H, Togo M, Hara M, Miyata T, Han K, Maekawa H, Ohno M, Hashimoto Y, Kurokawa K, Watanabe T: Advanced glycation endproducts stimulate mitogen-activated protein kinase and proliferation in rabbit vascular smooth muscle cells. *Biochem Biophys Res Commun* 239:111–115, 1997
11. Hangaishi M, Taguchi J, Miyata T, Ikari Y, Togo M, Hashimoto Y, Watanabe T, Kimura S, Kurokawa K, Ohno M: Increased aggregation of human platelets produced by advanced glycation end products in vitro. *Biochem Biophys Res Commun* 248:285–292, 1998
12. Lu M, Kuroki M, Amano S, Tolentino M, Keough K, Kim I, Bucala R, Adamis AP: Advanced glycation end products increase retinal vascular endothelial growth factor expression. *J Clin Invest* 101:1219–1224, 1998
13. Miyata T, Ishikawa S, Asahi K, Inagi R, Suzuki D, Horie K, Tatsumi K, Kurokawa K: 2-Isopropylidenehydrazono-4-oxo-thiazolidin-5-ylacetanilide (OPB-9195) treatment inhibits the development of intimal thickening after balloon injury of rat carotid artery: Role of glycoxidation and lipoxidation reactions in vascular tissue damage. *FEBS Lett* 445:202–206, 1999
14. Baynes JW: Role of oxidative stress in development of complications in diabetes. *Diabetes* 40:405–412, 1991
15. Ueda Y, Miyata T, Hashimoto T, Yamada Y, Izuhara Y, Sakai H, Kurokawa K: Implication of altered redox regulation by antioxidant enzymes in the increased plasma pentosidine, an advanced

glycation end product, in uremia. *Biochem Biophys Res Commun* 245:785–790, 1998
16. Yamanaka N, Oda O, Nagao S: Nitric oxide released from zwitterionic polyamine/NO adducts inhibits Cu^{2+}-induced low density lipoprotein oxidation. *FEBS Lett* 398:53–56, 1996
17. Seccia M, Perugini C, Bellomo G: The formation of some antigenic epitopes in oxidized human low-density lipoprotein is inhibited by nitric oxide. *Biochem Biophys Res Commun* 232:613–617, 1997
18. Hogg N, Kalyanaraman B, Joseph J, Struck A, Parthasarathy S: Inhibition of low-density lipoprotein oxidation by nitric oxide: Potential role in atherogenesis. *FEBS Lett* 334:170–174, 1993
19. Field L, Dilts RV, Ravichandran B, Lenhert G, Carnahan GE: An unusually stable thionitrite from N-acetyl-D,L-penicillamine: X-ray crystal and molecular structure of 2-(acetylamino)-2-carboxy-1,1-dimethylethyl thionitrite. *J Chem Soc Chem Commun* 1978: 249–250, 1978
20. Akaike T, Yoshida M, Miyamoto Y, Sato K, Kohno M, Sasamoto K, Miyazaki K, Ueda S, Maeda H: Antagonistic action of imidazolineoxyl N-oxides against endothelium-derived relaxing factor/NO through a radical reaction. *Biochemistry* 32:827–832, 1993
21. Miyata T, Taneda S, Kawai R, Ueda Y, Horiuchi S, Hara M, Maeda K, Monnier VM: Identification of pentosidine as a native structure for advanced glycation end products in β2-microglobulin-containing amyloid fibrils in patients with dialysis-related amyloidosis. *Proc Natl Acad Sci USA* 93:2353–2358, 1996
22. Hunt JV, Dean RT, Wolff SP: Hydroxyl radical production and autoxidative glycosylation: Glucose autoxidation as the cause of protein damage in the experimental glycation model of diabetes mellitus and ageing. *Biochem J* 256:205–212, 1988
23. Mitchel RE, Birnboim HC: The use of Girard-T reagent in a rapid and sensitive methods for measuring glyoxal and certain other alpha-dicarbonyl compounds. *Anal Biochem* 81:47–56, 1977
24. Wells-Knecht KJ, Zyzak DV, Litchfield JE, Thorpe SR, Baynes JW: Mechanism of autoxidative glycosylation: Identification of glyoxal and arabinose as intermediates in the autoxidative modification of proteins by glucose. *Biochemistry* 34:3702–3709, 1995
25. Grandhee SK, Monnier VM: Mechanism of formation of the Maillard protein cross-link pentosidine: Glucose, fructose, and ascorbate as pentosidine precursors. *J Biol Chem* 266:11649–11653, 1991
26. Dyer DG, Blackledge JA, Thorpe SR, Baynes JW: Formation of pentosidine during nonenzymatic browning of proteins by glucose: Identification of glucose and other carbohydrates as possible precursors of pentosidine in vivo. *J Biol Chem* 266:11654–11660, 1991
27. Miyata T, Wada Y, Cai Z, Iida Y, Horie K, Yasuda Y, Maeda K, Kurokawa K, van Ypersele de Strihou C: Implication of an increased oxidative stress in the formation of advanced glycation end products in patients with end-stage renal failure. *Kidney Int* 51:1170–1181, 1997
28. Miyata T, Maeda K, Kurokawa K, van Ypersele de Strihou C: Oxidation conspires with glycation to generate noxious advanced glycation end products in renal failure. *Nephrol Dial Transplant* 12:255–258, 1997
29. Knecht KT, Mason RP: The detection of halocarbon-derived radical adducts in bile and liver of rats. *Drug Metab Dispos* 19:325–331, 1991
30. Miyata T, Inagi R, Asahi K, Yamada Y, Horie K, Sakai H, Uchida K, Kurokawa K: Generation of protein carbonyls by glycoxidation and lipoxidation reactions with autoxidation products of ascorbic acid and polyunsaturated fatty acids. *FEBS Lett* 437:24–28, 1998
31. Oemar BS, Tschudi MR, Godoy N, Brovkovich B, Malinski T, Luscher TF: Reduced endothelial nitric oxide synthase expression and production in human atherosclerosis. *Circulation* 97:2494–2498, 1998
32. Miller FJ Jr, Gutterman DD, Rios CD, Heistad DD, Davidson BL: Superoxide production in vascular smooth muscle contributes to oxidative stress and impaired relaxation in atherosclerosis. *Circ Res* 82:1298–1305, 1998
33. Chakravarthy U, Hayes RG, Stitt AW, McAuley E, Archer DB: Constitutive nitric oxide synthase expression in retinal vascular endothelial cells is suppressed by high glucose and advanced glycation end products. *Diabetes* 47:945–952, 1998
34. Kari JA, Donald AE, Vallance DT, Bruckdorfer KR, Leone A, Mullen MJ, Bunce T, Dorado B, Deanfield JE, Rees L: Physiology and biochemistry of endothelial function in children with chronic renal failure. *Kidney Int* 52:468–472, 1997
35. Wever R, Boer P, Hijmering M, Stroes E, Verhaar M, Kastelein J, Versluis K, Lagerwerf F, van Rijn H, Koomans H, Rabelink T: Nitric oxide production is reduced in patients with chronic renal failure. *Arterioscler Thromb Vasc Biol* 19:1168–1172, 1999
36. Schmidt RJ, Yokota S, Tracy TS, Sorkin MI, Baylis C: Nitric oxide production is low in end-stage renal disease patients on peritoneal dialysis. *Am J Physiol* 276:F794–F797, 1999
37. Vallance P, Leone A, Calver A, Collier J, Moncada S: Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. *Lancet* 339:572–575, 1992