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Novel genomic targets in oxidant-induced vascular injury

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Abstract

To study the complex interaction between oxidative injury and the pathogenesis of vascular disease, vascular gene expression was examined in male Sprague–Dawley rats given 35 or 70 mg/kg allylamine, a synthetic amine converted to acrolein and hydrogen peroxide within the vascular wall. Vascular lesions and extensive vascular remodeling, coupled to increased production of 8-epi-PGF2α, nuclear localization of NFκB, and alterations in glutathione homeostasis, were observed in animals treated with allylamine for up to 20 days. Transcriptional profiling, immunohistochemistry, and in situ hybridization showed that genes involved in adhesion and extracellular matrix (ECM) (α_1 integrin, collagen), cytoskeletal rearrangements (α-smooth muscle actin, α-tropomyosin), and signal transduction (NFκB, osteopontin, and LINE) were altered by oxidant treatment. To evaluate mechanisms of gene dysregulation, cultured aortic smooth muscle cells were challenged with allylamine or its metabolites and processed for molecular analysis. These agents increased formation of reactive oxygen species and elicited changes in gene expression similar to those observed in vivo. Oxidative stress and changes in gene expression were inhibited by *N*-acetyl cysteine, a precursor of glutathione. These results indicate that genes along the ECM–integrin–cytoskeletal axis, in addition to LINE, are molecular targets in oxidant-induced vascular injury.

Keywords: Functional genomics; Gene expression; Oxidative stress; Vascular injury

1. Introduction

The development of atherosclerosis involves damage to endothelial cells and vascular smooth muscle cells (vSMCs), influx of inflammatory cells to sites of injury, release of inflammatory mediators, uncontrolled proliferation of vSMCs, and accumulation of lipids and matrix proteins. The uncontrolled proliferation of vSMCs is a major factor contributing to lesion progression, and involves reprogramming of phenotypic expression from a quiescent to a mitogen responsive, highly proliferative state [1]. This phenotypic switch has been extensively studied, but little is known about the complex gene–gene interactions that participate in phenotypic control. Of relevance is the finding that vascular oxidative stress caused by endogenous or exogenous reactive oxygen species (ROS) is a major contributor to phenotypic modulation and atherogenesis [2].

Many of the vascular disorders involving phenotypic modulation of vSMCs have roots that can be traced to ROS and its effects. For example, angiotensin-II induced hypertrophy of vSMCs is dependent on hydrogen peroxide produced intracellularly [3]. Likewise, there is a clear cut requirement for hydrogen peroxide in PDGF-induced vSMC proliferation [4]. However, much of the work completed to date examining the impact of oxidative stress on vascular cell proliferation has relied solely on the use of in vitro models, therefore hampering the study of the complex biological interactions in vivo.

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The study of vascular oxidative stress in vivo in this laboratory has been facilitated in experiments where oxidative injury is localized to cells of the cardiovascular system. To this end, a chemical model of vascular injury has been developed where allylamine, a synthetic aliphatic amine converted to acrolein and hydrogen peroxide, is administered to rats for varying times [5]. In this model, repeated cycles of injury promote the development of vSMC lesions and phenotypic modulation of vSMCs. The oxidative injury induced by allylamine is highly specific because metabolism of the parent amine is catalyzed by a vascular-specific amine oxidase expressed predominantly in vSMC [6]. Both acrolein and hydrogen peroxide promote peroxidative injury and activate the cellular stress response in vSMCs [7–9]. These metabolites induce phenotypic modulation of vSMCs in vivo, characterized by morphological and ultrastructural alterations [10], as well as changes in mitogenic signaling, extracellular matrix (ECM) production and integrin expression [11,12].

In this study we test the hypothesis that repeated cycles of oxidative injury alter the genomic circuitry of vSMCs. Evidence is presented that the production of reactive intermediates from allylamine leads to changes in gene expression caused by compromised redox homeostasis. As such, the injury induced by oxidative metabolites was fully preventable by *N*-acetyl cysteine, an intracellular precursor of glutathione. Our findings defined genes within the vessel wall affected by oxidative stress and showed that the adaptive response to oxidative injury involves disruption of growth regulatory genes and ECM–integrin–cytoskeletal associated genes.

2. Materials and methods

2.1. Animals

Six-week-old (175–180 g) male Sprague–Dawley rats were gavaged daily with 35 or 70 mg/kg per day allylamine (99% purity) (Sigma, St. Louis, MO), or water (1 ml/kg per day) for 20 days. All experimental procedures involving animals were in compliance with federal and institutional guidelines.

2.2. Antibodies

α_1 Integrin and NFκB were purchased from Chemicon (Temecula, CA), OPN from Santa Cruz (Santa Cruz, CA), α smooth muscle actin from Sigma (St. Louis, MO) and isoprostane from Assay Designs (Ann Arbor, MI).

2.3. Immunofluorescence

Vessels were removed, cut into 5 mm sections and placed in OCT embedding media (TissueTek, Torrence, CA), snap frozen in liquid nitrogen, and stored at –80 °C. Tissues were sectioned at 8–10 μm, fixed in –20 °C methanol for 15 min, and processed as described in Molecular Probes ELF-97 Protocol MP 06600 (Molecular Probes, Eugene, OR). Tissues were incubated for 5 min with 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI). Slides were mounted in ELF-97 immunohistochemical mounting medium from Molecular Probes.

2.4. Western analysis

Thoracic aortas were removed and completely stripped of adventitia. Vessels were placed into liquid N_2 and powdered using a mortar and pestle. Powdered aortas were then placed into T-PER (Pierce, Rockford, IL) containing 1× HALT protease inhibitor cocktail (Pierce), and proteins extracted according to a manufacturer's protocol. Protein concentrations were determined using a BioRad protein assay (Hercules, CA). 25 μg of protein per lane were loaded onto NuPage 4–12% Bis–Tris gels (Invitrogen, Carlsbad, CA), run at 10 V for 1 h and then transferred onto Invitrolon PVDF (Invitrogen). Gels were stained with GelCode Blue stain reagent (Pierce) and the membranes stained with Ponceau S to determine loading efficiency. Membranes were blocked overnight using 10% Blotto non-fat dry milk (Santa Cruz) and incubated with primary and secondary antibodies as noted below. Primary antibody concentrations were as follows; α-smooth muscle actin (0.4 μg/ml), osteopontin (0.4 μg/ml), alpha-1 integrin (1 μg/ml), and alpha-tropomyosin (0.67 μg/ml). Blots were developed using the Pierce SuperSignal West Dura substrate. Films were scanned and analyzed using 1D Image Analysis Software version 3.5 (Kodak, Rochester, NY).

2.5. Fluorescence in situ hybridization

The probe for the α-tropomyosin target (Tpm1) was a 50 nucleotide antisense oligo:5′ CAA GAC TCC TTC ATC AAG CCG GAT GTC CCA CCT CTC TGA GCT CTT TTT CG 3′. Sense probe was used as a control for non-specific binding. The oligos were designed according to manufacturer's recommendations, labeled with Alexa Fluor 488 (Genisphere, Hatfield, PA) and purified by PAGE. Pre-treatment and hybridization of sections was completed as specified in the Starfish (Genisphere, Hatfield, PA).

2.5.1. Microscopy

Labeled tissues were visualized using a BioRad RTS200MP Confocal Microscope (BioRad, Hercules, CA) equipped with 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) long-pass (LP) and fluorescein-5-isothiocyanate (FITC) filter sets. Sections were excited with the Tsunami Laser set to 790 nm. ELF-substrate excitation and emission wavelengths were 360 and 535 ± 18 nm, respectively. UV band-pass (BP) excitation filter 340–380 nm and LP suppression filter (425 nm) were used for the collection of DAPI images. For FISH experiments, the images were captured using a Zeiss Axiovert200 equipped with an Axiocam HRm cooled CCD camera (Zeiss, Thornwood, NY).