Free Radical Biology and Medicine 52 (2012) 2064–2071

Contents lists available at SciVerse ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Induction of lung glutathione and glutamylcysteine ligase by 1,4-phenylenebis(methylene)selenocyanate and its glutathione conjugate: Role of nuclear factor-erythroid 2-related factor 2

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ARTICLE INFO

Article history:
Received 23 August 2011
Received in revised form
7 March 2012
Accepted 24 March 2012
Available online 17 April 2012

Keywords:
Organoselenium
p-XSC
p-XSeSG
Glutathione
γ-GCL
Nrf2
MEF
Antioxidant response element
HepG2 ARE
Lung cancer
Fisher 344 rat
Free radicals

ABSTRACT

The synthetic organoselenium agent 1,4-phenylenebis(methylene)selenocyanate (*p*-XSC) and its glutathione (GSH) conjugate (*p*-XSeSG) are potent chemopreventive agents in several preclinical models. *p*-XSC is also an effective inducer of GSH in mouse lung. Our objectives were to test the hypothesis that GSH induction by *p*-XSC occurs through upregulation of the rate-limiting GSH biosynthetic enzyme glutamylcysteine ligase (GCL), through activation of antioxidant response elements (AREs) in GCL genes via activation of nuclear factor-erythroid 2-related factor 2 (Nrf2). *p*-XSC feeding (10 ppm Se) increased GSH (230%) and upregulated the catalytic subunit of GCL (GCLc) (55%), extracellular-related kinase (220%), and nuclear Nrf2 (610%) in lung but not liver after 14 days in the rat ($P < 0.05$). Similarly, *p*-XSeSG feeding (10 ppm) induced lung GCLc (88%) and GSH (200%) ($P < 0.05$), whereas the naturally occurring selenomethionine had no effect. Both *p*-XSC and *p*-XSeSG activated a luciferase reporter in HepG2 ARE-reporter cells up to threefold for *p*-XSC and greater than or equal to fivefold for *p*-XSeSG. Luciferase activation by *p*-XSeSG was associated with enhanced levels of GSH, GCLc, and nuclear Nrf2, which were significantly reduced by co-incubation with short interfering RNA targeting Nrf2. The dependence of GCL induction on Nrf2 was confirmed in Nrf2-deficient mouse embryonic fibroblasts, in which *p*-XSeSG induced GCL subunits in wild-type but not in Nrf2-deficient cells ($P < 0.05$). These results indicate that *p*-XSC may act through the Nrf2 pathway in vivo and that *p*-XSeSG is the putative metabolite responsible for such activation, thus offering *p*-XSeSG as a less toxic, yet highly efficacious, inducer of GSH.

Introduction

Lung cancer is the second most common cancer and is the leading cause of cancer deaths in the world. Because of difficulties associated with smoking cessation, early detection, and chemotherapeutic effectiveness, research into chemoprevention has become a priority. Selenium, in both organic and inorganic forms, has played a major role in the field of chemoprevention, particularly after the reporting of an almost 50% reduction in morbidity and mortality by major cancers after dietary supplementation with selenized brewer's yeast [1]. Various synthetic selenium compounds have been developed with goals of lowering toxicity while enhancing efficacy compared to other historic chemopreventive agents such as selenite, selenate, or selenomethionine [2]. The organoselenium agents 1,4-phenylenebis (methylene)selenocyanate (*p*-XSC) and its putative metabolic glutathione conjugate *p*-XSeSG have proven to be effective in preventing a variety of carcinogen-induced cancers in several animal models, including lung cancer [3–5].

We reported that *p*-XSC-mediated protection against lung tumors induced by the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in A/J mice was associated

Abbreviations: *p*-XSC, 1,4-phenylenebis(methylene)selenocyanate; *p*-XSeSG, glutathione conjugate of *p*-XSC or *N,N′*-[1,4-phenylenebis(methyleneselenothio((1*R*)-1-(((carboxymethyl)amino)carbonyl)-2,1-ethanediyl))]bis-L-glutamine; GSH, glutathione; GCL, γ-glutamylcysteine ligase; GCLc, catalytic subunit of γ-glutamylcysteine ligase; GCLm, modulatory subunit of γ-glutamylcysteine ligase; ARE, antioxidant response element; Nrf2, nuclear factor-erythroid 2-related factor 2; siNrf2, short interfering RNA targeting Nrf2; MEF, mouse embryonic fibroblasts; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; siControl, nontargeting control RNA; $Nrf2^{-/-}$, Nrf2-deficient

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http://dx.doi.org/10.1016/j.freeradbiomed.2012.03.018

with increased levels of lung GSH [5]. Also, selenized yeast supplementation enhanced levels of blood GSH in a clinical study of healthy adult men [6]. Glutathione plays a number of critical roles in protection against carcinogenesis through its activities as the most important and abundant endogenous antioxidant and in the detoxification of exogenous and endogenous carcinogens [7,8]. Thus, the maintenance of cellular GSH and/or induction of supranormal levels is likely to be part of an agent's chemopreventive potential.

Both the catalytic and the modulatory subunits of the rate-limiting enzyme for GSH synthesis, glutamylcysteine ligase (GCLc and GCLm, respectively), are regulated in part by the presence of antioxidant response elements (AREs) in the upstream promoter regions of each gene [9]. Enhanced nuclear translocation and subsequent binding of the nuclear factor-erythroid 2-related factor 2 (Nrf2) transcription factor to ARE-containing promoters activate a variety of chemoprotective phase II detoxification genes, including many in the GSH biosynthetic and homeostasis pathways [10].

Nrf2 is normally sequestered in the cytoplasm by the actin-bound protein Keap1, a substrate adapter for an E3 ubiquitin ligase, which targets Nrf2 for rapid turnover [11]. Keap1 contains multiple reactive cysteine residues that, when modified directly or indirectly by a variety of inducers, reduce its affinity for Nrf2 and promote the translocation of Nrf2 to the nucleus [12]. The synthetic organoselenium compound ebselen has been shown in a cell culture model to directly modify Keap1 [13]. Phosphorylation of Nrf2, mediated by extracellular signal-regulated kinase and c-Jun-NH_2-kinase pathways, has also been shown to disrupt its association with Keap1 [14].

The primary objective of the studies reported here was to determine if Nrf2 activation of AREs in GSH biosynthetic genes is responsible for GSH enhancement by *p*-XSC and its metabolite *p*-XSeSG in vitro and in vivo in rat lung.

Materials and methods

Reagents

Organoselenium compounds *p*-XSC and *p*-XSeSG were synthesized as described previously [15]. L-Selenomethionine was obtained commercially (Sigma, St. Louis, MO, USA). Antibodies (Nrf2, GCLc, GCLm, p-extracellular-related kinase (p-ERK), actin, lamin A) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Animals and diet preparation

Pathogen-free male Fisher 344 rats 4 weeks of age were purchased from Charles River Breeding Laboratory (Kingston, NY, USA). After a 1-week quarantine, the rats were weighed and placed into four treatment groups (control, *p*-XSC, *p*-XSeSG, and selenomethionine) of six rats each such that the average weights per group were similar. All rats were fed a semipurified diet (AIN-93 G) containing 0.1 ppm of selenium as sodium selenite throughout the study. At 6 weeks of age, the diets of the rats in the three selenium groups were supplemented with 10 ppm *p*-XSC, 10 ppm *p*-XSeSG, or 10 ppm selenomethionine. Levels of selenium (10 ppm) for incorporation into the diet and the time period for GSH induction were based on our previous studies [5,16]. The organoselenium compounds were blended into the diets by Harlan Teklad (Indianapolis, IN, USA), and stored at 4 °C. Rats were fed either the control or the supplemented diet for 2 weeks and were reweighed weekly. After the second week animals were sacrificed as described below. The 2-week period was based on preliminary studies (data not shown). All procedures involving animals were conducted in accordance with guidelines of the Institutional Animal Care and Use Committee of the Pennsylvania State University, as promulgated by the National Institutes of Health.

Necropsy and tissue harvesting and processing

Animals were euthanized by CO_2 inhalation between 9:00 and 10:00 AM to avoid the confounding effects of circadian fluctuations. Whole blood was collected into EDTA tubes by cardiac puncture and frozen at −80 °C for analysis of GSH. Lungs and livers were harvested, rinsed in saline, and blotted dry and then divided into portions and snap-frozen in liquid nitrogen. For Western blot analysis, nuclear and cytoplasmic extracts were obtained from frozen tissue using reagents supplied by Marligen (Madison, WI, USA) according to the manufacturer's protocol. Briefly, 0.15 g of frozen tissue was placed into 1 ml of ice-cold cytoplasm extract buffer containing EDTA and protease inhibitors, then homogenized using an all-glass Ten Broeck homogenizer, and centrifuged at 800*g* for 5 min at 0–4 °C. The resulting supernatant and nuclear pellets were stored at −80 °C until analysis.

Determination of total selenium in lung

Lung tissues were homogenized in 1.15% KCl (0.1 g/ml) using a glass hand homogenizer and digested in a MARS Xpress microwave digestion system (CEM Corp., Mathews, NC, USA) equipped with 55-ml Teflon PFA vessels and a turntable. The digestion was conducted in 50% nitric acid and then diluted to 20% before selenium analysis by atomic absorption spectroscopy. We used an AAnalyst 600 instrument from PerkinElmer with graphite furnace for total selenium analysis by measuring the absorbance peak area at 196 nm for each sample. Palladium matrix modifier was added along with each sample to the furnace. Reference standard solutions of selenium dioxide at various concentrations were used to construct a standard curve for comparison with all assays. Analysis was performed in duplicate for each sample and the average value was recorded. For each group at least six samples were analyzed and the results were expressed as the mean ± SE.

Blood and tissue glutathione

For GSH analysis, portions of lung (left lung) and liver (right lobe) (~0.1 g) were homogenized in 3 ml of 5% (w/v) metaphosphoric acid (MPA) using an all-glass Ten Broeck homogenizer at 0–4 °C, and whole blood (0.1 ml) was hemolyzed in 0.4 ml of 5% MPA. Precipitated protein was removed by centrifugation at 14,000*g* for 2 min and acid-soluble fractions were removed and stored at −80 °C until analysis for free glutathione by the enzymatic recycling method using Elman's reagent [17] with modifications [18]. Tissue GSH levels were normalized to tissue weight, and GSH in blood samples was normalized to hemoglobin levels.

Cell culture

HepG2 human hepatoma cells stably transfected with ARE-luciferase plasmid were obtained courtesy of Dr. Muriel Cuendet, Purdue University [19]. Cells were maintained in F-12 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, antibiotics, MEM amino acid solution, and insulin (1.45 μg/ml). Wild-type and Nrf2-deficient ($Nrf2^{-/-}$) mouse embryonic fibroblasts (MEFs) were obtained courtesy of Drs. Nobunao Wakabayashi and Thomas Kensler, Johns Hopkins University [20]. Cells were maintained in Iscove's MDM (Gibco12440;