EM-X Herbal Tea Inhibits Interleukin-8 Release in Alvelor Epithelial cells

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The aim of this study was to determine whether EM-X can inhibit the hydrogen peroxide (H_2O_2) and TNF-a-mediated release of the pro-inflammatory cytokine IL-8 in human alveolar epithelial cells (A549). Airway inflammation is a characteristic of many lung disorders including asthma, chronic obstructive pulmonary disease, adult respiratory distress syndrome and idiopathic pulmonary fibrosis. There is recruitment of immune and inflammatory cells, which are activated to produce mediators of inflammation including oxidants and cytokines, such as the pro-inflammatory cytokine tumor necrosis factor- a (TNF- a). Oxidative stress has been implicated in the pathogenesis of several inflammatory lung disorders. One consequence of this process is to enhance the expression of both pro-inflammatory lung disorders. One consequence of this process is to enhance the expression of both pro-inflammatory lung disorders. One consequence of this process is to enhance the expression of both pro-inflammatory and protective antioxidant genes. Oxidants and inflammatory mediators such as tumour necrosis factor-a (TNF- a) active transcription factors such as NF-?B and Activator Protein-1(AP-1). Interleukin-8 (IL-8) is a ubiquitous inflammatory chemokine that mediates a multitude of inflammatory events in the lung. Hydrogen peroxide (H_2O_2) (100 µM) and TNF- a (10 ng/ml) imposed oxidative stress in A549 cells as shown by depletion of the antioxidant reduced glutathione (GSH) concomitant with increased levels of oxidised glutathione (GSSG). EM-X inhibited both H_2O_2 and TNF-a mediated activation of NF-?B and AP-1. Both H_2O_2 and TNF- a significantly increased IL-8 release, which was inhibited by pre-treatment of A549 cells with EM-X compared to the control untreated cells. This study shows that EM-X inhibits both the oxidant H_2O_2 and the pro-inflammatory mediator, TNF- a induced IL-8 and suggests a mechanism for the anti-inflammatory effects of EM-X. We conclude that EM-X may have therapeutic potential in Neuroinflammation and inflammatory lung diseases.

INTRODUCTION

The general wellness of individuals and management of human clinical conditions in which a role for free radicals and oxidants are implicated continues to underpin the search for non-toxic antioxidants. Free radical reaction of lipid peroxidation is an important issue in the food industry where manufacturers minimize oxidation in lipid containing foods by use of antioxidants during the manufacturing process – foods are produced that maintain their nutritional quality over a defined shelf life. Biomedical scientists and clinicians are interested in antioxidants because they protect the body against damage by reactive oxygen species. The cocktail EM-X is a novel antioxidant drink derived from

rice bran and seaweed extracts that were fermented with Effective Microorgnaisms (EM). EM-x contains over 35 minerals, a-tocopherol, lycopene, ubiquinone, saponin and flavonoids (including quercetin, quercetin -3-0-glucopyranoside, quercetin-3-0-rhamnopyranoside). Interest in plant phenolics has increased greatly because of their roles as antioxidants and scavengers of free radicals and their potential effects on human health. Extensive studies on the acute and chronic toxicity of EM-x by EMRO have shown that the beverage is safe for oral administration.

ANTIOXIDANTS AND CELLULAR RESPONSES

TNF- a is a pleiotrophic protein that medicates a multitude of inflammatory events in the lung. The induction of inflammatory mediatory mediators can be regulated by the activation of redox-sensitive transcription factors Activator Protein-1 (AP-1), (c-Fos/cJun) and nuclear factor-kappa B (NF-?B) stimulated in response to oxidants and TNF-a. Reactive oxygen species (ROS) and cellular redox status, particularly intracellular thiol status can be directly involved in the activation of AP-1 and NF-?B. TNF-a increases AP-1 binding via the MAP kinase pathway. TNF-a has also been shown to affect the local tissue oxidant/antioxidant balance. Binding of AP-1 and NF-?B leads to the transcription of genes for both the protective antioxidants such as ?-glutamylcysteine synthetase (?-GCS), as well as several inflammatory mediators and chemokines, including IL-8.

Interleukin-8 is a major chemotactic and activating mediator for polymorphonuclear leukocytes (PMN) in the lungs. Thus modulation of its production may production may provide a therapeutic target in inflammatory lung diseases. Transcription factors such as NF-?B and AP-1 have been shown to be involved in the transcriptional activation of IL-8. These transcription factors may be activated by a variety of extra cellular signals, acting via surface receptors, and leading to kinase-mediated phosphorylation cascades. The transcription factors NF-?B and AP-1, which are activated by inflammatory stimuli, switch on inflammatory genes, including IL-8 leading to increased synthesis of inflammatory proteins.

Various approaches aimed at augmenting the lung's antioxidant shield have been used to increase cellular defences against oxidative stress, and hence to reduce lung inflammation. One potential therapy is to enhance thiol levels in lung cells. Thiol antioxidants such as N-acetylcysteine (NAC), that has been de-acetylated into cysteine can react directly with ROS, and also act as a precursor for glutathione synthesis. There is a possibility that NAC may have a deleterious effect on alveolar macrophages, neutrophils and lung epithelium. We were also interested in the effects of the natural antioxidant ergothioneine. Ergothioneine (2-mercaptoshistidine trimethylbetaine) (EGT) is a naturally occurring antioxidant that is abundant in most plants and animals, with human blood values reported as 4- 14mM. Humans can only absorb ergothioneine from a plant diet. Investigations in our laboratory have shown that NAC at concentrations above 3 mM is toxic to N-18-RE-105 (primary rat embryonic retinal cell crossed with mouse neuroblastoma cell), with cell viability averaging 50%.

Increasing concentrations of ergothioneine increased viability in cells supplemented with ergothioneine prior to the administration of 5 mM NAC. This suggests that ergothioneine can permit cellular tolerance of N-acetyl cysteine. This may be beneficial to pulmonary macrophages. It is therefore important to begin to understand the role of dietary antioxidants in the oxidative stress/signal transduction mechanisms involved in cellular responses.

Thus we hypothesise that EM-X regulates the TNF-a and H_2O_2-mediated release of the pro-inflammatory mediator IL-8 through a redox-mediated mechanism. Therefore, we studied the effect of EM-X on TNF-a and H_2O_2-induced release of IL-8 release in alveolar epithelial cells (A549) and compared it with that of ergothioneine.

MATERIALS AND METHODS

EM-X was provided by EMRO, Okinawa, Japan and ergothioneine was obtained from OXIS Health Products, Portland, Oregon, USA. The human type II alveolar epithelial cell line, A549 (ECACC No. 86012804) was maintained in continuous culture at 37^{o}C, 5% CO_2 in Dulbecco's modified minimum essential medium (DMEM) containing L-glutamine (mM), penicillin (100 U/ml), streptomycin (100 µg/ml) and 10% foetal bovine serum (FBS). Monolayers of A549 cells grown to approximately 80-90% confluency in 6 well plates containing 10% FBS were washed in CMF-PBS and exposed to the treatments in 2% serum-containing media. All treatments were performed in duplicate. The cells were treated with H_2O_2 (100 µM) or TNF-a (10 ng/ml) alone or with the addition of EM-X (100 µl/ml) the cells were incubated in 2 ml of 2% serum containing medium at 37^{o}C, 5% CO_2 for 1 hour and 20 hours. The monolayers were washed with cold sterile calcium and magnesium free PBS (Ca^{2+}/Mg^{2+} free PBS)and the cells scraped off the wells using a Teflon scrapper (Corning Coster, High Wycombe, UK). The culturemedia were used for IL-8 determination. An enzyme-linked immunosorbant assay (ELISA) was used to measure IL-8. All plates were read on a microplate reader (Dynatech MR 5000, Aldermaston, UK) and analyzed suing a computer-assisted analysis program (Assay ZAP, Blosoft, Cambridge, UK). Typically, standard curves generated with this ELISA were linear in the 50- to 2,500-pg IL-8 / ml range. Only assays having standard curves with a calculated regression line value >0.95 were used for further analysis. Il-8 levels were measured by the ELISA method. The data are expressed as mean ± S.E.M. Differences between values were compared by ANOVA and a two-way unpaired t-test.

RESULTS AND DISCUSSIONS

We sought to provide preliminary data to show the modulation by EM-X in these processes. Increased IL-8 release was observed in A549 cells following 20 hours of exposure to TNF-a and H_2O_2 compared to untreated cells ($p<0.01$) (Figure 1 and 2). Indeed EM-X (100µl/ml) inhibited both H_2O_2 and TNF-a-mediated activation of NF-?B and AP-1. Both H_2O_2 and TNF-a significantly increased IL-8 release, which was inhibited by pre-treatment of A549 cells with EM-X compared to the control untreated cells. EM-X inhibits both the oxidant H_2O_2 and the pro-inflammatory mediator TNF- a induced and IL-8 release, which is associated with decreased AP-1 and NF-?B activation. Fig. ? 1 shows the key result for EM-X. Pre-treatment of A549 cells for 2 hours with

ergothioneine inhibited both TNF-a and H_2O_2-induced IL-8 release in A549 cells ($p<0.01$).

Lung cells, in particular alveolar epithelial type II cells, are susceptible to stimuli such as oxidants and cytokines released into the local airspace environment. Reduced glutathione (GSH) is a tripeptide (L-?-glutamyl-L-cysteinly-glycine) containing a thiol (sulfhydryl) group. The GSH redox status is critical for various biological events that include transcriptional activation of specific genes, modulation of redox-regulated signal transduction, regulation of cell proliferation, apoptosis and inflammation. Maintenance of a high intracellular (GSH/GSSG) ratio (>90%) minimizes the accumulation of disulfides and provides and provides a reducing environment within the cell. However, if oxidative stress alters this ratio, this shift in the GSH/GSSG redox buffer influences a variety of cellular signaling processes, such as activation and phosphorylation of stress kinases (JNK, p38, MAPK, PI-3K) via sensitive cysteine rich domains; activation of the transcription factors AP-1 and NF-?B leading to increased gene transcription.

TNF-a and H_2O_2 increased the NF-?B and AP-1 activation and DNA binding in A549 cells. This suggests that alteration of GSH redox ratio may be sufficient to activate NF-?B and AP-1 in A549 cells.Co-treatment of A549 cells with ergothioneine and TNF-a or H_2O_2 inhibited NF-?B and AP-1 actiaavtion and DNA binding, suggesting that ergothioneine inhibits NF-?B and AP-1 actviation by a mechanism depending on its thiol-mediated antioxidant property. ROS and cellular redox status, particularly intracellular thiol status can be directly involved in the activation of AP-1 NF-?B. TNF-a increases AP-1 binding via the MAP kinase (stress activated protein kinase/JNK) signaling pathway, and activates NF-?B via the I?-B kinase pathway. The inhibitory effect on H_2O_2 and TNF-a-mediated release of IL-8 suggests that EM-X and ergothioneine may be acting to inhibit signal transduction pathway. IL-8 is a chemokine released during inflammation and is important in the recruitment and activation of immune and inflammatory cells. Moreover, oxidative stress has been shown to mediate IL-8 synthesis. IL-8 induction is associated with the activation of the nuclear transcription factors AP-1 and NF-?B.

Hence, it is likely that therapeutic thiol and dietary antioxidants have the potential to attenuate lung inflammation. The thiol compounds such as N-acetyl-L-cysteine also inhibits IL-1-induced activation of transcription factors AP-1 and NF-?B, and IL-8 release. In these cases, ROS and TNF-a would lead to an augmented increased inflammatory response. EM-X inhibits the H_2O_2 and TNF-a-mediated IL-8 release from alveolar epithelial cells suggesting that EM-X may be a potential antioxidant/anti-inflammatory therapy to inhibit the chronic inflammatory response, which occurs in the development of chronic inflammatory lung diseases.

The induction of IL-8 protein secretion and mRNA synthesis by H_2O_2 and TNF-a occur as a result of increased gene transcription in A549 cells and for other cell types. Therefore, we investigated the activation of the IL-8 promoter by measuring gene activity in a CAT reporter assay following transient transfection into A549 epithelial cells. Indeed H_2O_2 and TNF-a up-regulated the promoter region of the IL-8 gene, and this was

inhibitable by co-treatment with ergothioneine in A549 cells. We expect similar results with EM-X. This study has implications for inflammatory lung disease and other disease states where IL-8 in increased. Thus further studies are warranted.