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Original Contribution

Aldose reductase regulates acrolein-induced cytotoxicity in human small airway epithelial cells

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ABSTRACT

Aldose reductase (AR), a glucose-metabolizing enzyme, reduces lipid aldehydes and their glutathione conjugates with more than 1000-fold efficiency (K_m aldehydes 5–30 μM) relative to glucose. Acrolein, a major endogenous lipid peroxidation product as well as a component of environmental pollutants and cigarette smoke, is known to be involved in various pathologies including atherosclerosis, airway inflammation, COPD, and age-related disorders, but the mechanism of acrolein-induced cytotoxicity is not clearly understood. We have investigated the role of AR in acrolein-induced cytotoxicity in primary human small airway epithelial cells (SAECs). Exposure of SAECs to varying concentrations of acrolein caused cell death in a concentration- and time-dependent manner. AR inhibition by fidarestat prevented the low-dose (5–10 μM) but not the high-dose (> 10 μM) acrolein-induced SAEC death. AR inhibition protected SAECs from low-dose (5 μM) acrolein-induced cellular reactive oxygen species (ROS). Inhibition of acrolein-induced apoptosis by fidarestat was confirmed by decreased condensation of nuclear chromatin, DNA fragmentation, comet tail moment, and annexin V fluorescence. Further, fidarestat inhibited acrolein-induced translocation of the proapoptotic proteins Bax and Bad from the cytosol to the mitochondria and that of Bcl2 and Bcl_{XL} from the mitochondria to the cytosol. Acrolein-induced cytochrome *c* release from mitochondria was also prevented by AR inhibition. The mitogen-activated protein kinases (MAPKs), such as extracellular signal-regulated kinases 1 and 2, stress-activated protein kinase/c-Jun NH_2-terminal kinase, and p38MAPK, and c-Jun were transiently activated in airway epithelial cells by acrolein in a concentration- and time-dependent fashion, which was significantly prevented by AR inhibition. These results suggest that AR inhibitors could prevent acrolein-induced cytotoxicity in the lung epithelial cells.

Introduction

Acrolein is a ubiquitous environmental pollutant that arises from incomplete combustion of plastic materials, forest fires, cigarette smoke, and pyrolyzed animal and vegetable fats [1–3]. The presence of acrolein in the ambient air of urban atmospheres represents a considerable exposure hazard to humans, resulting in various health problems including atherosclerosis and airway disease such as asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis, and carcinogenesis [2,4–8]. Along with the strongly reactive aldehyde 4-hydroxy-2-nonenal (HNE), acrolein is produced in situ as the toxic by-product of oxidative stress-induced endogenous lipid peroxidation [9]. Among α,β-unsaturated aldehydes, acrolein is considerably the strongest electrophile and readily reacts with nucleophilic reactive groups of biomolecules, including the sulfhydryl group of cysteine, imidazole group of histidine, and amino group of lysine, resulting in the inactivation of the proteins [10].

A number of reports have described the damaging effects of acrolein on airway ciliary movement [11], airway inflammation, and infiltration of monocytes, macrophages, and lymphocytes into the interstitium, mucus-cell metaplasia, and airway enlargement. Acrolein is known to cause apoptosis in a number of cell types including keratinocytes, neutrophils, and airway cells [12–14]. However, the biochemical and molecular mechanisms involved in the cellular toxicity are not clearly understood.

Apoptosis is a programmed cell death mechanism and is known to play an important role in development as well as in maintaining tissue homeostasis [15]. Oxidant-induced apoptosis involves morphological and biochemical changes such as DNA fragmentation into 180–200 bp, externalization of phosphatidylserine, and cellular fragmentation, forming apoptotic bodies [16,17]. Triggered by various kinds of stimuli, the mitochondrial apoptosis pathway is thought to be regulated by antiapoptotic (Bcl2 and Bcl_{XL}) and proapoptotic (Bad and Bax) members of the Bcl2 family of proteins, which modulate the release of proapoptotic molecules such as cytochrome (Cyt) *c* [18].

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Translocation of Bad to the mitochondrial membrane eliminates the inhibitory effects of Bcl2 on Bax. Bax then forms Bax–Bax dimers, forming pores on the outer mitochondrial membrane, which may result in mitochondrial permeability transition leading to the release of proapoptogenic molecules including Cyt *c* [19]. Cyt *c* interacts with apoptosis protease-activating factor in the cytosol, which leads to activation of caspases in a sequential manner. The activated effector caspases cleave many cellular proteins including poly(ADP-ribose) polymerase (PARP) and inhibitor of DNase. This leads to characteristic morphological changes in cells akin to apoptosis, including chromatin condensation, nuclear membrane breakage, cell blebbing, and formation of apoptotic bodies. Further, at the molecular level apoptosis is regulated by oxidative stress-mediated activation of several signaling intermediates including the redox-sensitive stress kinases that activate transcription factors including nuclear factor-κB (NF-κB) and activator protein (AP)-1 [20]. The oxidative activation of Jun-N-terminal kinase (JNK) and mitogen-activated protein kinase (MAPK) phosphorylates and activates c-Jun, thereby activating AP-1 [21,22]. AP-1, a redox-sensitive transcription factor and known to regulate transcription of a number of inflammatory genes, utilizes cysteine residues as the redox switches [23]. Among other redox-sensitive targets, reactive oxygen species (ROS) directly activate certain protein tyrosine kinases, which then activate downstream signaling cascades [24]. Further, ROS also inactivate protein tyrosine phosphatases resulting in the persistent activation of tyrosine kinases [24,25]. Thus, oxidative stress induced by acrolein may activate redox signaling pathways that lead to enhanced expression of proapoptotic proteins, which could be responsible for acrolein-induced cell death [26].

We have shown that inhibitors of aldose reductase have anti-inflammatory and antioxidative properties [27,28]. Aldose reductase (AR) is a stress response protein and a metabolic enzyme that is known to detoxify aldehydes in addition to reducing glucose to sorbitol in hyperglycemia. Increased AR expression and activity have been linked with pathological conditions in which acrolein levels are known to be elevated, e.g., cigarette smoke exposure [29]. Although extensive literature on acrolein toxicity is available, the role of AR in acrolein-induced cytotoxicity remains controversial and at best unclear. Therefore, we investigated the modulatory role of AR on acrolein-induced cell death using primary human small airway epithelial cells (SAECs). Our hypothesis is that AR is the mediator of low-dose acrolein-induced cytotoxicity and thus AR inhibition could prevent acrolein-induced inflammatory signals and apoptosis in airway epithelial cells.

Our results show that inhibition of AR by fidarestat prevents acrolein-induced increased ROS levels, nuclear condensation and DNA degradation, imbalance in the translocation of pro- and antiapoptotic proteins, and phosphorylation and activation of stress kinases that lead to an increase in SAECs apoptosis.

Material and methods

Reagents

Small airway epithelial basal medium (SABM), small airway epithelial growth medium (SAGM Bullet Kit); and Reagentpack containing trypsin 0.025%/EDTA 0.01%, trypsin neutralizing solution, and Hepes-buffered saline solution were purchased from Lonza Walkersville (Walkersville, MD, USA). The AR inhibitor fidarestat was obtained as gift from Sanwa Kagaku Kenkyyusho Co. (Japan) and Livwell (USA). Acrolein was purchased from Sigma–Aldrich (St Louis, MO, USA). Antibodies against Bad, Bax, Bcl2, Bcl_{XL}, cytochrome *c*, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), cytochrome *c* oxidase unit IV (COX IV), PARP, and caspase-3 and those against phospho-JNK, phospho-extracellular signal-regulated kinases 1 and 2 (ERK1/2), phospho-p38, phospho-c-Jun, JNK, ERK1/2, p38, and c-Jun were obtained from Cell Signaling Technology (Danvers, MA, USA). Dihydroethidium (DHE) fluorescent dye was purchased from Molecular Probes/Invitrogen (Carlsbad, CA, USA). The CometAssay kit was purchased from Trevigen (Gaithersburg, MD, USA). The reagents and supplies used in the Western blot analysis were obtained from Bio-Rad (Hercules, CA, USA). All other reagents used were of analytical grade.

Cell culture

Primary human SAECs were obtained from Lonza Walkersville. The cells were cultured at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2 according to the supplier's instructions and used between passages 3 and 6. The cells were cultured in SABM as described [30].

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The SAECs were plated in a 96-well plate at the density of 5000 cells/well and growth-arrested for 24 h by replacing complete medium with fresh basal medium containing the AR inhibitor fidarestat (10 μM) or carrier (phosphate-buffered saline (PBS)). The cells were incubated with various concentrations of acrolein (0, 5, 10, 15, and 20 μM) for 48 h or with 5 μM acrolein for various time periods (0, 12, 24, 48, and 72 h) unless otherwise indicated. At the end of incubation 10 μl of MTT (5 mg/ml) was added to each well and incubated at 37 °C for 2 h. The medium was removed and 100% dimethyl sulfoxide was added to each well to dissolve the formazan granules. Absorbance was measured at 570 nm using a 96-well plate reader. Results are expressed as mean absorbance ± SD ($n=4$).

Detection of ROS

Approximately 1×10^5 SAECs were seeded on chambered slides and starved in serum-free SABM with or without fidarestat for 24 h. The cells were washed with Hanks' balanced salt solution (HBSS) and treated with acrolein (5 μM) for 2 h. SAECs were rinsed with HBSS and incubated with DHE (2.5 μM) at 37 °C for 15 min. Subsequently the cells were rinsed in HBSS and mounted with 4′,6-diamidino-2-phenylindole (DAPI)-containing mounting medium (Vector Laboratories, Burlingame, CA, USA). The cells were observed and photomicrographs were acquired with an Olympus camera fitted on an epifluorescence microscope with a 585-nm long-pass filter. The red fluorescence, indicative of superoxide in the cells, was quantified using AlphaEaseFC software of AlphaImager 2200 (Alpha Innotech Corp., Randburg, South Africa) and shown as a bar diagram ($n=3$). The DAPI staining was used to depict cell nuclei, which appear blue.

Determination of apoptosis by nuclear condensation assay

The SAECs were grown to 75–80% confluency in two-chambered slides and growth-arrested in 0.1% serum medium without or with the AR inhibitor fidarestat. After 24 h, acrolein (5 μM) without or with fidarestat was added to the basal medium and the cells were incubated for another 24 h. At the end of incubation, the cells were washed with cold HBSS and mounted with mounting medium containing DAPI (Vector Laboratories). The cells were subsequently observed and photomicrographs were acquired with an Olympus camera fitted on epifluorescence microscope. The cells with fragmented and/or condensed nuclei were classified as apoptotic cells and quantified by manual counting per viewing area. The mean values ± SD from each group ($n=3$) are presented as a bar diagram.