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SHORT COMMUNICATION

Increases in the mRNA Levels of γ-Glutamyltransferase and Heme Oxygenase-1 in the Rat Lung after Ozone Exposure

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ABSTRACT. γ-Glutamyltransferase (GGT) and heme oxygenase-1 (HO-1) are induced by chemical and physical stresses producing an oxidative burden on tissues and cells. Both enzymes are proposed to have an antioxidant role in protecting cells and tissues from oxidative burden. To explore the effects of ozone (O_3), the major oxidant in photochemical smog, on the expression of GGT and HO-1 genes in the lung, we exposed rats to 0.4 ppm O_3 for up to 7 days. After exposures, mRNA levels of GGT and HO-1 in the lung were measured by RNA blot analysis. Although a 1-day exposure did not change either GGT or HO-1 mRNA levels in the lung, both genes responded to prolonged exposure to O_3. GGT mRNA was increased to 149% ($P < 0.01$) and 158% ($P < 0.01$) of the control by 3- and 7-day exposures, respectively. HO-1 mRNA was also elevated to 174% ($P < 0.01$) and 184% ($P < 0.001$) of the control after 3- and 7-day exposures, respectively. The elevation of GGT and HO-1 mRNA after prolonged exposure to O_3 suggests that expression of these genes is not involved in the acute respiratory response, but in the recovery process from lung damage induced by O_3. BIOCHEM PHARMACOL 53;7:1061–1064, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. ozone; γ-glutamyltransferase; heme oxygenase-1; mRNA; rats

O_3‖ is a major oxidant in photochemical smog. When inhaled, O_3 reacts first with lipids, peptides, and proteins in bronchiolar and alveolar lining fluid that forms the lung-air interface [1]. Epithelial cells, located at the region where the lung-air interface is thin or absent, are attacked directly by O_3. Reaction of O_3 with biomaterials such as unsaturated fatty acids, cholesterol, and GSH in the target area produces ozonized derivatives or reactive oxygen intermediates [2]. These reactions and their products are responsible for the initiation of inflammation and cellular damage caused by O_3 inhalation [3].

GSH is one of the principal molecules in maintaining redox potential in both epithelial cells and epithelial lining fluid [4]. When the lung is exposed to O_3 at ambient levels, the GSH content in the lung tissue and bronchial-alveolar lining fluid initially decreases, followed by an increase over the control level after a continuous 3-day exposure to O_3 [5]. As GSH increases, several sets of antioxidant enzyme activities (superoxide dismutase, catalase, GSH reductase, and GSH peroxidase) are elevated [6, 7]. The elevation of GSH and antioxidant enzyme activities appears to imply a primary involvement of oxidative burden in the initiation of acute inflammatory response and the development of tissue damage during O_3 inhalation.

GGT (EC 2.3.2.2) is one of the rate-limiting enzymes of the proposed γ-glutamyl cycle, in which GSH is formed [8]. This ectoenzyme is localized mainly on the apical surface of epithelial cells and catalyzes the transfer of γ-glutamyl residues from donors, usually GSH, to acceptors. Resulting amino acids or dipeptides are transported across the cell membrane and used for the re-synthesis of GSH or other γ-glutamyl compounds [8]. In the lung, GGT is localized at the apical membrane of the bronchiolar nonciliated epithelial cell and the alveolar type 1 and type 2 cells [9, 10]. HO (EC 1.14.99.3) catabolizes heme into biliverdin, from which bilirubin is formed by biliverdin reductase. Biliverdin and bilirubin trap peroxiradicals and terminate radical chain reactions [11]. Hence, the induction of HO is thought to be a protective response to oxidative stress [12].

Recent accumulating evidence suggests that GGT and

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‖ *Abbreviations:* O_3, ozone; GGT, γ-glutamyltransferase; HO, heme oxygenase; and GSH, glutathione.

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HO-1, an inducible isozyme of HO, play important roles in protecting the lung against exposure to oxidative gas [13, 14]. However, Cosma *et al.* [15] observed no increase in HO-1 mRNA in the lung following a 6-hr exposure to 0.5 and 1 ppm O_3, nor did they examine the effects of prolonged exposure to O_3 on HO-1 mRNA level. Hence, we hypothesized that GGT and HO-1 may respond to prolonged exposure to ambient levels of O_3. In the present study, we exposed rats to 0.4 ppm O_3 for up to 7 days. Then, we confirmed that the levels of GGT and HO-1 mRNA in the rat lung increased after 3 days of exposure, and that elevated levels of these mRNAs were maintained for at least 7 days of exposure.

MATERIALS AND METHODS

Animal and Exposure

Specific pathogen-free male Jcl:Wistar rats were obtained from Clea Japan (Tokyo). The rats (N = 6), weighing 145–160 g, were exposed continuously to 0.40 ± 0.001 ppm O_3 or filtered clean air for 7 days. Details of the exposure chamber sets, gas control system, and animal care were the same as previously mentioned [16]. Animal experiments were performed under the approval of the institution's Animal Committee. After exposure, rats were anesthetized by i.p. injection of pentobarbital and the lungs were isolated. Total RNA was extracted from the lung using a guanidinium isothiocyanate-acid phenol solution.

Probe Preparation

A cDNA probe for GGT was prepared as described previously [17]. A DNA fragment (+56 to +616) of GGT cDNA was selected for the probe because it should hybridize to all GGT mRNA subtypes (GGT mRNAI–IV) with equal affinity. HO-1 cDNA [18] was obtained from the Riken Gene Bank (Tsukuba, Japan). Probes were labeled with [^{32}P]dCTP by random priming with the Klenow enzyme and used for RNA blot analysis. An oligonucleotide (5'-AACGATCAGAGTAGTGGTATTTCACC) was used as a 28S rRNA probe [19]. The oligonucleotide was labeled with [^{32}P]phosphate, which was transferred from [γ-^{32}P]ATP by T4 polynucleotide kinase.

RNA Blot Analysis

Messenger RNA levels were determined by the RNA blotting method. Five micrograms of lung total RNA was denatured in 1 M glyoxal, electrophoresed on a 1.0% agarose gel, using a comb with 3-mm wide lanes, and then transferred to a nylon membrane (Schleicher & Schuell, Dassel, Germany; NYTRAN) in 10x SSC [1x SSC contains 0.15 M NaCl and 15 mM sodium citrate (pH 7.0)] overnight. The membrane was hybridized in 50% formamide with the radiolabeled GGT probe at 42° for 16 hr. The non-specific binding of the probes was removed by washing sequentially with 6x SSPE [1x SSPE contains 0.18 M NaCl, 10 mM sodium phosphate (pH 7.7), and 1 mM EDTA], 1x SSPE, and 0.1x SSPE each containing 0.1% SDS, followed by a 3-day exposure to the Imaging-Plate of the BAS 2000 Image Analyzer for analysis of radioactivity (Fuji, Tokyo, Japan). The GGT probe was stripped with a low salt wash at 90°, and the membrane was reprobed for HO-1 and then 28S rRNA. A 28S rRNA probe hybridized at 42° without formamide and the probed membrane was washed with 2x SSC containing 1% SDS at 42° for 30 min. The membranes probed for HO-1 and 28S rRNA were exposed to the Imaging Plate for 2 days and 30 min, respectively.

Statistical Analysis

Analyses of significant differences between control and exposed animals were performed using selected multiple comparison procedures after one-way ANOVA.

RESULTS AND DISCUSSION

Rats were exposed to 0.4 ppm O_3 for up to 7 days. The levels of GGT mRNA and HO-1 mRNA were analyzed by

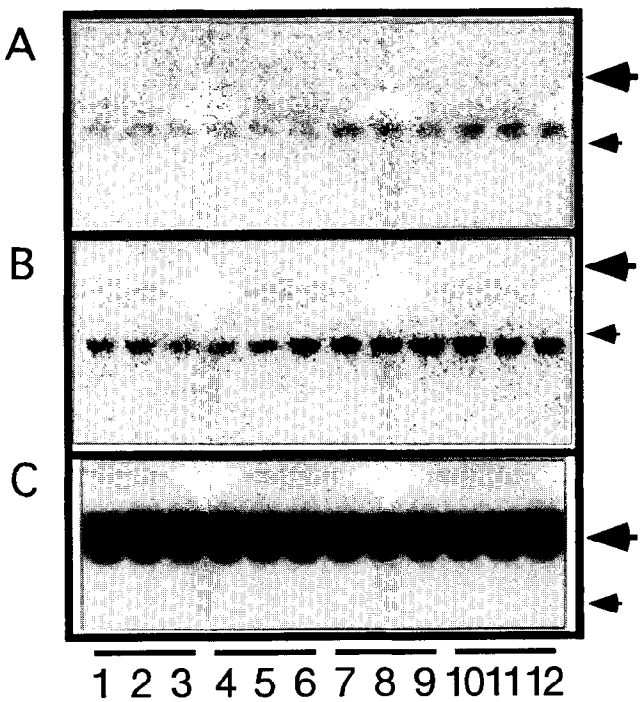

FIG. 1. RNA blot analysis of lung RNA. Rats (N = 6) were exposed to control clean air (lanes 1–3) or 0.4 ppm O_3 for 1 day (lanes 4–6), 3 days (lanes 7–9), and 7 days (lanes 10–12). After exposures, total RNA was extracted from the lung. Five micrograms of total RNA was separated in 1% agarose followed by transfer to a nylon membrane. Resulting membrane was hybridized to probes of GGT (panel A), HO-1 (panel B), and 28S (panel C) sequentially (large and small arrows indicate positions corresponding to 28S and 18S rRNA, respectively). Blots from half of the rats are shown in Fig. 1. Another set of blots showed the same pattern as Fig. 1 did.

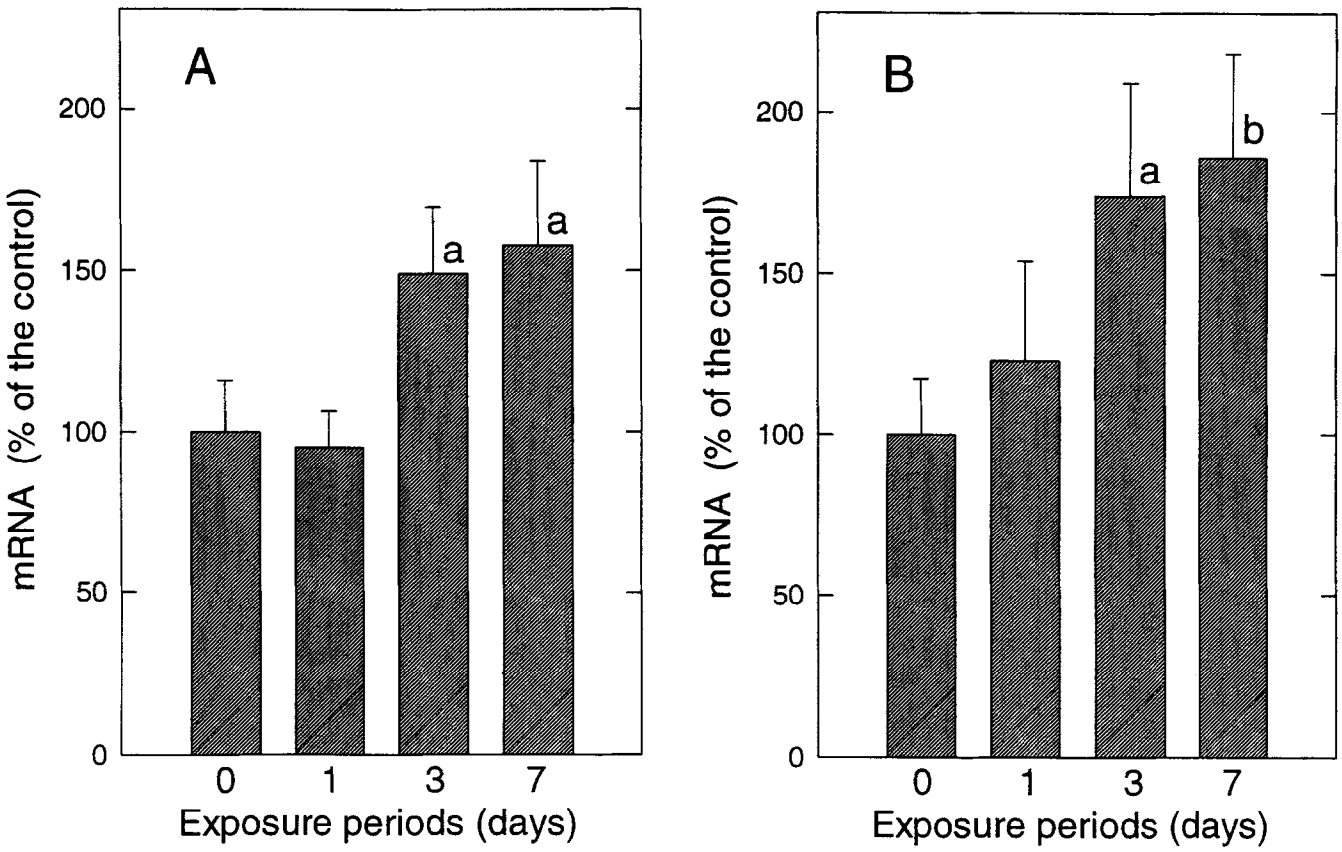

FIG. 2. Levels of GGT and HO-1 mRNA during O_3 exposure. Rats were exposed to 0.4 ppm O_3 for up to 7 days, and RNA blot analyses for GGT and HO-1 mRNA were performed as in Fig. 1. GGT (A) and HO-1 (B) mRNA levels normalized to 28S rRNA were calculated from radioactivity of each band as described in Materials and Methods. Values are means ± SD, N = 6. Key: (a) $P < 0.01$ vs 0 days; and (b) $P < 0.001$ vs 0 days.

the RNA blotting method. The GGT probe hybridized to a 2.2 kb message. Signal intensity was not affected by a 1-day exposure to 0.4 ppm O_3. However, it increased gradually from 3 to 7 days of exposure to 0.4 ppm O_3 (Fig. 1A). GGT signals normalized to those of 28S rRNA (Fig. 1C) were elevated significantly to 149% ($P < 0.01$) and 158% ($P < 0.01$) of the control after 3 and 7 days of exposures, respectively (Fig. 2A). Signals corresponding to HO-1 (1.7 kb) also increased gradually during the 7-day exposure to O_3 (Fig. 1B). Normalized HO-1 signals were elevated significantly to 174% ($P < 0.01$) and 184% ($P < 0.001$) of the control by 3- and 7-day exposures to 0.4 ppm O_3, respectively (Fig. 2B).

The present study clearly shows that GGT and HO-1 respond to O_3 exposure at the mRNA level. It has been reported that elevation of the mRNA level of Cu, Zn-superoxide dismutase, catalase, and GSH peroxidase was not observed until 3 days of continuous exposure to 0.7 ppm O_3 [20]. Cosma *et al.* [15] observed no increase in lung HO-1 mRNA following a 6-hr exposure to 0.5 and 1 ppm O_3. These observations are consistent with our results, indicating that elevation of GGT and HO-1 mRNA became evident after 3 days of exposure to 0.4 ppm O_3 and was maintained for at least 7 days of exposure.

O_3 exposure damages the epithelium mainly at the terminal bronchiole and the proximal alveolus [7, 16]. Ciliated cells on the terminal bronchiole and type 1 cells on the proximal alveolus are sensitive to O_3. These epithelial damages are repaired during subsequent exposure by proliferation of nonciliated cells and type 2 cells, progenitor cells of the bronchiolar and alveolar epithelium, respectively. This was followed by differentiation to ciliated cells and type 1 cells, matured cells of the epithelium [21]. Thymidine uptake into these cells reaches its peak after 2 days of exposure to O_3 under the same conditions as described in this paper [16]. *De novo* synthesis of glutathione is required for entry and progression of the cell cycle [22]. GGT participates in GSH metabolism, and catabolites of heme via heme oxygenase have an antioxidative property. Taken together, the elevation of GGT and HO-1 mRNA after 3 days of exposure suggests that these gene products may be involved in the recovery process from O_3-induced lung damage and may have an important role in protection against oxidative burden induced by O_3 exposure.

GGT gene expression is regulated by factors specific to tissue types, developmental stages, and environmental stresses [13]. Menadione, an H_2O_2 generator, and hyperoxia up-regulate the GGT gene in rat alveolar epithelial cell

lines [13, 23]. The HO-1 gene in various tissues is also activated by glutathione depletion [24], H_2O_2, sodium arsenite, and UVA radiation [25]. H_2O_2, a common inducer of GGT and HO-1 genes, is produced by reaction of O_3 with olefinic lipid in the presence of H_2O. Therefore, H_2O_2 and other reactive oxygen intermediates produced in the lung epithelium and epithelial lining fluid may play a role in the induction of expression of GGT and HO-1 genes through interactions with *trans*-acting factors, although the detailed mechanisms of elevation of GGT and HO-1 mRNA are unclear. We are currently studying the *cis*-acting DNA region that regulates GGT and HO-1 gene expression by O_3 exposure. So far as concerns the regulation of the heat shock protein genes [26], the induction of expression of various genes by some environmental stresses and toxic chemicals is regulated by a common specific transcription factor and its binding element in promoter and enhancer regions. Further examination of the molecular mechanisms of induction of expression of GGT and HO-1 genes will give us basic insight toward a better understanding of the effects of O_3 on biological systems.

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