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Adaptation to acrolein through upregulating the protection by glutathione in human bronchial epithelial cells: The materialization of the hormesis concept

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ABSTRACT

Acrolein is a thiol reactive compound present in cigarette smoke and plays a pivotal role in the deleterious effects of smoking. Acrolein causes toxicity in human bronchial epithelial cells in a dose dependent manner. GSH forms the first line of defense against acrolein-induced toxicity. At high doses of acrolein ($\geqslant$10 μM) the capacity of the cellular protection by GSH is overwhelmed and GSH is not able to quench all the acrolein, resulting in cytotoxicity.

At a relatively low dose of acrolein (3 μM), no cytotoxicity is observed due to protection by GSH. Moreover we found that exposure to a low dose of acrolein protects cells against the toxic effect of a second higher dose of acrolein. The adaptation to acrolein is induced via Nrf2 mediated gene expression of γ-glutamylcysteine synthetase leading to elevated GSH levels. This upregulation of the protection by GSH demonstrates a hormetic response to acrolein.

Hormesis is an adaptive or compensatory response induced by a relatively subtle challenge of homeostasis by a toxic compound. Insight into the mechanism of hormesis is mandatory for a more accurate societal regulation of toxic compounds.

1. Introduction

The human body is continuously exposed to a wide variety of compounds present in the environment that can have toxic effects at a relatively low dose, such as polycyclic aromatic hydrocarbons, aldehydes and metals [1]. These exogenous compounds can be inhaled by breathing polluted air, ingested by consuming food or contaminated water or absorbed via the skin. A relatively high dose of an exogenous compound results in damage to vital macromolecules including critical proteins and DNA, causing toxicity. This toxicity has been implicated in the etiology of cardiovascular, respiratory and neurodegenerative diseases [2]. In the attempt to prevent disease formation, governmental agencies regulate upper limits at which humans can safely be exposed.

The continuous exposure to toxicants seems to render us as relatively resistant to these substances, which evidences our ability to adapt. The extent of the ability to adapt defines the status of human health [3]. The phenomenon of inducing an adaptive or compensatory response by a relatively mild challenge on homeostasis is called hormesis [4]. To actually examine the concept of hormesis, we have selected acrolein as an exogenous compound. Acrolein, an α,β-unsaturated aldehyde, is implicated in the toxicity of cigarette smoke, traffic exhaust and other air pollution [5]. Acrolein can also directly induce cellular damage [6] due to its intrinsic reactivity [7,8]. According to the Hard Soft Acid Base concept acrolein is a soft electrophile that prefers to react to soft nucleophiles such as thiols [9]. The high thiol reactivity of acrolein results in Michael adducts of acrolein on cysteine residues of critical cellular proteins which can elicit cell toxicity [7]. Acrolein can be considered as a toxicophore, a characteristic moiety within molecules responsible for their toxic properties, and is therefore a model for electrophilic xenobiotics that display toxicity as a direct result of their electrophilicity, e.g. NAPQI [10]. In this study the concept of hormesis is investigated on a cellular level by assessing the adaptive response to acrolein in bronchial epithelial cells.

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2. Materials and methods

2.1. Chemical reaction acrolein and GSH

The amount of acrolein (Sigma–Aldrich, St. Louis, MO, USA) reacting with reduced glutathione (GSH) (Sigma–Aldrich, St. Louis, MO, USA) was determined by adding 0, 1, 3, 6, 9, 10 and 12 μM acrolein to 10 μM GSH at 37 °C. The amount of acrolein that has reacted with GSH is indirectly measured by quantifying the amount of GSH that has not reacted with acrolein. A concentration of 120 μM of DTNB (Sigma–Aldrich, St. Louis, MO, USA) is added and the absorbance is spectrophotometrically determined at a wavelength of 412 nm.

2.2. Cell culture

Adenovirus-12 SV40 hybrid virus transformed, non-tumorigenic human bronchial epithelial cells (BEAS-2B; ATCC, Manassas, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM/F-12; Gibco, Bleiswijk, The Netherlands) supplemented with 50 U/ml penicillin (Gibco, Bleiswijk, The Netherlands), 50 μg/ml streptomycin (Gibco, Bleiswijk, The Netherlands), 15 μg/ml bovine pituitary extract, 0.5 mg/ml bovine serum albumin (Invitrogen, Breda, The Netherlands), 10 ng/ml cholera-toxin (List Biological Laboratories, Inc., Campbell, California), 10 ng/ml epidermal growth factor (Merck Millipore, Darmstadt, Germany), 5 μg/ml insulin, 5 μg/ml transferrin and 0.1 μM dexamethasone (Sigma–Aldrich, St. Louis, MO, USA) in an environment containing 95% O_2 and 5% CO_2 at 37 °C. The maximal passage used in this experiment was 10.

2.3. Treatments

Cells were seeded in 6-well plates at a density of 400,000 cells per well and cultured overnight in 95% O_2 and 5% CO_2 at 37 °C. Treatments with acrolein were made in Hank's Balanced Salt Solution (HBSS; Gibco, Bleiswijk, The Netherlands). Cells were exposed to 0, 1, 3, 10, 30, 100 and 200 μM acrolein for 30 min and lysed immediately to determine GSH levels. To determine cellular toxicity HBSS was replaced with medium and 24 h post acrolein exposure medium was collected to measure lactate dehydrogenase (LDH) activity of the released LDH. In addition, cells were treated with 3 μM and 10 μM of acrolein and GSH levels were determined 4 and 6 h after exposure (Supplementary Fig. 1). Furthermore, 4 and 8 h after exposure RNA was isolated to determine gamma-glutamylcysteine synthetase (γGCS) expression (Supplementary Fig. 1). Finally, 3 μM of acrolein was added to the cells 4 h prior to 10 μM of acrolein treatment and GSH levels and cellular toxicity were measured (Supplementary Fig. 1).

2.4. Detection of cytotoxicity

LDH activity was measured by transferring 50 μl of the collected medium to a 96-well plate. A solution of 100 mM sodium pyruvate in 500 mM potassium phosphate buffer, pH 7.5 was prepared. After addition of 50 μl of 0.25 mg/ml NADH in sodium pyruvate solution, the change in absorbance at a wavelength of 340 nm was determined over 4 min using a Spectramax plate reader (SpectraMax M2 & M2e Multi-Mode Microplate Reader, Sunnyvale, United States). LDH activity of the samples was compared to the LDH activity measured after LDH leakage due to cell lysis achieved by addition of 3% Triton-X-100 (Sigma–Aldrich, St. Louis, MO, USA) for 15 min.

2.5. Analysis of cellular GSH levels

Cells were washed with 2 ml 1× Dulbecco's Phosphate Buffered Saline (DPBS; Gibco, Bleiswijk, The Netherlands). The cells were lysed with 500 μl 100 mM potassium phosphate buffer containing 10 mM EDTA disodium salt, pH 7.5 and 1% Triton-X-100 (Sigma–Aldrich, St. Louis, MO, USA). After 30 min of incubation on ice, cells were scraped and then centrifuged for 10 min at 14,000 rpm 4 °C to remove cellular debris. The protein content was measured using the bicinchoninic acid assay (BCA; Pierce, Thermo Fisher Scientific, Etten-Leur, The Netherlands). In addition, 300 μl of remaining supernatant was mixed 1:1 with 6% sulfosalicylic acid (Sigma–Aldrich, St. Louis, MO, USA) and samples were diluted 1:5 in 100 mM potassium phosphate buffer with 10 mM EDTA disodium salt, pH 7.5. Finally, GSH was determined using an enzymatic recycling method as previously described [11].

2.6. Measurement of gamma-glutamylcysteine synthetase expression

First cells were lysed using Qiazol (Qiagen, Venlo, The Netherlands). Second, 200 μl chloroform (Sigma–Aldrich, St. Louis, MO, USA) per ml of Qiazol was added to perform a phase separation. After incubation for 2–3 min at 20 °C and centrifugation for 15 min at 12,000*g* 4 °C, the colorless aqueous upper phase containing RNA was removed and added to 500 μl isopropanol per ml of Qiazol to allow the RNA to precipitate. The cells incubated at 4 °C overnight and after centrifugation the supernatant was removed. The pellet was washed with 1 ml 75% ethanol per ml of Qiazol. After centrifugation at 7500*g* for 5 min at 4 °C, the supernatant was removed and the pellet was allowed to dry for an hour. Hereafter, the pellet was resuspended in 50 μl of RNAse/DNAse free water and incubated for 10 min at 60 °C. The quantity of RNA was determined using the nanodrop (thermo scientific nanodrop 1000 spectrophotometer, isogen life science, De Meern, The Netherlands). Five hundred nanograms of RNA was converted into complementary DNA (cDNA) by using iScript cDNA synthesis kit (Biorad, Veenendaal, The Netherlands). Sensimix SYBR & Fluorescein kit (Bioline, Alphen aan de Rijn, The Netherlands) was used to perform quantitative RT-PCR measuring gamma-glutamylcysteine synthetase (γGCS; sense: 5′-GCACATCTACCACGCCGTC-3′ and antisense: 5′-CCACCTCATCGCCCCAC-3'). β-actin (β-actin; sense: 5′-CCTGGCACCCAGCACAAT-3′ and antisense: 5′-GCCGATCC ACACGGAGTACT-3′) was applied as housekeeping gene. Finally, the $2^{-\Delta\Delta CT}$ method was used to determine relative γGCS gene expression [12].

2.7. Statistics

All data are shown as mean ± SEM. A *t* test was performed for independent samples with equal variances to assess statistical significance between two individual groups. A $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. The reactivity of acrolein towards GSH

To investigate the reaction of acrolein towards GSH, various amounts of acrolein were added to a fixed amount of GSH (initial concentration of 10 μM in a volume of 1 ml) in a test tube. This revealed that acrolein reacts within seconds with GSH in stoichiometry of 1:1 (Fig. 1).

3.2. Acrolein depletes GSH in the cell

To investigate whether acrolein is quenched by intracellular GSH similarly as in the test tube, BEAS-2B cells were treated with acrolein (1, 3, 10 and 30 μM), and GSH was measured. Acrolein dose-dependently reduced intracellular GSH in BEAS-2B cells