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Decrease in brain POMC mRNA expression and onset of obesity in guinea pigs exposed to 2-chloroethyl ethyl sulfide, a mustard analogue

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Abstract

The full spectrum of physiological effects resulting from exposure to sulfur mustard and its analogs is currently unknown. In a guinea pig model, initially selected to study the role of an inflammatory cytokine cascade in mustard gas induced lung injury, we observed significant body weight gain in guinea pigs exposed to an intratracheally injected single dose of 2-chloroethyl ethyl sulfide, a mustard analog. The body weight gain was not associated with any apparent change in appetite. To further elucidate a molecular basis for the observed weight gain, we evaluated candidate genes for the obese phenotype by quantitative RT-PCR. We observed a time- and dose-dependent decrease in guinea pig pro-opiomelanocortin (POMC) message following treatment with mustard gas. This reduction in POMC message is consistent with the onset of obesity in the animals. We hypothesize that the POMC melanocortin pathway provides a mechanistic basis for the observed effects of sulfur mustard on body weight.

Keywords: 2-Chloroethyl ethyl sulfide; Obesity; POMC; Guinea pig

Mustard gas is a poisonous chemical agent that exerts a local action on eyes, skin, and respiratory tissue followed by impairment of nervous, cardiac, and digestive system in humans and laboratory animals [1–4]. Sulfur mustard disrupts and impairs a variety of cellular activities. Inhalation of mustard gas causes hemorrhagic inflammation to the tracheobronchial tree with severe pulmonary complications including adult respiratory distress syndrome (ARDS) [5]. Most deaths are due to secondary respiratory infections. Besides its use in World war I and World war II, sulfur mustard has been used on Iranian soldiers, on civilians during the Gulf war, and on the Iranian-occupied village of Halabja as a vesicant chemical warfare agent resulting in many civilian casualties [6,7]. Mustard agents are also harmful in long-term exposure at low doses. Long-term exposure to mustard gas may lead to lung cancer as indicated by the studies on Japanese who worked in poison gas factories [8]. Unfortunately, the molecular mechanisms of carcinogenesis in former poison gas workers remain unclear [9], and the attempts to seek confirmatory and substantial evidence in laboratory animals for links between mustard gas exposure and cancer have not yielded consistent results [10].

Several studies in rats and mice have shown that the mechanism of mustard gas action on lung, skin or other organs includes DNA alkylation; cross linking of DNA [11]; activation of proteases resulting in proteolysis of several vital intracellular enzymes and structural proteins [12]; production of free radicals and free radical-mediated oxidative stress [13,14]; and inflammation [15]. We have recently developed the guinea pig model to study the role of inflammatory cytokine cascade in mustard gas induced

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lung injury [16,17]. In these studies, we have observed activation of NF-κB and tumor necrosis factor (TNF-α), and accumulation of ceramides, a second messenger involved in cell apoptosis. Surprisingly, during the course of these investigations, we observed significant body weight gain in guinea pigs exposed to intratracheally injected single dose of CEES. This was interesting in view of the fact that there was no difference in food intake between control and experimental animals. To understand the phenotype better, we decided to monitor the status of some of the obesity-associated genes in guinea pig tissues in response to mustard gas exposure. To our knowledge, only three obesity genes [pro-opiomelanocortin (POMC), neuropeptide Y (NPY), and lipoprotein lipase (LPL)] have been characterized in guinea pigs, whereas at least 25 genes have been characterized in humans. Deficiency of POMC and peripheral melanocortins has been etiologically implicated in the initiation of obesity in mouse [18,19]. The objective of the present study was to determine whether the mustard gas-induced obesity in guinea pigs is associated with down-regulation of POMC in brain. Insights from these experiments will increase our understanding of the physiological effects of sulfur mustards and might unravel new mechanisms for the induction of obesity.

Materials and methods

Chemicals. 2-Chloroethyl ethyl sulfide [Cl-CH_2CH_2-S-CH_2CH_3, CEES] was obtained from Sigma Chemicals (St. Louis, MO).

Animals and CEES treatment. Male guinea pigs (Hartley strain, 5–6 weeks old, 400 g body weight) were obtained from Harlan Sprague–Dawley (Indianapolis, Indiana). Animals were injected intratracheally single doses of CEES (0.5–6.0 mg/kg body weight) in ethanol (total injection volume was 100 μl per animal). Control animals were injected with 100 μl ethanol in the same way intratracheally. The animals were sacrificed at different time intervals (1, 4, and 6 h, 1 day, 7 day, 14 day, and 21 day) after CEES injection. Excepting for timepoints up to 6 h, the animals were sacrificed at the same time each day to offset gene changes induced by diurnal variations. Body weight gain was recorded every week. The tissues were flash-frozen in liquid N_2 and kept at −70 °C for future use.

Preparation of RNA. Total RNA was isolated from frozen brain slices of male guinea pigs treated with varying doses of CEES (0.5–6.0 mg/kg body weight) for 7, 14 or 21 days. Untreated male animals were used as controls. Total RNA was isolated from guinea pig brain using TRI-Reagent supplemented with 1 μl/ml polyacryl carrier, according to the manufacturer's recommended protocol (Molecular Research Center, Cincinnati, OH, USA). Briefly, 50 mg tissue/ml of TRI-Reagent was homogenized for 45 s and then incubated at room temperature for 5 min. Following elution of RNA, the A_{260}/A_{280} ratio of the samples was determined by spectrophotometry. RNA integrity was confirmed by electrophoretic traces on a BioAnalyzer instrument (Agilent Technologies, Wilmington DE).

Preparation of cDNA templates. All RNA samples were DNased using the DNA-free kit (Ambion, Austin TX) according to protocol. The samples were then quantitated by RiboGreen (Molecular Probes). Glyceraldehyde-6-phosphate dehydrogenase (GAPDH) gene expression was analyzed using real-time PCR [PE Applied Biosystems (ABI), Foster City, CA, USA] in the absence of reverse transcriptase to ensure the samples were free of genomic DNA. The samples were then converted to cDNA using the High Capacity cDNA Archive Kit according to manufacturer's recommendations (Applied Biosystems, Foster City, CA, USA). Samples were diluted to a final concentration of 5 ng/μl of cDNA.

Analysis of mRNA gene expression. Guinea pig oligonucleotides were designed for use in real-time PCR using a modified version of Primer 3 (MIT, Cambridge, MA) and synthesized according to the protocols of Keystone labs (BioSource International, Camarillo, CA) (forward: 5′-CCTTGCTGCTTCAGATCTCCA-3′; reverse: 5′-AGGCACTCCAG-CAGGTGTCT-3′, encompassing nucleotides 44–140 of guinea pig POMC mRNA, GenBank Accession No. S78260). The synthesized oligonucleotides were diluted in 10 mM Tris–HCl, 0.1 mM EDTA, pH 8.0 buffer at a mix stock concentration of 3 μM each of forward and reverse primers. Pro-opiomelanocortin gene was assayed using real-time PCR, the SYBR Green 1 Double-stranded Binding Dye assay, and the ABI 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). Ten microliter of a mix containing final concentrations of 1× PCR SYBR master mix, 300 nM primer mix and water, was added to a well of a 384-well optical plate containing 12.5 ng of template (5 ng/μl). The PCR cycling conditions were 95 °C for 10 min and 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Dissociation curve analysis was performed for all experimental samples and the resulting melting temperature was generated to confirm the specificity of the product.

Statistical analysis. Data for biochemical and physiological measurements were analyzed by ANOVA and the significance level was set for $p < 0.05$. Gene expression results are normalized to expression of GAPDH gene in each sample. A non-parametric, Wilcoxon rank-sum test was used to evaluate the changes in POMC gene expression in control and CEES treated brains.

Results

Effects of CEES exposure on body weight gain

There was a gradual weight gain in guinea pigs exposed to varying doses of mustard gas. As shown in Fig. 1, body weight gain was significantly higher ($p < 0.05$) in the CEES exposed group at every time period tested (7, 14, and 21 days post-treatment at 0.5 mg/kg dose), as assessed by a two-tailed t test. No significant difference in feeding

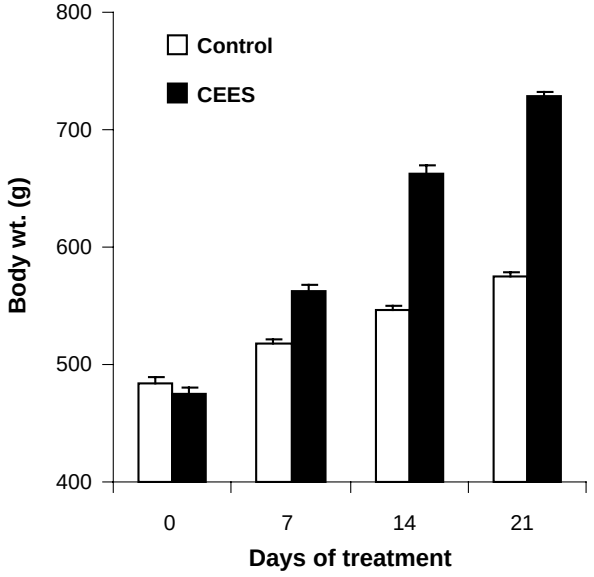

Fig. 1. Effect of CEES exposure on body weight gain in guinea pigs. CEES was injected at a dose of 0.5 mg/kg body weight intratracheally. Values reported are means ± SEM of six animals. Body weight gain was significantly higher ($p < 0.05$) in experimental group at each time periods (7, 14, and 21 days post-treatment), assessed by a two-tailed t test.

behavior was noted among the control and treated groups, suggesting that the weight gain was not related to food intake.

Effects of CEES exposure on POMC gene expression

Using real-time PCR, we investigated the levels of POMC gene expression in the brains of guinea pigs that had been exposed to mustard gas at various concentrations for various periods of time. The results from three independent studies are summarized in Figs. 2A–B. As shown in Fig. 2A, exposure of guinea pigs for 24 h with 1 mg/kg CEES resulted in a statistically significant reduction in POMC RNA levels in the brain (POMC message was unchanged in lung and liver tissues; also message levels for neuropeptide Y and lipoprotein lipase genes were unchanged in brain, lung, and liver tissues, data not shown) as estimated by Wilcoxon rank-sum test ($p < 0.03$) or two-tailed t test ($p < 0.06$). The reduction in POMC message did not occur until after 6 h of exposure to CEES and near maximal levels of reduction were achieved by 24 h of treatment when compared to 7 and 14 days of CEES exposure (Fig. 2B). Similar reduction in POMC gene expression was also observed for higher doses of CEES (data not shown).

Discussion

Pro-opiomelanocortin (POMC) is a prohormone yielding bioactive peptides that are generated in the hypothalamic neurons and act as endogenous ligands for the

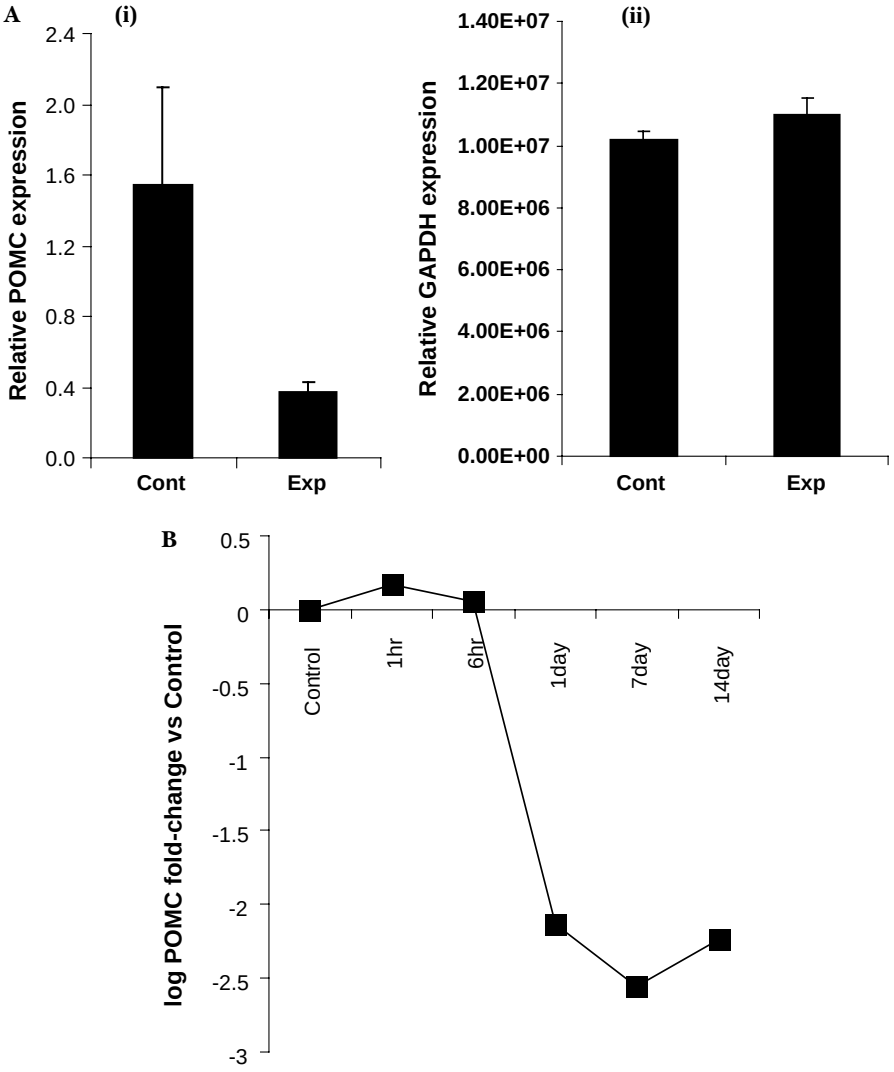

Fig. 2. Effect of CEES exposure on POMC RNA levels measured by quantitative RT-PCR. (A) Relative expression of (i) POMC and (ii) GAPDH in control and CEES treated guinea pig brains (1 mg/kg CEES for 1 day). POMC message levels were normalized to the expression of GAPDH in the same sample. Since POMC message levels were very low compared to GAPDH message levels, an arbitrary multiplier of 10,000 was applied to the normalized POMC values to arrive at the 'Relative POMC Expression' plotted on the y-axis. Mean expression values for the two groups are plotted, and the SEM for each group is shown ($n = 4$ in each group). (B) Time course of POMC expression in guinea pigs treated with 0.5 mg/kg CEES. POMC expression values were obtained by the method described in (A). The fold-change in POMC message levels compared to control are plotted for every time point (values expressed in the $\log_{10}$ scale).

melanocortin-4 receptor, a key molecule involved in appetite control and energy homeostasis. Several lines of evidence establish a role of POMC in the development of obesity. Although rare, inactivating mutations in POMC in severely obese patients have been found [20] and the phenotype displayed by POMC null mice is remarkably similar to that seen in human mutant POMC subjects [21]. POMC also serves as a quantitative trait loci in obesity and correlates, in part, with variation in serum leptin levels [22]. POMC messenger RNA levels are decreased in leptin deficient (ob/ob) and leptin receptor deficient (db/db) mice compared with controls [23]. Thus, the inactivation or reduction in POMC levels appears to be positively associated with the development of obesity. In this study, we report the unexpected finding relating a gradual weight gain in guinea pigs exposed to mustard gas. The biochemical mechanism for the observed weight gain appears to involve POMC since POMC levels decrease as the animals gain weight. Thus, the sulfur mustard, CEES, appears to act as a signaling trigger for the reduction in POMC. The exact mechanism of how CEES induces a decline in POMC message presently is not clear. One could hypothesize that CEES acts as a mutagen and reproducibly induces inactivating mutations in the POMC gene; alternatively, CEES may act as a cell signaling trigger that results in a decrease in POMC transcription in the guinea pig model. The magnitude of the decrease in POMC message varied considerably (approximately 3- to 100-fold over control) but consistently among independent experiments. It is also interesting to note that although POMC is traditionally related to feeding behavior, we observed no noticeable change in feeding in the animals despite decrease in their POMC transcripts. This is suggestive of additional functions of POMC in the control of body weight.

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