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Original Contribution

Peroxynitrite modifies the structure and function of the extracellular matrix proteoglycan perlecan by reaction with both the protein core and the heparan sulfate chains

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ABSTRACT

The heparan sulfate (HS) proteoglycan perlecan is a major component of basement membranes, plays a key role in extracellular matrix (ECM) structure, interacts with growth factors and adhesion molecules, and regulates the adhesion, differentiation and proliferation of vascular cells. Atherosclerosis is characterized by chronic inflammation and the presence of oxidized materials within lesions, with the majority of protein damage present on ECM, rather than cell, proteins. Weakening of ECM structure plays a key role in lesion rupture, the major cause of heart attacks and strokes. In this study peroxynitrite, a putative lesion oxidant, is shown to damage perlecan structurally and functionally. Exposure of human perlecan to peroxynitrite decreases recognition by antibodies raised against both the core protein and heparan sulfate chains; dose-dependent formation of 3-nitrotyrosine was also detected. These effects were modulated by bicarbonate and reaction pH. Oxidant exposure resulted in aggregate formation, consistent with oxidative protein crosslinking. Peroxynitrite treatment modified functional properties of perlecan that are dependent on both the protein core (decreased binding of human coronary artery endothelial cells), and the HS chains (diminished fibroblast growth factor-2 (FGF-2) receptor-mediated proliferation of Baf-32 cells). The latter is consistent with a decrease in FGF-2 binding to the HS chains of modified perlecan. Immunofluorescence of advanced human atherosclerotic lesions provided evidence for the presence of perlecan and extensive formation of 3-nitrotyrosine epitopes within the intimal region; these materials showing marked co-localization. These data indicate that peroxynitrite induces major structural and functional changes to perlecan and that damage to this material occurs within human atherosclerotic lesions.

Introduction

Perlecan is a large multi-domain heparan sulfate proteoglycan (HSPG), which is found in basement membranes and the pericellular environment [1]. The perlecan protein core (mass 470 kDa) consists of five distinct domains. Domains II – V display homology to the low-density lipoprotein receptor, laminin A chain, neuronal cell adhesion molecule and epidermal growth factor [2]. The N-terminal domain, domain I, is the main region of glycosaminoglycan substitution, with a cluster of three attachment sites; an additional two potential attachment sites exist on domain V [3,4]. Endothelial cell-derived perlecan is substituted exclusively by heparan sulfate (HS) at the domain I attachment sites [5]; chondroitin sulfate and keratan sulfate variants have also been isolated from chondrogenic and epithelial cell sources [6]. Perlecan is the major HSPG expressed in the vascular wall and plays a key role in vascular homeostasis via the stabilization and organization of vascular extracellular matrix (ECM), and the regulation of adhesion, differentiation and proliferation of vascular cells [4,7]. These functions are mediated by interactions of its protein core and HS chains with a variety of extracellular matrix molecules, growth factors and adhesion

Abbreviations: ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); dONOO, decomposed peroxynitrite; ECM, extracellular matrix; FGF-2, fibroblast growth factor 2; HCAEC, human coronary artery endothelial cells; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; MTT, 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan; 3-nitroTyr, 3-nitrotyrosine; $ONOO^-$, peroxynitrous acid anion; ONOOH, peroxynitrous acid; TCA, trichloroacetic acid.

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molecules. Homozygous-null mutations in the perlecan gene are embryonically lethal and associated with cardiovascular malformations and rupture of the heart and large blood vessels [8].

Atherosclerosis is a multi-factorial disease characterized by the accumulation of lipids in the artery wall and chronic inflammation [9]. Lesions contain large numbers of activated monocytes and macrophages that are capable of generating reactive oxidants, and it has been demonstrated that human atherosclerotic lesions, of all degrees of severity, contain oxidized lipids and proteins. The levels of these materials are significantly elevated, and antioxidant levels significantly decreased, compared to healthy tissue (reviewed [10]). Previous studies have shown that the majority of oxidative damage to proteins detected in atherosclerotic lesions is associated with ECM, rather than intracellular, proteins [11]. The contribution of oxidative damage to vascular ECM to the progression of atherosclerosis is currently unclear, however it may be of considerable importance. Modification of the ECM of the artery wall is believed to play a key role in both the development of atherosclerosis [12], and the subsequent rupture of lesions, which is the major cause of heart attacks and strokes [13]. The composition of the ECM within the artery wall alters during lesion development, and is markedly different at site of lesion erosion and rupture, with alterations in the type and distribution of proteoglycans, collagens, and hyaluronan [14–16]. These changes in composition and properties may contribute to endothelial cell loss and dysfunction, altered vascular smooth muscle cell phenotypes and a decrease in the mechanical stability of the lesion cap, thereby increasing the propensity of lesions to undergo rupture. Notably, perlecan has been shown to play a key role in modulating smooth muscle cell proliferation and migration, a key feature of developing lesions and a major response to vascular injury [17].

Considerable evidence supports a role for peroxynitrite ($ONOO^-$/ONOOH; this equilibrium mixture is termed peroxynitrite from hereon) in the initiation of oxidation in atherosclerotic lesions and ECM modification (reviewed in [18]). Peroxynitrite is generated as a result of the diffusion-controlled reaction (k ca. 7×10^9 M^{-1} s^{-1} [19]) of superoxide anion radicals ($O_2^{\bullet-}$; generated by the respiratory burst of activated leukocytes) with nitric oxide radical ($^{\bullet}NO$, generated by the inducible nitric oxide synthase enzyme of macrophages) [20]. Other sources of $O_2^{\bullet-}$ and $^{\bullet}NO$ present at sites of inflammation, such as in atherosclerotic lesions that may also contribute to peroxynitrite formation, include the activities of xanthine oxidase [21] and endothelial nitric oxide synthase [22]. Considerable data supports an increased rate of generation of these radicals, and hence peroxynitrite, at sites of inflammation, including within the diseased artery wall [20]. Thus extensive antibody staining for 3-nitrotyrosine (3-nitroTyr), a stable modification to Tyr residues formed on exposure to peroxynitrite and other reactive nitrogen species, has been detected throughout human atherosclerotic lesions [23], and high- and low-density lipoproteins isolated from atherosclerotic lesions contain elevated levels of 3-nitroTyr when compared to circulating lipoproteins [24,25].

Perlecan is likely to be an important target for damage by peroxynitrite in the vascular wall, however the effects of this oxidant on its structure and function are unknown. Data obtained with isolated glycosaminoglycans, and intact ECM, indicate that HS chains of perlecan are potential targets for modification and fragmentation by peroxynitrite. Thus, peroxynitrite can fragment *isolated* glycosaminoglycan chains (e.g. [26–28]), with this occurring in a site-specific manner as a result of damage being induced by both hydroxyl ($HO^{\bullet}$) and carbonate ($CO_3^{\bullet-}$) radicals (but not to any great extent by the peroxynitrite anion or nitrogen dioxide radical; $NO_2^{\bullet}$) [27,28]. Treatment of cell culture-derived matrix or isolated arterial matrix with peroxynitrite results in the release of matrix fragments that include both protein and carbohydrate components [29], consistent with damage to perlecan. Concomitant generation of 3-nitroTyr in ECM proteins was observed [29], however the susceptibility of perlecan to this modification and the potential roles of protein versus HS modification in the degradation of this target is unclear. Perlecan is also implicated as a target for the myeloperoxidase-derived oxidants HOCl and HOBr [11,30,31], and recent studies have established that HOCl can selectively modify and functionally impair the cell adhesive function of the protein core, without impairing the ability of its heparan sulfate chains to promote FGF-2-dependent cellular proliferation [32].

To further elucidate the potential role of damage to perlecan by peroxynitrite in vascular disease, the mechanisms and functional consequences of exposure of human arterial endothelial cell-derived perlecan (the major proteoglycan component of the arterial subendothelial matrix), to peroxynitrite have been examined in detail. In particular, these studies have sought to resolve the role of damage to the protein versus the HS chains, the potential role of bicarbonate and reaction pH in modulating these reactions, and whether such oxidant damage alters the biological activities of this important vascular macromolecule.

Materials and Methods

Chemicals

Solutions were prepared using water purified through a four-stage Milli Q system (Millipore-Waters) treated with washed Chelex resin (Bio-Rad) to remove contaminating trace metal ions. pH control was achieved using phosphate buffer (0.1 M), with pH adjustments made using sodium monophosphate (0.1 M), sodium diphosphate (0.1 M), or small quantities of concentrated HCl or NaOH.

Peroxynitrite was synthesized as previously [33]. Stock concentrations were determined spectrophotometrically using $\varepsilon_{302\ nm}$ 1670 M^{-1} cm^{-1} [33]. Stock solutions of peroxynitrite anion (pH ca. 12) were prepared by dilution of the synthesized material into 0.1 M NaOH. Due to the high concentration and pH of the stock solutions, small volumes were added to strongly buffered solutions to minimize pH changes; the reported pH values are determined for the final reaction mixtures. "Decomposed" peroxynitrite (dONOO) was prepared by incubation overnight at 37 °C in 0.1 M phosphate buffer, pH 6.

Cell culture

Human coronary artery endothelial cells (HCAECs) were cultured in HCAEC Growth Medium (Cell Applications) at 37 °C in a humidified atmosphere containing 5% CO_2. Cell-conditioned medium was aspirated from near confluent flasks of cells and stored at -80 °C, for subsequent perlecan purification. For cell adhesion studies, cells were cultured to approximately 90% confluence, washed with PBS (pH 7.4) containing 1 mM $MgCl_2$/1 mM $CaCl_2$ then incubated with 1% trypsin for 3 min at 37 °C. Cell-conditioned media was added to neutralize trypsin. Cells were washed twice with Medium-199 (Sigma) containing 1% BSA and resuspended in Medium-199 containing 1% BSA at a cell concentration of 2.5×10^5 cells mL^{-1}.

BaF3 cell culture

The ability of perlecan to promote cellular proliferation in response to exogenous fibroblast growth factor-2 (FGF-2) was investigated using a heparan sulfate proteoglycan-deficient myeloid cell line (BaF3) expressing the FGF receptor isotype 1c (FGFR1c), essentially as described previously [34]. Briefly, BaF3 cells (10^5 cells mL^{-1}) were incubated in RPMI-1640 medium with perlecan (1.25 nM) or heparin (0.3 – 30 nM) in the presence of FGF-2 (0.3 nM) for 72 h at 37 °C and proliferation was then determined by the MTT assay using CellTiter 96® AQueous One Solution (10% v/v, 6 h, 37 °C).