Sulforaphane protects against acrolein-induced oxidative stress and inflammatory responses: modulation of Nrf-2 and COX-2 expression

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Abstract

Introduction: Acrolein (2-propenal) is a reactive α, β-unsaturated aldehyde which causes a health hazard to humans. The present study focused on determining the protection offered by sulforaphane against acrolein-induced damage in peripheral blood mononuclear cells (PBMC).

Material and methods: Acrolein-induced oxidative stress was determined through evaluating the levels of reactive oxygen species, protein carbonyl and sulfhydryl content, thiobarbituric acid reactive species, total oxidant status and antioxidant status (total antioxidant capacity, glutathione, superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase activity). Also, Nrf-2 expression levels were determined using western blot analysis. Acrolein-induced inflammation was determined through analyzing expression of cyclooxygenase-2 by western blot and PGE2 levels by ELISA. The protection offered by sulforaphane against acrolein-induced oxidative stress and inflammation was studied.

Results: Acrolein showed a significant ($p < 0.001$) increase in the levels of oxidative stress parameters and down-regulated Nrf-2 expression. Acrolein-induced inflammation was observed through upregulation ($p < 0.001$) of COX-2 and PGE2 levels. Pretreatment with sulforaphane enhanced the antioxidant status through upregulating Nrf-2 expression ($p < 0.001$) in PBMC. Acrolein-induced inflammation was significantly inhibited through suppression of COX-2 ($p < 0.001$) and PGE2 levels ($p < 0.001$).

Conclusions: The present study provides clear evidence that pre-treatment with sulforaphane completely restored the antioxidant status and prevented inflammatory responses mediated by acrolein. Thus the protection offered by sulforaphane against acrolein-induced damage in PBMC is attributed to its anti-oxidant and anti-inflammatory potential.

Key words: oxidative stress, acrolein, sulforaphane, inflammation.

Introduction

Acrolein (2-propenal-1-al) is a highly reactive ubiquitous environmental pollutant. Its exposure to humans occurs through multiple entries, including food, petroleum fuels, biodiesel and smoking tobacco products [1, 2]. The maximum human exposure results from smoking tobacco products. Acrolein is formed as a byproduct during heating and combustion of food and petroleum products respectively. Chemical reactions involved in acrolein production include heat-induced dehydration of glycerol, retro-aldol cleavage of dehydrated carbohydrates, lipid peroxidation...
Acrolein exposure results in various health hazards which are primarily mediated through oxidative stress and inflammation. However, the end result leads to cellular damage which occurs through apoptosis/necrosis. Acrolein-induced cellular damage mediates disease conditions such as chronic obstructive pulmonary disease, atherosclerosis, and Alzheimer’s disease [4–6]. Acrolein-induced cellular damage has been shown to be mediated through apoptosis in various cells, including neutrophils [7], neurons [6], Chinese hamster ovary cells [8], and hepatocytes [9]. Cellular depletion of glutathione is one of the well-established mechanisms by which acrolein induces toxic effects [10, 11]. It is known that acrolein is metabolized through sulfhydryl groups of glutathione forming 3-hydroxypropyl mercapturic acid which is excreted through urine [1]. In addition, circulating levels of acrolein in the bloodstream cause oxidative damage to the endothelial cells which ends in damage to the vascular wall. There are not many reports on acrolein-induced effects on peripheral blood mononuclear cells (PBMC). The main objective of the present study was to investigate the oxidative stress and cell death induced by acrolein on PBMC.

Since oxidative stress is mainly mediated through a reduction in the cellular antioxidant pool, the possible protective effect against acrolein-induced toxicity can be mediated by therapeutic intervention with antioxidants. Sulforaphane is an excellent antioxidant which is abundantly present in cruciferous vegetables [12]. Abundant data suggest the multiple potential of sulforaphane in different disease conditions including inhibition of phase II drug metabolizing enzyme, suppression of cell cycle progression and angiogenesis, apoptosis induction and anti-inflammatory properties. It has been well studied for its chemopreventive and protective properties, which are mainly attributed to its nuclear factor erythroid 2-related factor 2 (Nrf-2) induction. Nrf-2 is a redox sensitive transcription factor activated by antioxidants and upregulation of various phase II antioxidant enzymes. Recently Nrf-2 has gained importance as a therapeutic target in cytoprotection against oxidative stress. Sulforaphane is involved in regulating the glutathione levels through upregulating γ-GCS expression [13]. The protective effects of sulforaphane against various disease conditions such as cancer, neurodegenerative disorder, cardiovascular disease and diabetes have been reported previously [14–20]. Since oxidative stress and the downstream effects play a major role in acrolein-induced toxicity, the present study was aimed at investigating the protective effect of sulforaphane on acrolein-induced oxidative stress in human PBMC.

**Material and methods**

**PBMC isolation and treatment**

Peripheral blood mononuclear cells were isolated from whole blood from healthy volunteers using Ficoll-Paque PLUS (GE Healthcare Life Sciences). The study was conducted with the approval of the Henan Provincial People’s Hospital Institutional Review Board for research on human subjects. Informed consent was acquired from all donors, and this study was carried out in compliance with the Declaration of Helsinki for ethical principles of conducting medical research. The blood was collected in EDTA tubes. The blood was diluted in PBS at a 1 : 1 ratio. An equal volume of diluted blood and Ficoll-Hypaque solution was added and centrifuged at 1500 rpm (30 min; RT). After centrifugation, the PBMC layers (found at the interface between the plasma and the Ficoll-Paque Plus solution) were collected and washed twice in RPMI medium. The cells were washed three times with PBS (centrifuged at 1500 rpm for 10 min each). The supernatant was discarded and the pellet was resuspended in PBS and the cell count was carried out by the trypan blue exclusion method in a hemocytometer. The cell viability was observed to be > 95%.

**Cell viability – MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay**

The cells were seeded at a density of $1 \times 10^5$ cells/well in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 U/ml streptomycin. The cells were treated with different concentrations of acrolein (10–60 µM) to determine the cytotoxic effects. The cells were allowed to stand for 24 h after acrolein treatment. After the complete treatment schedule the cells were treated with MTT (4 mg/ml) for 3 h. The formazan crystals were dissolved using DMSO and the absorbance was measured at 540 nm in a spectrophotometer [21]. The IC$_{50}$ value (30 µM) was calculated and used for the further studies.

The further cytoprotective effect of sulforaphane against acrolein-induced toxicity was determined. Cells were pretreated with sulforaphane at a concentration 1, 5 and 10 µM for 24 h followed by acrolein treatment for 24 h. Cells pretreated with 5 and 10 µM showed a better protective effect. So, further studies were carried out...
out with a low concentration of sulforaphane, i.e. 5 µM. Treatment groups for the further studies were: group I – control; group II – sulforaphane (5 µM); group III – acrolein (30 µM); group IV – sulforaphane (5 µM) + acrolein (30 µM).

**Lactate dehydrogenase (LDH) assay**

Lactate dehydrogenase activity was determined using the LDH cytotoxicity kit (Bayer Diagnostics, France). The assay was performed according to the manufacturer’s instructions. The absorbance was measured spectrophotometrically at 520 nm. The results are expressed as % LDH levels compared to the control.

**Reactive species generation (ROS)**

The cells at a density of 2 × 10^5 cells were used. The cells were incubated with 2,7'-dichloroethylfluorescein diacetate (DCF-DA) for 30 min. Cells were treated with acrolein in the presence/absence of sulforaphane. For protective studies cells were treated with sulforaphane for 24 h. Then sulforaphane was removed and treated with acrolein for 24 h. At the end of the treatment schedule, all the treatment groups were centrifuged (1500 rpm, 10 min) and re-suspended in PBS. The fluorescence intensity was measured at (excitation wavelength 480 nm, emission wavelength 520 nm). The results were formulated as % DCF fluorescence by comparing the values to those of control cells [22].

**Lipid peroxidation**

After the treatment schedule the cells from all the groups were sonicated and used for the lipid peroxidation assay. To the cell extract 8% SDS and 0.8% TBA in 20% acetic acid was added. The final volume of the assay was made up with water to 4 ml. The extract was boiled for 60 min at 90°C. After cooling to room temperature, 2 ml of butanol/pyridine mixture was added and shaken vigorously. It was centrifuged at 4000 rpm for 10 min and the organic layer was read at 532 nm. The lipid peroxide content was expressed as nanomoles of TBA reactants/mg of protein [23]. Protein concentration was determined as previously described [24].

**Protein carbonyl and sulfhydryl content**

**Protein carbonyls**

The protein carbonyls formed were measured as described by Dalle-Donne et al. [25]. Briefly, the reaction between carbonyl groups in the sample and 2,4-dinitrophenylhydrazine (DNPH) results in the formation of 2,4-dinitrophenylhydrazone, which is quantified spectrophotometrically at 365 nm. Carbonyl content was calculated using the extinction coefficient of 22 × 10^3 M⁻¹cm⁻¹. The results are expressed as nmoles of protein carbonyls/mg of protein.

**Protein sulfhydryls**

The protein sulfhydryls were determined using Ellman’s reagent (5,5-dithiobis-2-nitrobenzoic acid), and absorbance was measured at 412 nm [26]. Sulfhydryl content was calculated using 13,600 × 10^3 M⁻¹cm⁻¹. The results are expressed as nmoles protein sulfphydryls/mg protein. Protein concentration was determined as previously described [24].

**Total antioxidant capacity (TAC) and total oxidant status (TOS)**

Total antioxidant capacity was determined as described by Erel [27]. The principle involves determination of hydroxyl radical formation between the reactions of antioxidants in the sample against free radicals. The results are expressed as millimoles of Trolox equiv/l. TOS of serum was determined as described by Erel [28]. The basic principle involves oxidation of ferrous ion-o-dianisidine complex to ferric ion by the oxidants. The colored complex formed in the presence of xylene orange is measured spectrophotometrically. The absorbance is directly proportional to the amount of oxidant molecules. The results are expressed as µmol H₂O₂ equiv/l.

**Antioxidant enzymes**

**Glutathione (GSH) content**

The principle involves reaction between 5,5 dithiobis (2-nitrobenzoic acid) (DTNB) and reduced GSH to form a yellow compound which is measured at 405 nm spectrophotometrically (Moren et al., 1979) [29].

**Superoxide dismutase (SOD) activity**

The assay is based on reduction of nitroblue tetrazolium (NBT). Auto-oxidation of pyrogallol was measured by the increase in absorbance (420 nm) at 30-second intervals for 3 min. 1 U of SOD activity = amount required for 50% inhibition of NBT reduction. The SOD activity is expressed as U/mg of protein [30].

**Glutathione-S-transferase (GST) activity**

The reaction between 1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione results in formation of dinitrophenylthioether which is measured at 340 nm for 3 min at 30 s intervals [31]. The enzyme activity was calculated using the extinction coefficient of E340 = 0.0096 µM⁻¹ cm⁻¹ and expressed as units/mg of protein.
1 U = amount of enzyme producing 1 mmol of CDNB-GSH conjugate/min.

**Glutathione peroxidase (GPx) activity**

The GPx activity was determined as described by Pagia and Valentine [32]. The oxidized glutathione (GSSG) is reduced by glutathione reductase and NADPH. The oxidation of NADPH to NADP⁺ is measured by a decrease in absorbance at 340 nm. GPx activity is expressed as U/mg of protein.

**Catalase (CAT) activity**

The reaction mixture contained the sample and 30 mM H₂O₂ in a 50 mM phosphate buffer, pH 7.0. The activity was estimated by the decrease in absorbance of H₂O₂ at 240 nm [33].

**Western blot analysis**

After the appropriate treatment schedule, cells were treated with lysis buffer (50 mM Tris–HCl (pH 7.4), 1 mM NaF, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethanesulfonyl fluoride; 1% NP-40 and 10 mg/ml leupeptin). The tubes were incubated on ice for 30 min, which was followed by centrifugation at 12,000 rpm for 30 min at 4°C. The supernatant containing proteins were aliquoted and analyzed for protein content [24]. The samples containing 50 µg of proteins were separated on 8–12% SDS-polyacrylamide gels and transferred onto polyvinylidene fluoride (PVDF) membranes using glycine transfer buffer (192 mM glycine, 25 mM Tris-HCl (pH 8.8), 20% MeOH (v/v)). Following membrane transfer, it was treated with 5% nonfat dried milk for 1 h at RT for blocking non-specific sites. After membrane wash, the membrane was probed with specific primary mouse monoclonal anti-COX-2 Ab (1 : 1000, BD Biosciences Pharmingen, San Jose, CA, USA) and anti-Nrf-2 (1 : 1000, Calbiochem, La Jolla, CA, USA) at 4°C overnight. The membrane was washed and incubated for 30 min with secondary peroxidase-conjugated anti-rabbit IgG (1 : 5000). The bands were visualized with an enhanced chemiluminescence (ECL) system. Densitometry analyses of the Western blot bands were performed using ImageJ software.

**ELISA**

Prostaglandin E2 (PGE2) levels in the medium were measured using an enzyme linked immunosorbent assay (ab133021, Abcam, USA). A mouse IgG antibody was pre-coated onto 96-well plates. Prostaglandin E2 standards from the manufacturer kit or test samples were added to the wells, along with an alkaline phosphatase (AP) conjugated-prostaglandin E2 antibody. After incubation the excess reagents were washed away and pNpp substrate was added and was catalyzed by AP to produce a yellow color. The intensity of the yellow coloration is inversely proportional to the amount of PGE2 captured on the plate. The PGE2 levels were expressed as ng/mg of protein.

**Statistical analysis**

The statistical analysis was determined using the Statistical Program of Social Sciences (SPSS), version 10.0 (IBM, Armonk, New York, USA) [34]. The data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test.

**Results**

**Protective effect of sulforaphane against acrolein-induced toxicity**

Acrolein-induced toxicity was determined by MTT assay. The results show that acrolein caused dose-dependent cell death in PBMC. The IC₅₀ value was found to be 30 µM (p < 0.01) (Figure 1 A). Cytoprotection offered by sulforaphane against acrolein-induced toxicity was tested by pre-treating with different concentrations (1 µM, 5 µM and 10 µM). Cell viability was observed to be 87%, 96% and 95% during pretreatment with 1 µM, 5 µM and 10 µM sulforaphane respectively. Maximum cytoprotection (p < 0.001) was achieved at 5 µM and 10 µM pre-treatment. Thus the 5 µM pre-treatment was used for determining oxidative stress and inflammation caused by acrolein in PBMC (Figure 1 B). Figure 1 C shows that sulforaphane treatment significantly reduced (p < 0.001) the LDH levels when compared to acrolein-treated cells. There was a non-significant difference in LDH release in the sulforaphane group compared to control cells.

**Sulforaphane inhibits acrolein-induced oxidative stress**

Sulforaphane-induced ROS generation was measured by DCF-DA. The results showed that acrolein caused a significant increase (p < 0.001) in ROS generation, lipid peroxide levels, protein carbonyl and sulfhydryl content when compared to control cells. Pre-treatment with sulforaphane showed a significant decline in the oxidative stress markers compared to cells treated with acrolein (Figure 2 A–D).

**Effect of sulforaphane on TAC, TOS and antioxidant status**

Acrolein caused a significant increase (p < 0.001) in TAC levels and a decline (p < 0.001) in the TAC and antioxidant status when compared to control cells.
Sulforaphane protects against acrolein-induced oxidative stress and inflammatory responses: modulation of Nrf-2 and COX-2 expression

Sulforaphane showed a statistically significant increase in total antioxidant levels, GSH and non-enzymic antioxidants (SOD, CAT, GST and GPX) and a significant decrease \( (p < 0.001) \) in total oxidant levels when compared to acrolein-treated cells (Table I).

Sulforaphane suppresses COX-2 expression and upregulates Nrf-2 levels

The effect of sulforaphane on acrolein-mediated COX-2 and Nrf-2 levels was determined by western blot analysis. Densitometric analysis shows significant upregulation \( (p < 0.001) \) of COX-2 and downregulation \( (p < 0.001) \) of Nrf-2 during acrolein treatment when compared to control cells. The sulforaphane pre-treatment showed increased expression \( (p < 0.001) \) of Nrf-2 and inhibition of COX-2 levels \( (p < 0.001) \) in acrolein-treated cells when compared to acrolein alone treated cells (Figure 3).

Sulforaphane downregulates PGE2 levels

Figure 4 shows a significant increase in PGE2 levels in cells treated with acrolein when compared to controls. Also, sulforaphane treatment resulted in downregulation of PGE2 compared to acrolein alone treated cells. There was a non-significant level of PGE2 in cells treated with sulforaphane alone when compared to control cells (Figure 4).

Discussion

Acrolein, a highly reactive \( \alpha, \beta \)-unsaturated aldehyde, is exposed to humans from industrial and environmental sources and remains in the body for several days in the active form [35]. Acrolein is metabolized in the presence of glutathione, which ultimately results in loss of endogenous antioxidant defense and thereby enhances oxidative stress. Acrolein can form adducts, and increased cellular levels have been identified in patients

Figure 1. Sulforaphane protects against acrolein-induced toxicity in PBMC. A – Acrolein-induced cytotoxicity: MTT assay was performed to determine the cell viability. Cells were treated with acrolein (10–60 \( \mu \)M) for 24 h. IC\(_{50} \) value was found to be 30 \( \mu \)M. Results are expressed as % cell viability when compared to control cells (* \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \); NS – non-significant when compared to control). B – Sulforaphane protects against acrolein-induced cytotoxicity: cells were treated with sulforaphane (1 \( \mu \)M, 5 \( \mu \)M and 10 \( \mu \)M) in the presence or absence of acrolein. Cell viability was determined by MTT assay. Results are expressed as % cell viability when compared to control cells. (** \( p < 0.01 \), *** \( p < 0.001 \) in comparison to acrolein-treated cells. NS represents non-significant in comparison to control cells). C – Sulforaphane reduces LDH release: results are expressed as % LDH leakage when compared to group I. Results shown are mean ± SEM. ** \( p < 0.01 \); NS – non-significant compared to control and *** \( p < 0.001 \) in comparison to group III. Groups I, II, III and IV indicate control, sulforaphane, acrolein, and sulforaphane + acrolein respectively. Results shown are mean ± SEM (one-way ANOVA followed by Tukey’s multiple comparison tests)
with various disease conditions [36–38]. A substantial increase in serum acrolein levels has been observed in smokers [39] and exposure with cyclophosphamide (anticancer drug) [40]. The highest levels were found in a patient after consuming allyl alcohol (a herbicide), which leads to death through acute cardiotoxic effects [41]. The toxic effects of acrolein are mediated through its soluble nature in both water and alcohol, by which it readily crosses the cell membrane [42].

The present study showed that acrolein induced oxidative stress by significantly increasing Table I. Effect of sulforaphane and acrolein on antioxidant status.

| Parameter | Group I | Group II | Group III | Group IV |
|-----------|---------|----------|-----------|----------|
| TAC       | 4.2 ±0.02 | 4.0 ±0.02   | 1.9 ±0.01 *** | 3.8 ±0.01 *** |
| TOS       | 10.18 ±1.10 | 10.0 ±1.13 NS | 24 ±1.90 *** | 15 ±1.19 *** |
| GSH       | 41 ±1.11 | 42 ±1.03 NS | 15 ±1.11 *** | 34 ±1.09 *** |
| GPX       | 6.89 ±0.001 | 6.82 ±0.002 NS | 2.14 ±0.001 *** | 5.15 ±0.001 *** |
| GST       | 45 ±1.10 | 43 ±1.19 NS | 12.98 ±1.87 *** | 37 ±1.19 *** |
| SOD       | 156 ±3.02 | 154 ±2.87 NS | 56 ±2.62 *** | 132 ±2.13 *** |
| CAT       | 1.71 ±0.001 | 1.63 ±0.002 NS | 0.82 ±0.001 *** | 1.54 ±0.002 *** |

Results are given as mean ± SEM for 6 rats in each group. Groups I, II, III and IV indicate control, sulforaphane, acrolein, and sulforaphane + acrolein respectively. Results shown are mean ± SEM (one-way ANOVA followed by Tukey’s multiple comparison tests). ***p < 0.001 when compared to group I and **p < 0.01, +++p < 0.001 in comparison to group III.
Sulforaphane protects against acrolein-induced oxidative stress and inflammatory responses: modulation of Nrf-2 and COX-2 expression

Various stress markers such as reactive oxygen species, lipid peroxide, protein carbonyl and sulfhydryl content. Oxidative stress and subsequent damage to proteins and lipids are well established [43]. Acrolein-induced lipid peroxide and oxidative stress have been documented in primary neonatal cultures [44] and human umbilical vein endothelial cells [45]. Acrolein specifically attacks the thiol groups of amino acids forming adducts and inactivates the functions of proteins by forming carbonyl contents [46]. In addition, oxidative stress induced by acrolein in most of the conditions is mediated through GSH depletion [47, 48]. Acrolein treatment resulted in depletion of GSH and antioxidant defense mechanisms, thus exacerbating the oxidative damage in PBMC. Imbalance in redox homeostasis leads to subsequent induction of apoptosis [49, 50]. Controlled levels of oxidant production in the cells regulate various essential signaling pathways. However, under uncontrolled conditions, reactive oxidants exceed the capacities of antioxidant defense mechanisms and initiate redox signaling. Antioxidants are well established in cytoprotection by regulating antioxidant status and inflammation [51, 52]. Expression of phase II antioxidant enzymes is regulated by nuclear factor erythroid 2-related factor 2 (Nrf-2), a redox-sensitive transcription factor [53, 54]. Protection through Nrf-2 induction against various stress mediators and diseased conditions has been documented [55–58]. However, we could observe that sulforaphane treatment completely offered a protective effect by decreasing the oxidative stress markers through upregulating Nrf-2 levels and enhancing the antioxidant status in PBMC. Significant upregulation of Nrf-2 and downstream antioxidant enzymes by sulforaphane has been reported to function in reducing oxidative stress during renal ischemia reperfusion injury [59]. The cardioprotective effect was mediated through Nrf-2 activation and subsequent decline in oxidative stress and DNA fragmentation [60]. The sulforaphane-induced decline in reactive oxygen species and oxidative stress has also been shown to involve inhibition of CYP activity [16, 61].

Acrolein-mediated oxidative stress and its associated inflammatory responses are well documented [62]. Two distinct isozymes of COX (COX-1 and COX-2) have been identified in mammalian cells. COX-1 is involved in housekeeping functions and is constitutively expressed. Under inflammatory conditions COX-2 (inducible), which catalyzes synthesis of prostaglandins, is expressed [63]. Prostaglandins are involved in normal biological functions, but their biosynthesis is found to be higher in oxidative stress conditions, ultimately leading to acute and chronic inflammation and thereby mediating disease outcome [64]. In PBMC, acrolein showed significant upregulation of COX-2 and PGE2 levels. Acrolein-induced atherosclerosis and its associated endothelial dysfunction were mediated through COX-2 expression [5, 65]. Park et al. [66] reported that MAPK kinases mediated COX-2 and downstream expression of prostaglandins in acrolein-induced toxicity in human
umbilical vein endothelial cells. A sulforaphane-induced anti-inflammatory effect has been reported by suppression of lipopolysaccharide-induced COX-2 levels [67]. In addition, sulforaphane potentially inhibits PGE2 synthesis by inhibiting micromolar prostaglandin E synthase 1 and exerts therapeutic control against inflammation and cancer [68]. Sulforaphane-induced anti-inflammatory mechanisms have been reported through inhibition of NF-κB and inflammatory cytokines [69, 70]. The present study shows that sulforaphane, an isothiocyanate compound, offers a protective effect against oxidative stress and inflammatory responses mediated by acrolein in PBMCs. Furthermore, therapeutic and dietary intervention using sulforaphane might be an excellent strategy to prevent oxidative stress-induced cellular damaging effects.

Conflict of interest

The authors declare no conflict of interest.

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