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Original Contribution

Peroxynitrite-mediated glyoxalase I epigenetic inhibition drives apoptosis in airway epithelial cells exposed to crystalline silica via a novel mechanism involving argpyrimidine-modified Hsp70, JNK, and NF-κB

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ABSTRACT

Glyoxalase I (Glo1) is a cellular defense enzyme involved in the detoxification of methylglyoxal (MG), a cytotoxic by-product of glycolysis, and MG-derived advanced glycation end products (AGEs). Argpyrimidine (AP), one of the major AGEs coming from MG modification of protein arginines, is a proapoptotic agent. Crystalline silica is a well-known occupational health hazard, responsible for a relevant number of pulmonary diseases. Exposure of cells to crystalline silica results in a number of complex biological responses, including apoptosis. The present study was aimed at investigating whether, and through which mechanism, Glo1 was involved in Min-U-Sil 5 crystalline silica-induced apoptosis. Apoptosis, by TdT-mediated dUTP nick-end labeling assay, and transcript and protein levels or enzymatic activity, by quantitative real-time PCR, Western blot, and spectrophotometric methods, respectively, were evaluated in human bronchial BEAS-2B cells exposed or not (control) to crystalline silica and also in experiments with appropriate inhibitors. Reactive oxygen species were evaluated by coumarin-7-boronic acid or Amplex red hydrogen peroxide/peroxidase methods for peroxynitrite ($ONOO^-$) or hydrogen peroxide (H_2O_2) measurements, respectively. Our results showed that Min-U-Sil 5 crystalline silica induced a dramatic $ONOO^-$-mediated inhibition of Glo1, leading to AP-modified Hsp70 protein accumulation that, in a mechanism involving JNK and NF-κB, triggered an apoptotic mitochondrial pathway. Inhibition of Glo1 occurred at both functional and transcriptional levels, the latter occurring via ERK1/2 MAPK and miRNA 101 involvement. Taken together, our data demonstrate that Glo1 is involved in the Min-U-Sil 5 crystalline silica-induced BEAS-2B cell mitochondrial apoptotic pathway via a novel mechanism involving Hsp70, JNK, and NF-κB. Because maintenance of an intact respiratory epithelium is a critically important determinant of normal respiratory function, the knowledge of the mechanisms underlying its disruption may provide insight into the genesis, and possibly the prevention, of a number of pathological conditions commonly occurring in silica dust occupational exposure.

Introduction

Glyoxalase I (EC 4.4.1.5) is a ubiquitously expressed cytosolic enzyme that catalyzes the conversion of methylglyoxal (MG) into the corresponding α-hydroxyacid, using reduced glutathione (GSH) as a cofactor. In particular, Glo1 catalyzes the formation of *S*-D-lactoylglutathione from the hemithioacetal that is formed through the nonenzymatic conjugation of MG and GSH. MG, mainly generated as a by-product of glycolysis [1], is a highly reactive dicarbonyl compound and one of the most powerful glycating agents. It specifically reacts with arginine, lysine, and cysteine residues of proteins, nucleic acids, and lipids [1], generating stable advanced glycation end products (AGEs). The reaction of MG with protein arginine residues generates stable MG moieties, called argpyrimidine (AP) [2]. Despite being a physiological compound, MG, either directly or, more frequently, through AGE formation, is a potent cytotoxic agent, mainly via apoptosis induction, that occurs through multiple mechanisms, including the accumulation of AP [2–4]. As is known, apoptosis can be induced or

Abbreviations: Glo1, glyoxalase I; MG, methylglyoxal; AGE, advanced glycation end product; AP, argpyrimidine; ROS, reactive oxygen species; RNS, reactive nitrogen species; miRNA, micro-RNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; L-NAME, l-ω-nitroarginine methyl ester; AG, aminoguanidine bicarbonate; CBA, coumarin-7-boronic acid; mTempol, Mito-TEMPO-H; MnTBAP, [tetrakis(4-carboxylatophenyl)porphyrinato]manganese(III) chloride.

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mediated by reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) [5,6]. However, despite active research, the molecular mechanisms underlying ROS/RNS involvement in apoptosis induction are still far from being completely understood, because these reactive species can induce a vast array of physiological and pathological effects often dependent on the extent, timing, and location of their production [7].

Several lines of evidence suggest that microRNAs (miRNAs) are novel factors through which ROS regulate apoptosis [8,9]. MiRNAs can be modified structurally by ROS and RNS either when produced in cells, as a consequence of aerobic metabolism, or when induced by environmental factors [10]. As is known, miRNAs comprise a large family of noncoding single-stranded RNA molecules whose major function is to negatively regulate gene expression at the posttranscriptional level [11]. In mammals, miRNAs are predicted to control the activity of 30–50% of all protein-coding genes, which makes their biogenesis and function a serious player in cell fate decisions [12,13].

Crystalline silica is a well-known occupational health hazard, responsible for a relevant number of pulmonary diseases such as silicosis, chronic bronchitis, and lung cancer [14,15]. Substantial evidence exists that among the mechanisms in the genesis of the aforementioned pathologies, apoptosis plays an important role [16,17]. It has been largely described, in fact, that crystalline silica is a potent inducer of such programmed cell death in various types of cells [18–21] and that such an apoptogenic function can occur, through oxygen- and nitrogen-based free radicals, in either in vitro or in vivo models [14,15,22]. However, much still remains to be explored about the molecular events and signaling pathways involved in the apoptosis induced by ROS/RNS after crystalline silica exposure. Emerging evidence suggests that miRNAs also may play an important role, as shown in the altered miRNA expression profiling observed in experimental silicosis rats [23]. Hence, the mechanisms by which crystalline silica exposure induces ROS/RNS-dependent apoptosis remain an understudied area that is of obvious relevance to silica-induced lung diseases. The elucidation of such mechanistic events may, in fact, provide a rationale for developing prevention or amelioration strategies in crystalline silica exposure-related diseases. In particular, such strategies are urgently needed for silicosis, one of the most important occupational diseases worldwide [24], progressing even after the end of occupational exposure, for which no curative treatments exist.

In general, the primary responses of the pulmonary epithelium are believed to play a key role in the onset and development of crystalline silica-induced lung diseases, along with those of the alveolar macrophages [14]. However, considerable attention should be paid also to the bronchial cells lining the airway epithelium, which, earlier than pulmonary cells or macrophages, come into contact with respirable silica particles and whose maintenance in an intact state is a critically important determinant of normal respiratory function. So far, very few data are available on silica-induced apoptosis in human bronchial cells [18]. In the present study, we wanted to investigate whether, and through which mechanism, the antiglycation enzymatic defense protein Glo1 might play a role among the molecular events involved in the apoptosis induced by ROS/RNS after crystalline silica exposure, possibly with the involvement of miRNAs, in BEAS-2B human bronchial epithelial cells. To this aim cells were exposed to crystalline silica Min-U-Sil 5 particles and the induced biological effects, as well as the related molecular mechanisms, were studied after an acute exposure. We previously correlatively described Glo1 involvement in the cytotoxicity induced by crystalline silica Min-U-Sil 5 exposure in human bronchial BEAS-2B cells [25] and, more recently, described a role for this enzyme in the apoptosis induced by wood dust exposure in the same cell model [4]. Therefore, to further investigate whether and how Min-U-Sil 5-induced cytotoxicity occurs via Glo1 modulation-dependent apoptosis, we used the same cell model here. In addition, BEAS-2B cells represent the normal cell model employed in a large part of in vitro studies on environmental exposures [26–30], because they are derived from the bronchial epithelium, the first site of in vivo injury [31], and retain the capability of fiber phagocytosis [32].

Material and methods

Materials

The commercially available crystalline silica Min-U-Sil 5 ground silica was kindly provided by U.S. Silica Co. (Berkeley Springs, MD, USA) [33]. Reagents included MTT, L-NAME, uric acid, AG, FeTPPS, U-0126, and (+)-catechin hydrate (Sigma–Aldrich, Milan, Italy); Lipofectamine and Laemmli buffer (Invitrogen, Milan, Italy); CBA (Cayman Chemical, Rome, Italy); mTempol and MnTBAP (Enzo Life Sciences, Vinci, Florence, Italy); transfection-ready plasmids pCMV-Glo1 and control DNA plasmid pCMV-GFP (OriGene, Rockville, MD, USA); Roti-Block (Roth, Germany); BCA kit (Pierce, USA); and BAY 11-7082 (Santa Cruz Biotechnology, Heidelberg, Germany). The antibodies used in this study included mouse anti-Glo1 monoclonal antibody (mAb; BioMac, Leipzig, Germany); mouse anti-AP mAb (Antibodies-Online, Aachen, Germany); rabbit anti-Bcl-XL polyclonal Ab, rabbit anti-Bax (N20) polyclonal Ab, mouse inhibitor of caspase-activated DNase (ICAD) mAb (Santa Cruz Biotechnology); rabbit anti-phospho-IκBα (Ser32) (14D4) mAb, rabbit anti-caspase-3 polyclonal Ab, mouse anti-human cytokeratin-18 (CK-18)-Asp396 mAb (M30 CytoDEATH) (Alexis Biochemicals, Florence, Italy); mouse anti-Bcl-2 mAb (DAKO, Milan, Italy); mouse anti-cytochrome *c* (Cyt *c*) mAb, mouse anti-Apaf-1 mAb (clone 24), phospho-c-Jun N-terminal kinase (JNK) (clone 41) (BD Pharmingen, Milan, Italy); mouse anti-Cyt *c* oxidase subunit IV (Cox IV) mAb (Molecular Probes, Monza, Italy); phospho-p44/42 mitogen-activated protein kinase (MAPK) (aka extracellular signal-regulated kinase 1/2 (ERK1/2)) (Thr202/Tyr204) mAb, rabbit cleaved ICAD mAb (Cell Signaling Technology, Milan, Italy); and mouse anti-SOD2 mAb (Abcam, Milan, Italy).

Cell culture and Min-U-Sil 5 particles exposure

Human bronchial epithelial cells (BEAS-2B) were obtained from the American Type Culture Collection (Manassas, VA, USA). BEAS-2B were cultured at the appropriate number in serum-free bronchial epithelial cell basal medium (BEBM) (Clonetics, San Diego, CA, USA) supplemented with epidermal growth factor (0.5 ng/ml), insulin (5 μg/ml), hydrocortisone (0.5 μg/ml), transferrin (10 μg/ml), epinephrine (0.5 μg/ml), tri-iodothyronine (6.5 ng/ml), bovine pituitary extract, retinoic acid (0.1 ng/ml), gentamicin (50 μg/ml), and amphotericin-B (50 μg/ml). Cells were incubated in a humidified atmosphere at 37 °C with 5% CO_2. Subconfluent cells [34] were treated with crystalline silica Min-U-Sil 5, prepared as previously described [25], at the final concentrations of 5, 10, 60, and 100 μg/cm^2 for 6, 12, and 24 h. Because 100 μg/cm^2 silica 24 h postexposure yielded the major studied biological effects, mechanistic studies were carried out under such experimental conditions. Untreated cells, serving as controls, were incubated for the same time periods. Independent experiments were also performed by pretreating cells with the appropriate inhibitors or scavenging agents and then treating under the conditions described above. These reagents were used at concentrations producing no significant toxicity to BEAS-2B cells. Control cells for the experiments with nonaqueous-medium-dissolved agents did not show any significant difference with respect to control