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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

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Abstract We have pursued the hypothesis that the carbonyl modification of proteins by glycoxidation and lipoxidation reactions plays a role in atherogenesis. Human atherosclerotic tissues with fatty streaks and uremic arteriosclerotic tissues were examined, with specific antibodies, to detect protein adducts formed with carbonyl compounds by glycoxidation or lipoxidation reactions, i.e. advanced glycation end products (AGEs) or glycoxidation products, such as carboxymethyllysine (CML) and pentosidine, and lipoxidation products, such as malondialdehyde (MDA)-lysine and 4-hydroxy-nonenal (HNE)-protein adduct. All the four adducts were identified in the proliferative intima and in macrophage-rich fatty streaks. If the carbonyl modification is not a mere result but is a contributor to atherogenesis, inhibition of glycoxidation and lipoxidation reactions might prevent vascular tissue damage. We tested this hypothesis in rats following balloon injury of their carotid arteries, a model exhibiting a remarkable intimal thickening, which are stained positive for all the four adducts. Oral administration of 2-isopropylidenehydrazono-4-oxo-thiazolidin-5-ylacetanilide (OPB-9195), an inhibitor of both glycoxidation and lipoxidation reactions, in rats following balloon injury effectively prevented the intimal thickening. These data suggest a role for the carbonyl modification of proteins by glycoxidation and lipoxidation reactions in most, if not all, types of vascular tissue damage ('carbonyl stress'), and the usefulness of inhibitors of carbonyl reactions for the treatment of vascular tissue damage.

Key words: Advanced glycation end product; Glycoxidation; Lipoxidation; Carbonyl compound; Intimal thickening; Rat carotid artery balloon injury

1. Introduction

Oxidative stress has been implicated in tissue damage associated with several diseases including atherosclerosis. Oxidation conspires with non-enzymatic glycation to form advanced glycation end products (AGEs) [1], which lead to tissue damage through the alteration of structure and function of matrix proteins [2] and stimulation of pathogenic cellular responses [3,4]. Among AGE structures are the glycoxidation products, N^{ε}-carboxymethyllysine (CML) [5] and pentosidine [6]. Several intermediate carbonyl compounds resulting from the oxidation of glucose have been identified as efficient precursors of CML and pentosidine, such as glyoxal, arabinose, methylglyoxal, and glycolaldehyde [7–9], as well as dehydroascorbate formed on oxidation of ascorbate [10,11].

Lipid peroxidation of polyunsaturated fatty acids, such as arachidonate, yields other carbonyl compounds: some are identical to those formed from carbohydrates [12], such as glyoxal and methylglyoxal, and others are characteristic of lipids, such as malondialdehyde (MDA) and 4-hydroxy-nonenal (HNE) [13]. The latter carbonyl compounds produce lipoxidation products, such as MDA-lysine and HNE-protein adduct.

We therefore hypothesized that under oxidative stress in vascular lesions, carbohydrates, ascorbate, and polyunsaturated fatty acids may be converted to carbonyl intermediates, which, in turn, react with tissue proteins, leading to formation of glycoxidation and lipoxidation products. In the present study, in order to characterize carbonyl modification of proteins by glycoxidation and lipoxidation reactions in vascular lesions, we immunohistochemically examined human atherosclerotic tissues and arteriosclerotic tissues from uremic patients.

If the carbonyl modification is not merely a result of oxidative stress but a contributor to atherogenesis, inhibition of glycoxidation and lipoxidation reactions might prevent vascular tissue damage. During our search for compound(s) that inhibit glycoxidation and lipoxidation reactions, we found that 2-isopropylidenehydrazono-4-oxo-thiazolidin-5-ylacetanilide (OPB-9195) is a potent inhibitor for both glycoxidation and lipoxidation reactions ([14,15], and manuscript submitted). We therefore tested whether the inhibition of glycoxidation and lipoxidation reactions by OPB-9195 prevents the development of vascular lesions in rats following balloon injury of their carotid arteries, a model exhibiting a remarkable intimal thickening.

2. Materials and methods

2.1. Human tissue specimens

Arterial tissue sections were obtained at autopsy from six (age 46–56) non-diabetic patients with hypercholesteremia (>250 mg/dl) and ischemic heart diseases, such as myocardial infarction or angina, with angiographic evidence of coronary artery disease and from 12 non-diabetic patients with end-stage renal failure due to chronic glomer-

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Abbreviations: AGE, advanced glycation end product; CML, N^{ε}-(carboxymethyl)lysine; HNE, 4-hydroxy-nonenal; MDA, malondialdehyde; OPB-9195, (±)-2-isopropylidenehydrazono-4-oxo-thiazolidin-5-ylacetanilide

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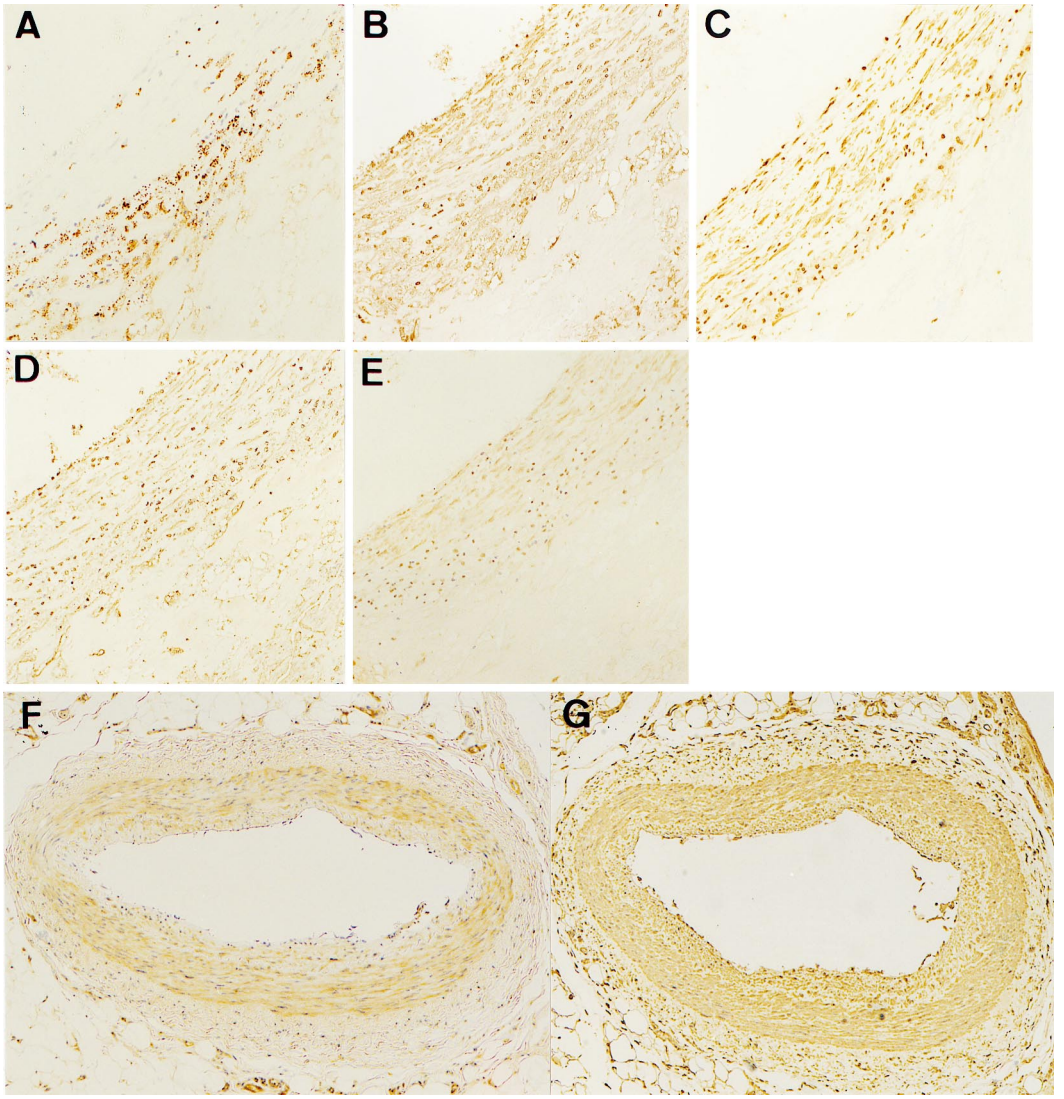

Fig. 1. Immunohistochemical detection of glycoxidation and lipoxidation products in human atherosclerosis and uremic arteriosclerosis. Arterial tissue specimens from a 55-year-old non-diabetic male (A–E) and from a 60-year-old non-diabetic uremic male who had undergone hemodialysis for 11 years (F and G). Immunostaining for CD68 (A), CML (B and F), pentosidine (C), MDA-lysine (D and G), and HNE-protein adduct (E). Nuclei were counterstained with Meyer's hematoxylin. Glycoxidation and lipoxidation products are present in the proliferative intima (B–G) and in macrophage-rich fatty streaks (B–E). A–E, ×36; F and G, ×18.

ulonephritis who had undergone regular hemodialysis (seven males, age range: 52–81 years old, hemodialysis duration range: 4–16 years). The samples were fixed in 10% formalin and embedded in paraffin.

2.2. Antibodies

Anti-pentosidine rabbit IgG [16], anti-HNE-protein adduct rabbit IgG (kindly provided by Dr. K. Uchida) [17], and anti-MDA-lysine mouse monoclonal IgG (kindly provided by Dr. J.L. Witztum) [18,19] were used for immunohistochemistry. Anti-AGE rabbit IgG [20], the major epitope-structure of which was identified as CML [20], was also used.

2.3. Immunohistochemistry

Tissue sections (5 μm thickness) were mounted on a slide coated with 3-aminopropyltriethoxy silane (Sigma, St. Louis, MO), deparaffinized, rehydrated in distilled water, and then incubated with either anti-pentosidine, anti-AGE, anti-HNE-protein adduct, or anti-MDA-lysine antibody overnight in humid chambers at room temperature. The sections were washed and incubated with 1:100 diluted goat anti-rabbit or -mouse IgG conjugated with peroxidase or alkaline phosphatase (Dako, Glostrup, Denmark) for 2 h, followed by the detection with 3,3′-diaminobenzidine solution containing 0.003% H_2O_2 (for peroxidase) or with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (for alkaline phosphatase). The specificity of immunostainings was confirmed by competition experiments by incubating anti-AGE, anti-pentosidine, anti-HNE, or anti-MDA antibody for 4 h at 37°C with an excess of CML-, pentosidine-, MDA-, or HNE-BSA, respectively, as described previously [16,20,21]. Non-immune rabbit or mouse IgG was used as a negative control.

2.4. Experimental animals

Male 6-week-old normotensive, euglycemic Sprague-Dawley rats weighing 180–200 g were used in studies conducted in accord with the NIH Guide for the Care and Use of Laboratory Animals. Protocols were approved by the Institutional Animal Care and Use Subcommittee. The animals were housed under controlled temperature and lighting conditions with access to food and water ad libitum.

Endothelial denudation and injury to the vascular wall were achieved in the left common carotid artery of rats as described previously [22]. The rats were anesthetized with intraperitoneal administration of sodium pentobarbital and Fogarty arterial embolectomy balloon catheter (model 12-060-2F, Cv-1035: Baxter Health Care, Santa Ana, CA) was inserted through the right iliac artery and advanced to left carotid artery via the aorta. The catheter was then

inflated with saline and gently withdrawn through the entire length of the artery. After this procedure was repeated five times, the catheter was removed, the wound was ligated and closed. The right carotid artery remained untreated and served as an intraindividual control.

In order to evaluate the inhibitory effect of OPB-9195 on intima thickening, rats were given orally OPB-9195 suspended in 0.5% carboxymethyl cellulose sodium salt solution, twice daily (30 mg/kg of rat weight) for 14 days following vascular injury. Control rats were given orally the same amount of 0.5% carboxymethyl cellulose sodium salt solution without OPB-9195.

Both carotid arteries were excised 14 days after the balloon injury, adventitia removed with forceps, then washed three times in PBS. Sections (10 mm length) proximal to the bifurcation of the carotid arteries were fixed and sectioned for immunohistochemical analysis and for light microscopy for morphometric analysis. The intimal/media ratio was measured as the ratio of the distance from the lumen to the internal elastic lamina to the distance from the internal to the external elastic lamina. The distances were measured by micrometry on microscopic photographs of each vessel. Determinations were performed on four different cross-sections of the vessel.

2.5. Statistical analysis

Data are expressed as means ± S.E.M. Student's *t*-test was used for a statistical evaluation of significant difference between groups.

3. Results

3.1. Immunohistochemical detection of glycoxidation and lipoxidation products in human atherosclerosis and uremic arteriosclerosis

Early atherosclerosis is characterized by the diffuse intima thickening and by fatty streaks composed primarily of multiple layers of macrophage-derived foam cells. Fatty streaks were stained positive for CD68 (Fig. 1A), i.e. macrophage-derived foam cells. Both the glycoxidation products, CML (Fig. 1B) and pentosidine (Fig. 1C), and lipoxidation products, MDA-lysine (Fig. 1D) and HNE-protein adduct (Fig. 1E), were stained positive in the proliferative intima and macrophage-rich fatty streaks. Immunostainings were specific as demonstrated by their complete inhibition in the presence of an excess of competitors (data not shown).

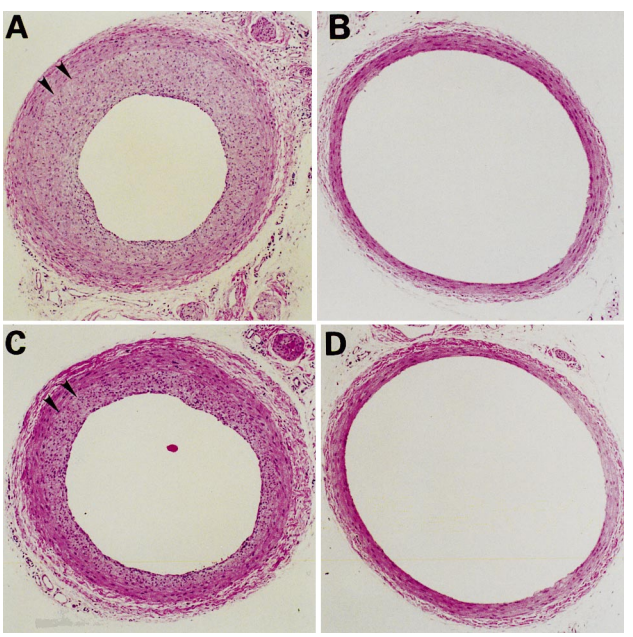

Fig. 2. Inhibitory effect of OPB-9195 on intimal thickening of rat carotid artery following balloon injury. Section of left carotid artery (injured and non-treated with OPB-9195) showing the intimal response 14 days after balloon injury (A). The effect of OPB-9195 treatment on the left (injured and treated) vessel is shown in C. Contralateral (non-injured) vessels in a rat without or with OPB-9195 treatment are shown in B and D, respectively. Arrowsheads indicate the internal elastic lamina. ×16.

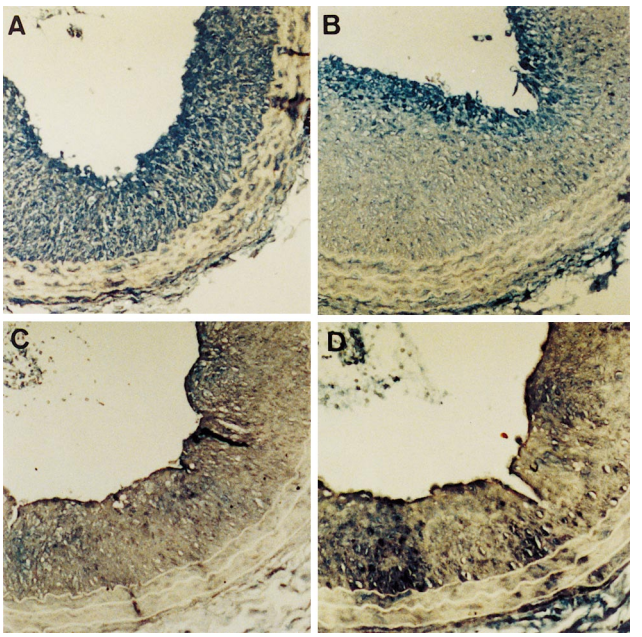

Fig. 3. Immunohistochemical detection of glycoxidation and lipoxidation products in a rat carotid artery following balloon injury. Tissue specimens from rat carotid artery 14 days after balloon injury. Immunostaining for CML (A), pentosidine (B), MDA-lysine (C), and HNE-protein adduct (D). Nuclei were counterstained with Meyer's hematoxylin. Glycoxidation and lipoxidation products are present in the intima. ×35.

Uremic arteriosclerosis, characterized by the intimal thickening of arterial walls, was then examined for glycoxidation and lipoxidation products. Both glycoxidation and lipoxidation products were stained positive in the proliferative intima (exemplified for CML, Fig. 1F and for MDA-lysine, Fig. 1G).

3.2. Inhibitory effect of OPB-9195 on intimal thickening of rat carotid artery following balloon injury

In order to test the efficacy of OPB-9195 in the prevention of vascular lesions, we utilized an experimental model of traumatic vascular lesion of the carotid artery in rats. Rat left carotid arterial tissues obtained after balloon injury, exhibited a remarkable intimal thickening (Fig. 2A), which is an early and major event in the development of human vascular lesions, whereas the non-injured right carotid artery exhibited no intimal thickening (Fig. 2B). The proliferative intima contains glycoxidation and lipoxidation products: CML (Fig. 3A), pentosidine (Fig. 3B), MDA-lysine (Fig. 3C), and HNE-protein adduct (Fig. 3D).

Daily oral administration of OPB-9195 in rats following balloon injury markedly reduced intimal thickening in the injured left carotid artery as shown in Fig. 2C and Table 1. Immunohistochemical study revealed the positive stainings for glycoxidation and lipoxidation products in the reduced intima (data not shown). The non-injured right carotid artery of OPB-9195-treated rats exhibited no intimal thickening (Fig. 2D).

Table 1
Inhibitory effect of OPB-9195 on intimal thickening of the rat carotid artery following balloon injury

	Injured carotid artery with OPB-9195 treatment ($n = 8$)	Injured carotid artery without OPB-9195 treatment ($n = 6$)
Intimal/media ratio[a]	$1.11 \pm 0.15^{*}$	1.68 ± 0.10

Values are mean ± S.E.M.
[a]Intimal/media ratio is the ratio of the distance from the lumen to the internal elastic lamina to the distance from the internal to the external elastic lamina.
$^{*}P < 0.05$.

4. Discussion

The immunohistochemical studies disclosed the co-distribution of protein adducts formed by glycoxidation and lipoxidation reactions, not only in vascular lesions in aged subjects (Fig. 1), but also in uremic patients (Fig. 2), in diabetic patients [14], and in a rat carotid artery following balloon injury (Fig. 3). Our data are in agreement with previous studies by others [23,24] demonstrating the presence of lipoxidation products in atherosclerotic tissues. Therefore, the formation of glycoxidation and lipoxidation products in vascular lesions might be a phenomenon common in most if not all types of vascular damage, irrespective of whether the stimulation of vascular injury is metabolic or mechanical.

CML and pentosidine are combined products of glycation and oxidation of proteins, whereas MDA-lysine and HNE-protein adduct are oxidation-dependent lipid-protein adducts. The antibodies used in the present study recognize distinct structures, and apparently do not cross-react with the other structures based on inhibition by modified BSA competitors [16,20,21]. Therefore, the distribution of the four adducts, which are formed on proteins with carbonyl compounds derived from the oxidation of carbohydrates and lipids, in vascular lesions provide independent evidence for a localized oxidative stress and carbonyl modification by glycoxidation and lipoxidation reactions ('carbonyl stress').

Polyunsaturated fatty acids are undoubtedly the primary sources of MDA-lysine and HNE-protein adduct. However, the primary source of CML and pentosidine in vivo remains inconclusive. In vitro incubation of BSA yielded pentosidine from glucose and ascorbate, but not from arachidonate [14]. Of interest is our recent observation that in uremic patients, the plasma levels of pentosidine were well correlated with those of dehydroascorbate, but not with those of glucose [11]. The primary source of CML in vivo is more complicated. CML can be formed on BSA by incubation with glucose, ascorbate, and arachidonate [14]. As previously suggested by Fu et al. [12], CML in atherosclerotic fatty streaks in normoglycemic subjects could thus result not only from glycoxidation reaction but also from lipoxidation reaction. In agreement with this speculation, the plasma CML levels in normoglycemic uremic patients on hemodialysis are correlated with those of MDA-lysine, but not with those of fructoselysine, a marker of prevailing glucose concentration [25].

The present results that oral administration of OPB-9195 effectively inhibited the intimal thickening of rat carotid artery following balloon injury, suggests a link of carbonyl modification by glycoxidation and lipoxidation reactions to the development of vascular lesions in rats. Whether it also applies to humans remains to be investigated. OPB-9195 inhibits most, if not all, carbonyl reactions with proteins including not only glycoxidation but also lipoxidation reactions ([14,15], and manuscript submitted). A plausible explanation for the inhibitory mechanism of OPB-9195 on the carbonyl reactions might be the trapping of reactive carbonyl compounds. The hydrazine nitrogen atom of OPB-9195 is capable of reacting with carbonyl groups, directly or via the free base upon hydrolysis, by the hydrazone formation. We therefore speculate that OPB-9195 inhibits intimal thickening by entrapping reactive carbonyl compounds and quenching carbonyl reactions in vascular lesions. The reduced intima of injured arterial walls in OPB-9195-treated rats still contained glycoxidation and lipoxidation products. The partial inhibitory effect of OPB-9195 on intimal thickening in the present model thus might be ascribed to incomplete inhibition of carbonyl reactions by OPB-9195.

However, other effects of OPB-9195 independent of its activity as carbonyl trap, e.g. inhibition of oxidative stress, remain to be examined. In this regard, recent studies demonstrated that antioxidant butylated hydroxytoluene [26] or probucol [27] treatment of hypercholesterolemic rabbits reduced intimal thickening after balloon injury.

Two possible mechanisms may be considered regarding the role of carbonyl stress in vascular tissue damage. First, the final glycoxidation and lipoxidation products, such as AGEs, have biological activities, e.g. stimulation of monocyte chemotaxis [3,28,29], secretion of inflammatory cytokines from macrophages [3,30,31], proliferation of vascular smooth muscle cells [32], and quenching of nitric oxide activity [33]. Secondly, as intermediate carbonyl compounds are capable of cross-linking covalently with proteins, they might cross-link matrix tissue or cell surface proteins, leading to alterations of structure and function of matrix proteins or stimulation of cellular responses [34,35]. Indeed, exposure of murine thymocytes or fibroblasts to carbonyl compounds, such as glyoxal, MDA, or HNE, resulted in the phosphorylation of the tyrosine residues of a number of intracellular proteins, which was completely inhibited by pretreatment with OPB-9195 [36].

In conclusion, the present study suggests a link of increased carbonyl modification by glycoxidation and lipoxidation reactions to the development of vascular lesions. Further studies will be undoubtedly required to evaluate the usefulness of inhibitors of carbonyl reactions for the treatment of vascular tissue damage.

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