

Repeated Exposure to Cruciferous Allyl Nitrile Protects against Chemically Induced Skin Inflammation in the Mouse

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ABSTRACT

Repeated exposure to cruciferous allyl nitrile can induce antioxidant and phase 2 detoxification enzymes in various tissues. In the present study, we examined the effect of five days repeated exposure to allyl nitrile at subtoxic levels (0 - 400 $\mu\text{mol/kg/day}$) on the mouse ear. There was an increase in catalase activity in the ear at 100 - 400 $\mu\text{mol/kg/day}$, while elevated quinone reductase activity was observed at 400 $\mu\text{mol/kg/day}$ only. Next, after repeated allyl nitrile exposure (0 - 400 $\mu\text{mol/kg/day}$), the skin irritant croton oil was applied to the ear to induce skin acute inflammation (oedema). Compared with the 0 $\mu\text{mol/kg/day}$ group, animals in the 100 and 400 $\mu\text{mol/kg/day}$ pre-treatment groups showed reduced oedematous response to croton oil. The reduced oedematous response was inversely associated with enhanced myeloperoxidase activity used as index of the presence of neutrophils. These data suggest that repeated exposure to allyl nitrile at subtoxic levels contributes to protection against croton oil-induced ear dermatitis, potentially through decreasing reactive oxygen species and through infiltration of neutrophils.

Keywords: Allyl Nitrile; Croton Oil; Catalase; Quinone Reductase; Skin; Inflammation

1. Introduction

Cruciferous vegetables are consumed throughout the world. Food preparation and eating cause release of the plant enzyme myrosinase, which causes hydrolysis of glucosinolates into numerous breakdown products including nitriles, isothiocyanates, and indoles [1-3]. Hydrolysis of the glucosinolate sinigrin leads to formation of allyl nitrile and allyl isothiocyanate in fermented cabbage [4,5]. We reported similar breakdown products following incubation of cabbage homogenates in water or in buffer at pH 1.0 [6], and following *in vitro* digestion of sinigrin by *Bifidobacteria* sp. [7]. Thus, allyl nitrile absorbed by the body may have relevance to human health.

Rodent studies have demonstrated that exposure to high levels of allyl nitrile induces behavioural abnormalities [8,9], apoptosis in the brain [9], and alterations in the γ -aminobutyric acid system [10]. Allyl nitrile exposure can also cause vestibular and auditory hair cell degeneration, corneal opacity, and gliosis in the retina and olfactory bulbs [11]. At subtoxic levels, repeated exposure to allyl nitrile increases antioxidant and phase 2 detoxification enzymes in mice [12-14]. In those studies,

quinone reductase (QR) was up-regulated in the stomach, small intestine, urinary bladder, kidneys, lungs and brain, glutathione peroxidase was upregulated in the kidneys and small intestine, thioredoxin reductase was upregulated in the liver, kidneys and rectum, glutathione S-transferase (GST) was upregulated in the stomach, rectum, kidneys, lungs and brain, and glutathione was upregulated in the stomach, rectum, urinary bladder and brain. The increased expression of antioxidant and phase 2 enzymes are of particular interest to epidemiological studies indicating an inverse association between crucifer intake and the incidence of various cancers [15-19].

The skin is a major site of defence against pathogenic agents and is exposed to many pro-inflammatory environmental agents. The potential for skin irritation or skin sensitization from workplace chemicals is a great concern for individuals. However, it remains unknown whether repeated exposure to this allyl nitrile can upregulate antioxidant and phase 2 enzymes in the skin to suppress inflammation produced by environmental agents.

In the present study, we examined the effect of five days repeated allyl nitrile exposure at subtoxic levels on the mouse ear skin by measuring the activities of catalase, glutathione peroxidase, QR, and GST. Furthermore, we

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evaluated anti-inflammatory effect of allyl nitrile exposure by measuring oedematous response and leukocyte infiltration following inflammation induction using the croton oil-induced mouse ear dermatitis, a model of skin acute inflammation [20].

2. Materials and Methods

2.1. Materials

Nicotinamide adenine dinucleotide phosphate (NADPH), 2,6-dichloroindophenol and dicumarol were purchased from Sigma-Aldrich Ltd. (St. Louis, MO, USA), croton oil, hexadecyltrimethylammonium bromide (HTAB) and allyl nitrile (3-butenenitrile, CAS No. 109-75-1, purity > 98%) from Tokyo Kasei Organic Chemicals (Tokyo, Japan), rabbit anti-myeloperoxidase polyclonal antibody (Ab-1) from Thermo Fischer Scientific (Fremont, CA, USA), Vectastain ABC kit (peroxidase rabbit IgG) from Vector Laboratories (Burlingame, CA, USA), and sodium pentobarbital from Dainihon Seiyaku Co. (Tokyo, Japan). All other chemicals were purchased from Nacalai Tesque (Kyoto, Japan).

2.2. Animals and Treatments

All animal experiments were performed according to the Guidelines of the Committee on Animal Experimentation of Kanazawa University. Male ddY mice weighing 26 - 30 g obtained from Japan SLC Co. (Shizuoka, Japan) were used. Animals were maintained at 22°C ± 2°C under a 12:12 h light/dark cycle and allowed free access to tap water and laboratory food (CRF-1; Charles River Japan Inc., Yokohama, Japan).

Groups of 3 - 4 animals were administered allyl nitrile at subtoxic levels (100, 200, or 400 µmol/kg) or vehicle-distilled water (control; 4 mL/kg) daily for five days by gastric intubation, based on our previous findings [13]. For antioxidant and phase 2 enzyme analyses, the animals were sacrificed on the sixth day. For induction of dermatitis, the animals were given croton oil on the sixth day as described in Section 2.4.

2.3. Tissue Preparation and Biochemical Assays

Mice were anesthetized with 100 mg/kg sodium pentobarbital and perfused transcardially with 1.15% KCl. An ear plug was immediately taken and stored at -80°C until analysis. Cytosolic supernatant for enzyme analyses was prepared as previously described [12]. Protein concentrations were measured according to the method of Bradford [21] using bovine serum albumin as the standard. QR activity was measured according to the spectrophotometric method of Ernster [22], and GST activity according to the spectrophotometric method of Habig *et al.*

[23] using 1-chloro-2,4-dinitrobenzene as the substrate. Catalase activity was measured using the method of Abei [24] using H₂O₂ as the substrate, and glutathione peroxidase activity was measured according to the spectrophotometric method of Paglia and Valentine [25] using H₂O₂ as the substrate and NADPH as the source of reducing equivalents.

2.4. Croton Oil-Induced Dermatitis

Skin acute inflammation was induced on the right ear by application of 40 µg of croton oil dissolved in 15 µL of acetone, while 15 µL of acetone was applied on the left ear. At selected times following the croton oil application, animals were sacrificed and a 63.3 mm² tissue punch was taken from both the right and left ears to evaluate oedema and the leukocyte infiltrate. Quantitative assessment of oedema was performed by measuring the difference in weight between the portions taken from the right and left ears. The effect of repeated exposure to allyl nitrile on oedema development was quantified up to 48 h following croton oil application.

2.5. Assessment of Neutrophilic Granulocyte Infiltrate

Quantitative assessment of the cellular infiltrate was performed by measuring myeloperoxidase activity, an index of the presence of neutrophilic granulocytes [20]. Myeloperoxidase was extracted by HTAB according to Bradley *et al.* [26]. A portion of ear (63.6 mm²) was suspended in 1 mL of 0.1 M phosphate buffer pH 6.0, containing 0.1% HTAB, and then homogenized by Polytron (Kinematica GmbH, Switzerland) for 1 min setting at 9. The homogenates were centrifuged at 15,000 g at 4°C for 20 min, and the resulting supernatant was used for the enzymatic assay. Myeloperoxidase activity was measured according to the method of Maehly and Chance [27] with guaiacol as the substrate, and was expressed as µmol of guaiacol oxidized/min/1mL of supernatant.

2.6. Immunohistochemistry

Ear biopsies, fixed in 4% paraformaldehyde followed by in 30% sucrose, were sectioned by cryostat (16 µm, performed as previously described [10]). Tissue sections were incubated with primary antibody (rabbit anti-myeloperoxidase, 1:800) for four days at 4°C. Sections were then incubated in Tris-HCl buffered saline (pH 7.4) containing 0.04% diaminobenzidine, 0.08% nickel ammonium sulfate, and 0.003% H₂O₂ for 10 min for visualization.

2.7. Statistics

Statistical analyses were performed by analysis of vari-

ance followed by the Dunnett's test for multiple comparisons. The level of significance was set at $P < 0.05$.

3. Results

3.1. Induction of Antioxidant and Phase 2 Detoxification Enzymes in the Ear

Exposure to five days repeated allyl nitrile at 100, 200, and 400 $\mu\text{mol/kg/day}$ levels resulted in an increase in catalase activities in the ear ($P < 0.05$) (Figure 1(a)). Allyl nitrile at 100 and 200 $\mu\text{mol/kg/day}$ had no effect on QR activity, while QR activity was elevated at 400 $\mu\text{mol/kg/day}$ ($P < 0.05$) (Figure 1(b)). The activities of GST and glutathione peroxidase in the ear were too low to measure at any dose of allyl nitrile (0 - 400 $\mu\text{mol/kg/day}$).

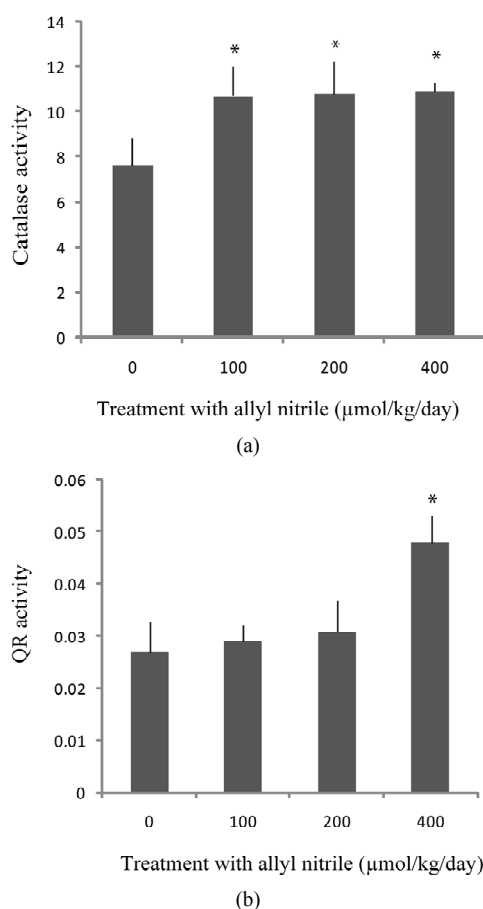


Figure 1. Catalase and quinone reductase (QR) activities in the ear following repeated allyl nitrile exposure. Mice were administered oral allyl nitrile at 0, 100, 200, or 400 $\mu\text{mol/kg}$ for five days. On the sixth day, catalase (a) and QR (b) activities were assessed. Catalase activity was defined as μmol of H_2O_2 consumed/min/mg of protein, and QR activity as μmol of 2,6-dichloroindophenol reduced/min/mg of protein. Values represent mean \pm SD of three animals. Values with asterisk are significantly different ($P < 0.05$) from the control (0 μmol allyl nitrile/kg/day).

3.2. Effect of Allyl Nitrile on Oedematous Response following Croton Oil-Induced Dermatitis

Control animals at 0 $\mu\text{mol/kg/day}$ showed oedema after croton oil application, peaking at 6 h post-dosing, and then decreasing 24 and 48 h post-dosing (Figure 2). There was a significant reduction in oedema at 24 h post-dosing in the 100 $\mu\text{mol/kg/day}$ allyl nitrile group ($P < 0.05$), and at 6 and 24 h post-dosing in the 400 $\mu\text{mol/kg/day}$ group ($P < 0.05$). There was no effect of 200 $\mu\text{mol/kg/day}$ allyl nitrile on oedema throughout the observation period.

3.3. Myeloperoxidase Activity after Croton Oil Application

The recruitment of leukocytes in the inflamed ear tissue was measured as myeloperoxidase activity. In control animals exposed to 0 $\mu\text{mol/kg/day}$ allyl nitrile, myeloperoxidase activity appeared at 6 h post-dosing with croton oil, reached a peak at 24 h, and decreased at 48 h (Figure 3). There was a significant increase in myeloperoxidase activity at 24 h post-dosing in the 100 $\mu\text{mol/kg/day}$ allyl nitrile group ($P < 0.05$), and at 6 h post-dosing in the 400 $\mu\text{mol/kg/day}$ ($P < 0.05$), while there was no effect in the 200 $\mu\text{mol/kg/day}$ group.

3.4. Myeloperoxidase Immunostaining in the Ear

Immunostaining with antimyeloperoxidase revealed evidence of tissue oedema and leukocyte infiltration after

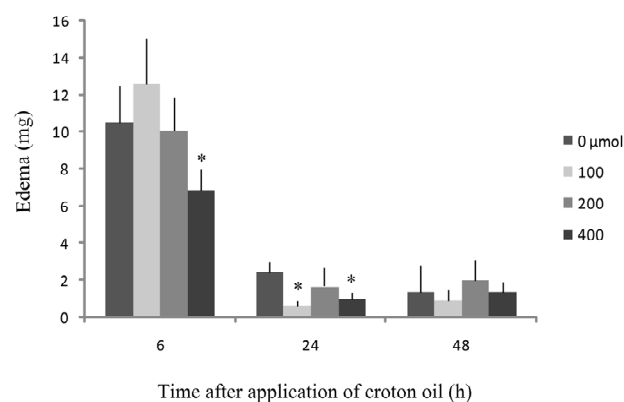


Figure 2. Oedematous response to croton oil application in the ear. Mice were administered oral allyl nitrile at 0, 100, 200, or 400 $\mu\text{mol/kg}$ for five days. On the sixth day, croton oil was applied on the right ear, and acetone on the left ear. Oedema (difference in weight between 63.6 mm^2 punches from the treated and untreated ears) was assessed up to 48 h following croton oil application. Values represent mean \pm SD of four animals. Values with asterisk within hours after the application are significantly different ($P < 0.05$) from the control (0 μmol allyl nitrile/kg/day).

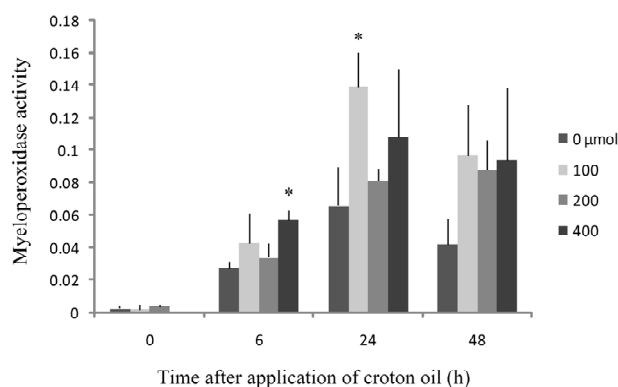


Figure 3. Myeloperoxidase activity in the ear after croton oil application. Mice were administered oral allyl nitrile at 0, 100, 200, or 400 $\mu\text{mol/kg}$ for five days. On the sixth day, croton oil was applied to the right ear. Myeloperoxidase activity (μmol of guaiacol oxidized/min/ 63.6 mm^2 tissue) in the right ear was assessed up to 48 h following croton oil application. Values represent mean \pm SD of four animals. Values with asterisk within hours after the application are significantly different ($P < 0.05$) from the control (0 μmol allyl nitrile/kg/day).

croton oil application. The ear from animals pre-treated with 0 $\mu\text{mol/kg}$ allyl nitrile for five days showed the oedematous response and leukocyte infiltration at 6 h post-dosing with croton oil (Figure 4(A)), while there was no response at 6 h after acetone only application (Figure 4(E)). At 24 h post-dosing the oedema had recovered while leukocyte infiltration was still evident (Figure 4(B)). The ear from the 400 $\mu\text{mol/kg/day}$ group also displayed the oedematous response and leukocyte infiltration at 6 h post-dosing with croton oil (Figure 4(C)), while at 24 h the oedema had recovered and there was evidence of myeloperoxidase-positive cells (Figure 4(D)).

4. Discussion

The present study showed that repeated exposure to allyl nitrile induced antioxidant and phase 2 enzymes in the skin, which resulted in reduced oedema and increased neutrophils (enhanced myeloperoxidase activity) after croton oil application. The induction of catalase and QR in the skin following repeated exposure to cruciferous allyl nitrile is consistent with previous reports showing a similar induction of antioxidant and phase 2 enzymes in other tissues [12–14]. These data suggest that allyl nitrile exposure is protective against chemically induced inflammation.

We employed the croton oil-induced ear oedema for a model of skin acute inflammation. Croton oil contains phorbol myristate acetate, which triggers keratinocytes in the skin to produce soluble mediators important in contact irritancy including interleukin- 1α , interleukin-8, tumour necrosis factor- α , and granulocyte/macrophage

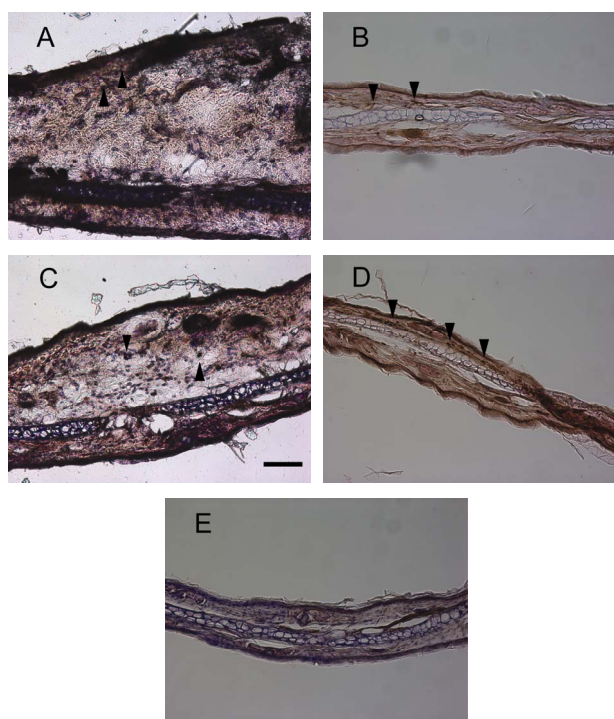


Figure 4. Mouse ear after the induction of the croton oil dermatitis. Mice were administered oral allyl nitrile at 0 (A), (B), and (E) or 400 (C) and (D) $\mu\text{mol/kg}$ for five days. On the sixth day, croton oil (A), (B), (C), and (D) or acetone alone (E) was applied to the right or left ears, respectively. At 6 h (A), (C), and (E) and 24 h (B) and (D) after croton oil application, immunostaining of infiltrated leukocytes with antimyeloperoxidase antibody was performed. Arrowheads indicate representative positive immunostained cells. Scale bar: 100 μm .

colony stimulating factor [28]. Phorbol myristate acetate also activates protein kinase C, resulting in activation of the reactive oxygen species-generating NADPH-dependent oxidase in polymorphonuclear neutrophils [29]. These reactive oxygen species can exaggerate contact irritancy. Catalase and QR induced by allyl nitrile exposure can decrease the reactive oxygen species formed in neutrophils, which may contribute to the reduced oedema observed with allyl nitrile treatment in response to croton oil exposure. Interestingly, there is growing evidence for a role of nuclear factor-erythroid 2-related factor-2 (Nrf2) in inflammatory disorders [30]. Nrf2 is a transcription factor that plays an important role in cellular defence against oxidative insults by inducing antioxidant and phase 2 detoxification enzymes, and has been suggested to have a protective role in inflammation-mediated disorders.

Myeloperoxidase activity was used as index of the presence of neutrophils in this study. Neutrophils are known to play an important role in resistance against extracellular pathogens and in acute inflammation, and

act as phagocytic cells, releasing lytic enzymes and producing reactive oxygen species [31]. Additionally, recent evidence suggests that neutrophils can reduce inflammation through the production of anti-inflammatory molecules [32]. In this study, the degree of oedema was inversely associated with myeloperoxidase activity, suggesting that that increased neutrophils (enhanced myeloperoxidase) may have contributed to resolution of inflammation. Nevertheless, it is unclear why allyl nitrile exposure increased neutrophils recruitment after croton oil application, although repeated exposure to the breakdown product allyl isothiocyanate, an Nrf2 activator, was reported to induce neutrophil recruitment in blood from rats [33,34].

In conclusion, repeated exposure to allyl nitrile at subtoxic levels can induce catalase and QR in the skin of the ear. Further, this pre-treatment was protective against the oedematous response to the irritant croton oil, likely via a reduction in reactive oxygen species and through infiltration of neutrophils.

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