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Acrolein activates cell survival and apoptotic death responses involving the endoplasmic reticulum in A549 lung cells

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ABSTRACT

Acrolein, a highly reactive α,β-unsaturated aldehyde, is a product of endogenous lipid peroxidation. It is a ubiquitous environmental pollutant that is generated mainly by smoke, overheated cooking oil and vehicle exhaust. Acrolein damages cellular proteins, which could lead to accumulation of aberrantly-folded proteins in the endoplasmic reticulum (ER). This study determines the mechanisms involved in acrolein-induced apoptosis mediated by the ER and possible links with the ER stress response in human A549 lung cells. The exposure of cells to acrolein (15–50 μM) for shorter times of 15 to 30 min activated several ER stress markers. These included the ER chaperone protein BiP and the three ER sensors: (i) the survival/rescue molecules protein kinase RNA (PKR)-like ER kinase (PERK) and eukaryotic initiation factor 2 alpha (eIF2α) were phosphorylated; (ii) cleavage of activating transcription factor 6 (ATF6) occurred, and (iii) inositol-requiring protein-1 alpha (IRE1α) was phosphorylated. Acrolein (25–50 μM) caused apoptotic cell death mediated by the ER after 2 h, which was characterised by the induction of CHOP and activation of ER proteases calpain and caspase-4. Calpain and caspase-7 were the initiating factors for caspase-4 activation in acrolein-induced apoptosis. These results increase our knowledge about cellular responses to acrolein in lung cells, which have implications for human health.

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1. Introduction

Acrolein is a highly reactive, electrophilic α,β-unsaturated aldehyde to which humans are exposed in environmental and industrial settings, particularly as a component of smoke from house and forest fires, cigarettes, cooking oil, smog and automobile exhaust [1,2]. It is also among the toxic by-products of lipid peroxidation [3,4]. Acrolein has been found in water and several foods, and levels are increased further by cooking. Aldehydes such as acrolein, formaldehyde and acetaldehyde have been identified among the most hazardous toxicants in cigarette smoke [5]. Given that inhalation is the major route of exposure, acrolein has been associated with acute lung injury, chronic obstructive pulmonary disease (COPD), asthma, and lung carcinogenesis [6]. Several studies suggest that acrolein could be involved in Alzheimer's [7–9], Parkinson's [10], atherosclerosis and cardiovascular diseases [11–13], diabetes [14,15] and bladder cancers [16].

The high reactivity of acrolein makes it a dangerous substance for living cells. Acrolein forms adducts with cellular targets such as proteins and DNA, and disrupts multiple biochemical pathways [1]. Moreover, acrolein increased the production of reactive oxygen species (ROS) in human cell types such as A549 [17], macrophages [18], IMR fibroblasts [19] and HepG2 [20]. Together, these events can lead to cell death by apoptosis and/or necrosis [21–24], which is dependent on thiol availability [25], dose (exposure time, concentration) of acrolein, cell type and cellular environment [1,2,26,27].

The chronic exposure to low doses of toxic compounds often leads to induction of adaptive survival responses, which allow cells and organisms to survive a toxic insult [28,29]. Adaptive responses usually involve changes in gene and protein expression. Organisms have developed elaborate defences against toxic stresses. These include antioxidants, heat shock proteins (HSP), anti-apoptosis proteins (IAPs), and cell survival signalling pathways involving phosphatidylinositol 3-kinase (PI3K)/AKT, Ras/mitogen-activated protein kinases (MAPKs), ER stress and autophagy [30]. If the level of adverse stimulus is low, cells attempt

Abbreviations: AFC, amino trifluorocoumarin; AKT, protein kinase B; AMC, amino methylcoumarin; ARE, antioxidant response element; ATF6, activating transcription factor-6; BAPTA-AM, 1,2-Bis(2-aminophenoxy)ethane-N,N,N,N-tetraacetic acid tetrakis (acetoxymethyl ester); Bax, Bcl-associated X protein; Bcl-2, B-cells lymphoma 2; Bim, Bcl-2-interacting mediator of cell death; cIAP, cellular inhibitors of apoptosis; COPD, chronic obstructive pulmonary disease; EDTA, ethylene diamine tetraacetic acid; eIF2α, eukaryotic initiation factor 2 alpha; ER, endoplasmic reticulum; ERAD, ER-associated protein degradation; FACS, fluorescence activated cell sorter; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; FMK, fluoromethyl ketone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Grp, glucose related protein; HO-1, heme oxygenase 1; Hsp, Heat shock protein; IRE1α, inositol-requiring protein-1 alpha; JNK, c-Jun N terminal kinase; MEMα, minimum essential medium alpha; Nrf2, nuclear factor (erythroid-derived 2)-like 2; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; PERK, protein kinase RNA (PKR)-like ER kinase; PI, propidium iodide; PI3K, phosphatidylinositol 3-kinase; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SEM, Standard error of mean; UPR, unfolded protein response

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to survive by activating stress responses that protect crucial biochemical functions such as the elimination of damaged proteins and repair of DNA damage. When an adverse stimulus is too severe or prolonged, the defences become overwhelmed and cells are eliminated by processes such as apoptosis and/or necrosis.

The endoplasmic reticulum (ER) is a key organelle where newly synthesized proteins form their correct tertiary structure by post-translational modification, folding and oligomerization [31]. When the folding capacity of the ER is exceeded, misfolded proteins accumulate and cause ER stress. The unfolded protein response (UPR) is an adaptive mechanism that cells use to mitigate ER stress and to restore homeostasis [31]. Eukaryotic cells possess at least three different mechanisms to adapt to ER stress and attempt to survive: (1) translational attenuation to limit further accumulation of misfolded proteins through the protein kinase-like ER resident kinase (PERK) and eukaryotic initiation factor 2 alpha (eIF2α)-mediated pathway; (2) activation of transcription factor 6 (ATF6) and genes encoding ER-resident chaperones such as glucose-regulated protein-78 (Grp78)/immunoglobulin binding protein (BiP); and (3) ER-associated degradation (ERAD), which reduces the stress by directing misfolded ER proteins into the cytosol for degradation by the 26S proteasome. The ERAD pathway is mediated by activation of inositol-requiring enzyme 1 alpha (IRE1α), which is triggered by the dissociation of BiP from the ER luminal domain of IRE1α. If these compensatory mechanisms fail to facilitate the adaptation of cells to ER stress, induction of the UPR can lead to apoptosis [32].

Acrolein causes protein damage [1,2,33,34], which could lead to accumulation of misfolded proteins in the ER and induce ER stress. The A549 cell line was selected for this study due to its human pulmonary origin because the major route of exposure to acrolein is through inhalation. This study aims to advance our knowledge about this toxic compound and investigates whether acrolein can activate the ER stress response and/or apoptosis mediated by the ER. The improved understanding of acrolein-induced cellular responses is important given the widespread chronic exposure of humans to acrolein and the potential for adverse effects on human health.

2. Material and methods

2.1. Cell culture

A549 cells (#CCL-185, ATCC) were grown in Dulbecco's modified Eagle's medium (Invitrogen Canada, Burlington, ON, Canada) containing 10% fetal bovine serum (FBS) (Invitrogen Canada), 2 mM L-glutamine, 3.7 g/l sodium bicarbonate and 1 mM sodium pyruvate in tissue culture flasks (Sarstedt, St-Laurent, QC, Canada) [17]. Near confluent cells were harvested using 0.25% (w/v) trypsin–0.02% (w/v) EDTA, centrifuged (1000 *g*, 3 min) and resuspended in minimum essential medium alpha (MEMα) containing 10% FBS at pH 7.4.

2.2. Treatment with acrolein

A549 cells (10^6/ml) were incubated with acrolein (15–50 μM) (Sigma-Aldrich, Canada, ON, Canada) for different times from 15 min to 2 h at 37 °C, relative to untreated controls. Where indicated, cells were pretreated for 1 h with 50 μM calcium chelator BAPTA-AM (Sigma-Aldrich), 20 μM calpain inhibitor I (Ac-LLnL-CHO) (Sigma-Aldrich), 50 μM caspase-7/3 inhibitor I (5-[(S)-(+)-2-(methoxymethyl) pyrrolidino]sulfonylisatin) (Calbiochem) or 20 μM caspase-4 inhibitor (LEVD-CHO). Cells were washed by centrifugation (1000 ×*g*, 3 min) to remove acrolein/inhibitors.

2.3. Western blotting

Whole cell lysates were prepared using washing buffer A (100 mM sucrose, 1 mM EGTA, 20 mM MOPS, pH 7.4) and lysis buffer B [buffer A plus 5% Percoll, 0.01% digitonin and cocktail of protease inhibitors [17,35]. Proteins (40 μg) were quantified [36], separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (10% for PERK, p-PERK, IRE1α, p-IRE1α, BiP, calpastatin; 15% for eIF2α, p-eIF2α, ATF6, CHOP; 8% for PARP) [37], and transferred to a polyvinylidene difluoride membrane using a MilliBlot Graphite Electroblotter I apparatus (Millipore, Bedford, MA, USA) [19]. The blots were probed with primary antibodies to ER proteins and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and then with horseradish peroxidase (HRP)-conjugated anti-mouse, anti-rabbit, or anti-goat IgG (Biosource, Camarillo, CA, USA). Protein levels were analysed using a scanning laser densitometer (Molecular Dynamics, Sunnyvale, CA, USA) relative to GAPDH [17].

2.4. Caspase and calpain enzymatic activities

Protease activities were detected by fluorescence spectroscopy using a Quadruple Monochromator Microplate Reader (Infinite M1000, Tecan US, NC, USA) [23]. The following substrates were used: Ac-DEVD-AMC for caspase-3, Ac-LEVD-AFC for caspase-4, MCA-Val-Asp-Gln-Val-Gly-Trp-Lys-(DNP)-NH_2 for caspase-7 and Suc-LY-AMC for calpain (Calbiochem, La Jolla, CA, USA). Enzymatic activities are represented as relative cumulative fluorescence during the 30 min kinetic reaction and compared to untreated controls. The activation of calpain, caspases-3, -4 and -7 was confirmed by detection of their cleavage fragments by Western blotting (data not shown).

2.5. Annexin V-FITC staining

Cells (10 000) were labelled with Annexin V-FITC (FL-1) (BD Biosciences Canada, Mississauga, ON, Canada) and propidium iodide (PI) (FL-3) (Sigma Chemical Co.) and then analysed by flow cytometry using a FACScan equipped with an argon laser (488 nm) (Becton Dickinson, Oxford, UK) [17]. The results are represented as the fraction of total apoptotic cells (early and late stage apoptosis: Annexin V+/PI±) and necrotic (Annexin V−/PI+) cells.

2.6. Hoechst 33258 staining

Nuclear chromatin condensation was analysed using Hoechst 33258 (Sigma) by fluorescence microscopy (Carl Zeiss Canada Ltd., St. Laurent, QC) [23]. Necrotic cells were labelled with PI. Photographs were taken by digital camera (camera 3CCD, Sony DXC-950P, Empix Imaging Inc., Mississauga, ON) and images analysed using Northern Eclipse software. The fractions of apoptotic (blue fragmented chromatin) and necrotic (red fluorescence) cells were determined relative to total cell number. A minimum of 300 cells was counted per sample.

2.7. Statistics

Data represent means ± SEM from at least 3 independent experiments performed in duplicate. Comparisons among multiple groups were made by one-way ANOVA, which measures the linear contrast of means. The Bonferroni–Holmes adjustment was used to control for the Family-wise error rate at a desired level ($\alpha = 5\%$). Software used was JMP Statistical Discovery 4.0 (SAS Institute Inc., Cary, NC).

3. Results

3.1. Acrolein activates the ER stress survival response in A549 lung cells

3.1.1. Acrolein causes phosphorylation of ER stress sensors PERK, eIF2α and IRE1α

The ER stress response is mediated by three membrane receptors, PERK, IRE1α and ATF6. The exposure of cells to acrolein (15–50 μM) caused phosphorylation of PERK after 15–30 min (Fig. 1A, B). Acrolein did not alter total cellular expression of PERK (Fig. 1C, D). Activated PERK phosphorylates eIF2α, thus attenuating translation to reduce the

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