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Ebselen a Seleno-organic Molecule Inhibits Alteration in the Biological Responses in Hypoxic Human Alveolar Lung Epithelial Cells

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Authors' contributions

Author NK designed the study, managed the literature search and wrote the first draft of the manuscript with assistance from author KR. Authors NK and AP performed the experiment. Authors SBS and DP edited the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Ebselen is a lipid-soluble seleno-organic compound whose benefits have been shown in a variety of diseases and in experimental studies including anti-inflammation, anti-proliferation, anti-angiogenesis, anti carcinogenesis and anti-oxidation properties. This study aimed to evaluate the effect of ebselen against hypoxia-induced biological responses in human alveolar epithelial cells (A549 cells). Hypoxia treatment increased the generation of reactive oxygen species, proinflammatory cytokines/chemokines, cell death and proliferation in A549 cells in a time-dependent manner. Notably, Ebselen treatment significantly reduced the production of reactive oxygen species and levels of biochemical markers for lipid peroxidation. Ebselen treatment increased the antioxidant enzyme superoxide dismutase activity as well. This seleno-organic compound also reduced the production of proinflammatory chemokines IL-8 and proinflammatory cytokine TNF-α. It is worth noticing that, Ebselen enhanced the viability as well as proliferation of

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hypoxic A549 cells. Collectively, these data demonstrate that the Ebselen attenuates the alterations in biological responses in hypoxic human alveolar epithelial cells. Our results further suggest that that ebselen could be used as a potential chemopreventive compound to restore the normal biological response in hypoxic cells and thus inhibiting the progression of the lung carcinoma.

Keywords: Hypoxia; oxidative stress; ebselen; inflammation; alveolar lung epithelial cells; A549 cells; reactive oxygen species; lung carcinoma.

1. INTRODUCTION

Oxidative stress, the imbalance between antioxidant defence and oxidant production inside the cell, is implicated in the onset and progression of many health problems. One of the important effects of oxidative stress and free radical generation is the decreased levels of cellular antioxidants. Along with regulating many biological functions such as cell death, proliferation and release of proinflammatory cytokines/chemokines in epithelial cells, hypoxia increases reactive oxygen species (ROS) production and causes degradation of polyunsaturated fatty acids. ROS leading to oxidative stress in the biological system, has been involved in the pathogenesis of various human inflammatory diseases [1-3].

Hypoxia induced oxidative stress increases the production of proinflammatory cytokine TNF-α which modulates the epithelial cell biology [3,4]. TNF-α has been shown to disrupt tight junctions and perturbate cell proliferation and cell migration [5-7]. Additionally, hypoxia triggers an increased release of chemokine IL-8 that causes recruitment of tumour associated macrophages. In lung carcinogenesis, infiltration of macrophages to the tumour site is an essential process for progression of the disease. Tumour associated macrophages increase cancer cell invasion, migration, angiogenesis, tumor progression or metastasis [8,9].

Ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one) a synthetic lipid-soluble seleno-organic compound that has been shown to possess anti-inflammation, anti-proliferation, anti-angiogenesis, anti carcinogenesis and anti-oxidation properties. Many epidemiological and experimental studies have also suggested the beneficial effect of ebselen in a variety of diseases including neurological disorders, acute pancreatitis, noise-induced hearing loss, and and in cardiotoxicity [10-17]. In addition ebselen has been proved to exhibit antiatherosclerotic, antithrombotic and cytoprotective properties [18-21]. The high electrophilicity of ebselen enables it to react with multiple cysteine residues of various proteins [22]. Despite extensive research on ebselen, its target molecules and mechanism of action remains obscure. In contrast to inorganic selenium, the toxicity of ebselen is minimal as it cannot enter selenium metabolism in the organism [23]. However, some reports had indicated that ebselen can also induce cell death in human tumor cell lines, suggesting a potential application of ebselen as an antitumor agent as well.

In the present study, we hypothesized that since administration of ebselen helped to avoid cardiotoxicity induced by daunorubicin by altering the redox state, it could have a similar effect in alveolar epithelial cells. A preliminary investigation was carried out using human epithelial cells. The levels of inflammatory cytokines-IL6, IL-8 and TNF alpha were also determined. To investigate the protective role of ebselen, the study was repeated after preincubating the cells with ebselen. The results indicate that hypoxia-induced cytotoxicity in human alveolar A549 cells is reduced by ebselen antioxidant and cytoprotective properties

2. MATERIALS AND METHODS

2.1 Chemicals

Dulbecco's Modified Eagle's Medium, fetal bovine serum, penicillin/streptomycin, trypsin EDTA, cell culture reagents were procured from Invitrogen, (Carlsbad, CA). 2′, 7′ dichlorofluorescein diacetate (DCFHDA), o-phthaldehyde (OPT) and bovine serum albumin (BSA) were obtained from Sigma-Aldrich, (St. Louis, MO). Lactate dehydrogenase (LDH) assay kits were procured from Randox laboratories, UK. Cytokines ELISA kits were purchased from BD Biosciences, (Franklin Lakes, NJ). Ebselen was obtained from (M P Biomedicals, USA). All other chemicals were of reagent grade or the highest quality available from Merck.

Cell Culture and Hypoxic Exposure: Human alveolar adenocarcinoma epithelial A549 cells (National Centre for Cell Science, Pune, India) were cultured in Dulbecco's Modified Eagle medium (Gibco, USA) supplemented with 2 mM L-glutamine, 10% FCS, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37°C in an incubator with 5% CO_2. The cells were exposed to hypoxia for 0, 6, 24 and 48h by transferring the culture flasks to a humidified incubation chamber maintained at 37°C and flushed with a gas mixture consisting of 0.5% O_2, 5% CO_2 balanced with N_2. The controls were maintained under normoxic conditions throughout the experiments.

2.2 Cytotoxicity/Cell Viability Assays

2.2.1 LDH assay

The cytotoxicity of cells was estimated by measuring the lactate dehydrogenase (LDH) released in the culture medium with LDH assay kit (Randox Laboratory, UK) as per manufacturer's instructions. The enzyme activity was estimated as (U/ mg protein) and expressed as a percentage, with normoxic control values set at 100%.

2.2.2 MTT assay

MTT 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan) assay was employed to quantitatively evaluate cell viability. The MTT assay was performed 6, 24 and 48 h after hypoxia with and without ebselen treatment. In brief, viable adherent cells were stained with MTT (2 mg/ml) for 2 h. The media were removed, and the formazan crystals produced were dissolved by adding 200 µl of dimethyl sulfoxide. Then, the absorbance at 540 nm was determined using Synergy H4 hybrid reader from BioTek.

2.3 Free Radical Estimation

Reactive oxygen species (ROS) levels were measured with DCFH-DA. ROS assay was done by the modified method described earlier [24]. Briefly, 150 µl sample was mixed with 10 µl of 100 µM DCFHDA and incubated for 40 min in the dark at 37°C. After incubation a sample total volume of 3ml was made up by adding PBS. Fluorescence was monitored at λ_{Ex}485 nm and λ_{Em} 530 nm. The data were expressed as percentage of the unexposed control.

2.4 Measurement of Malondialdehyde

Malondialdehyde (MDA), one of the end products of lipid peroxidation was analyzed by the modified method of Utley et al. [25]. Briefly, Equal volume of sample and 10% TCA in dH_2o was mixed and centrifuged at 3000 rpm for 10 min, supernatant was collected. Equal volume of supernatant and 0.67% TBA in 0.05 N NaOH was mixed and boiled for 15 min. Absorption was recorded at 532 nm and TEP was used for calculation of standard curve.

2.5 Determination of Superoxide Dismutase

SOD assay was performed as described before (Marklund & Marklund, [26]). In brief, 800 µl of dH_2o were added to 1.5 ml of 50 mM SOD buffer (Tris- Cacodylate buffer, 60% Tris buffer + 40% Cacodylate buffer). 100 µl of sample was added followed by 300 µl Triton (0.01%), 10 µl Pyrogallol (60 mM) and 30 µl NBT 1mM). OD was taken at 540 nm for 3 minutes at the interval of 31 seconds using Synergy H4 hybrid reader from BioTek.

2.6 Measurement of Inflammatory Cytokines

Supernatants were withdrawn from the wells and measured for various cytokines, IL-8 and TNF-alpha by the ELISA kits according to the manufacturer's protocols (BD optEIA, BD Biosciences, USA).

2.7 Statistical Analyses

GraphPad Prism 5 statistical analysis software was used for all statistical analyses performed in this work (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, California, USA). Data were analyzed by one-way ANOVA and compared to the corresponding control by the Bonferroni post test. Differences between treated samples and controls were considered statistically significant for P-values <0.05(*) and non-significant for P-values >0.05.

3. RESULTS

Several aspects of the antioxidant role of ebselen have been studied previously [12,13,14] however, an extensive study comparing the antioxidant potential of ebselen in A549 cell lines has not been reported. Here, we report the

antioxidant prospective of ebselen in hypoxia induced oxidative stress in A549 cells. The A549 epithelial cell line has been used in many studies as an experimental model of alveolar type II epithelial cells. A major advantage of these cells is that they can be grown in large numbers and manipulated under reproducible conditions.

3.1 Ebselen Attenuates Hypoxia Induced Oxidative Stress in lung Epithelial Cells

To test the effect of ebselen on hypoxia induced ROS generation in lung carcinoma cell line A549, the cells were pretreated with ebselen for 1h before hypoxia exposure. The 0.5% hypoxia exposure resulted in increased the levels of ROS by 40% which further increased to 105% with extended exposure of up to 24h (Fig. 1). The levels of ROS remained unchanged between 24 to 48h exposures. DMSO, which is a vehicle for ebselen had no significant effect on hypoxia induced ROS generation at any time point. Ebselen pretreatment significantly attenuated the increase in ROS levels observed during hypoxia exposure.

3.2 Degradation of Polyunsaturated Fatty Acids is Inhibited by Ebselen in Hypoxic lung Epithelial Cells

Since we observed that hypoxia exposure resulted in increased levels of ROS and this increase was completely attenuated with pretreatment of ebselen, we sought to determine the levels of polyunsaturated lipids oxidation by measuring the levels of MDA with ebselen pre-treatment in lung epithelial cell exposed to 0.5% hypoxia. As expected the hypoxia exposure significantly increased the levels of MDA (by 30%) after 6h (Fig. 2). We did not observe any further increase in the levels of MDA at 24 or 48h after hypoxia exposure. Interestingly, ebselen pre-treated cells exposed to 0.5% hypoxia had attenuated levels of MDA.

3.3 Ebselen Increases Dismutase Activity in Hypoxic Lung Epithelial Cells

We observed that hypoxic exposure decreased the SOD activity (around 50%), whereas ebselen pre-treatment abrogated the effect of hypoxia on SOD activity and restored the SOD activity to the levels of control cells (Fig. 3). The increased levels of ROS during hypoxia exposure could be due to decrease in the activity of SOD. Once ebselen increased the activity of SOD to the levels of control, we observed a concomitant decrease in the levels of ROS as well as MDA.

3.4 Ebselen Inhibits Chemokine IL-8 Production in Hypoxic lung Epithelial Cells

It has been shown that hypoxia increases levels of chemokine IL-8 in variety of cells [31]. We sought to determine if hypoxic exposure because of increased ROS production also contributed towards increase in IL-8 levels in lung cancer cell line A549. Exposure to hypoxia resulted in an increased amount of IL-8 production from 60±10.42 pg/mg protein in control to 350.72±47.52, 797.47±96.91, 323.95±108.91 after, 6, 24 and 48h of hypoxia, respectively (Fig. 4). This increase was significantly reduced when cells were pretreated with ebselen, further supporting the anti-inflammatory potential of this molecule.

3.5 Ebselen attenuates of tumour narcosis factor production in hypoxic lung epithelial cells

In epithelial cells and more specifically in lung cancer cell lines the effect of hypoxia and ROS on TNF-α is not known. We observed that exposure of hypoxia in A549 cells increased the amount of TNF-α production 2562.44±125.70 pg/mL protein in control to 3659.30±1428.23, 8567.51±1068.49, and 5605±203.64 with hypoxic exposure to 6, 24 and 48h, respectively (Fig. 5). In consistency with the IL-8 data, TNF-α level were significantly compromised with pretreatment of ebselen.

3.6 Ebselen Inhibits Both Apoptosis and Proliferation in Hypoxic Lung Epithelial Cells

Controlled levels of apoptosis and proliferation in epithelial cells are essential for the normal function of epithelial cells. Any alterations in homeostasis could lead to cancer [3,4]. As we observed increased levels of TNF-α during hypoxia and subsequent inhibition with ebselen during hypoxia, we sought to determine the levels of apoptosis and proliferation. MTT is the basis of a commonly used cell viability assay that is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenase. Our data with both MTT and LDH release convincingly showed that hypoxic exposure

resulted in an increase of cell death rate. The latter was significantly inhibited by ebselen pretreatment. Hypoxic exposure increased the released levels of LDH from control 20.2±0.79 U/mg protein to 28.89±5.5, 35.77±3.6 and 39.44±2.8 U/mg protein, after 6, 24, 48h, respectively (Fig. 6). The ebselen pretreatment decreased the level of LDH to 21.62±3.78, 21.74±3.96 and 25.56±7.36 respectively. Similar results were obtained in by MTT assay where the cells exposed to hypoxia demonstrated an increased cell death as compared with ebselen pre-treated cells (Fig. 7). Both assays suggested that the pre-treatment of ebselen inhibited the effect of hypoxia on proliferation of A549 epithelial cell.

4. DISCUSSION

Oxidative stress, the imbalance between antioxidant defense and oxidant production in cells, is implicated in the onset and progression of many health problems [27,28]. One of the important effects of oxidative stress and free radical generation is the decreased levels of cellular antioxidants. Changes in the redox state could affect signalling pathways for biological processes and disrupt cellular functions. The results of the present study demonstrate that the synthetic seleno-organic compound, ebselen, exerts a potent inhibitory activity against hypoxia induced oxidative stress markers and inflammatory biochemical events A549 cells that are primarily associated with lung tumor progression.

Hypoxic condition has been shown to increase the levels of reactive oxygen species in epithelial cells [29,30,31]. Lung carcinoma is tumor where hypoxia is commonly observed, playing a major role in progression of the disease and failure to treatment [32]. A substantial part of the pharmacological profile of ebselen appears to be due to its action as an antioxidant with a unique mode of action. Ebselen is an effective scavenger of organic hydroperoxides, in particular, of lipid hydroperoxides [18]. Polyunsaturated fatty acids oxidation is frequently detected in hypoxic condition. Ebselen pre-treatment completely attenuated the increase in levels of ROS generated during hypoxia exposure. The DCFA A549 with the structural high electrophilicity of ebselen enables it to react with ROS and mimics glutathione peroxidase (GPx) activities [18,33]. Pre-treatment of ebselen might be reacting in spontaneous reaction and quenching the nucleophilic ROS. Another possibility is that these electrophilic centres can also react with multiple cysteine residues of various proteins could lead to loss of hyperactivated proteins activity involved in generation of ROS during hypoxic condition [22].

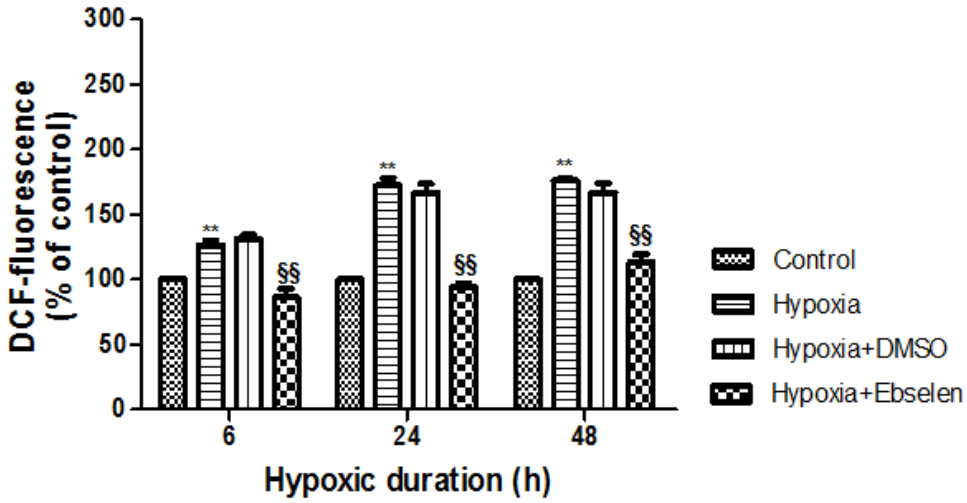

Fig. 1. Intracellular ROS levels in A549 cells

*ROS levels were measured either with or without treatment of ebselen followed by 0.5% hypoxic exposure for 6, 24 and 48h. Treatment of A549 cells with 20 μM ebselen significantly decreased the ROS levels in all groups. Values represent mean±SD (n=6). ** P < 0.01 compared with normoxic control, * P < 0.05 compared with normoxic control. §§ P < 0.01 hypoxia compared to hypoxia+ebselen*

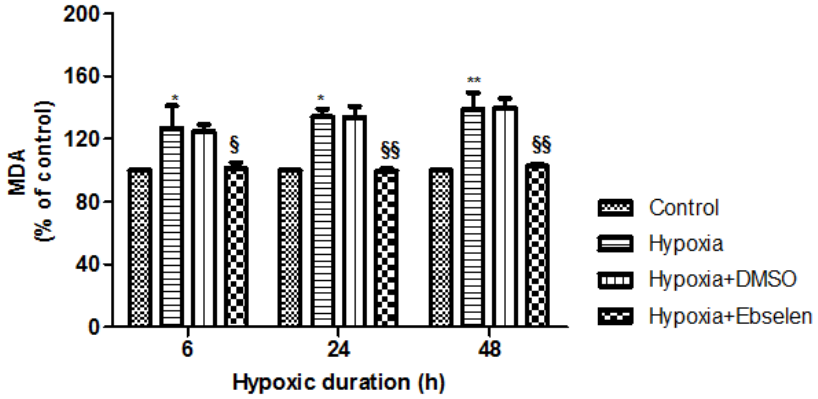

Fig. 2. Effect of hypoxia and ebselen on MDA level in A549 cells

A549 cells were exposed to 0.5% hypoxia for 0, 6, 24 and 48h. Percent MDA levels of indicated group of cells are expressed relative to non-treated control cells. Data are presented as the mean±SD of three independent experiments. § P < 0.05, §§ P < 0.01 hypoxia compared to hypoxia+ebselen

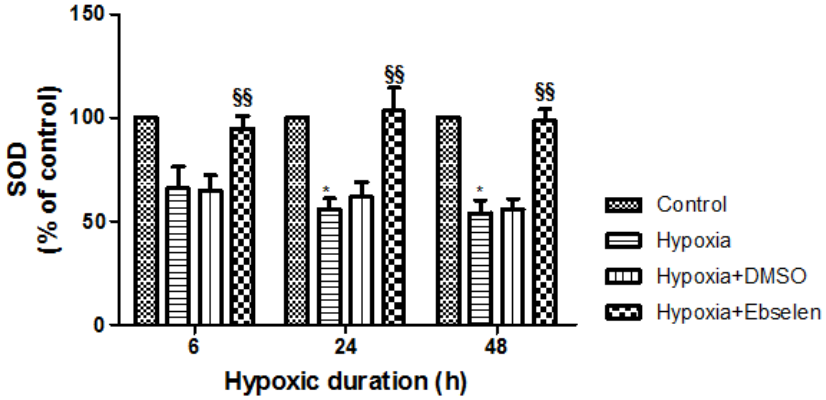

Fig. 3. Effect of hypoxia and ebselen on SOD level in A549 cells

*A549 cells were exposed to 0.5 % hypoxia 0, 6, 24 and 48h. The percent SOD levels is expressed as compared to non-treated control cells. Data are presented as the mean±SD of three independent experiments. *P < 0.05 compared with control, §§ P < 0.01 hypoxia compared to hypoxia+ebselen*

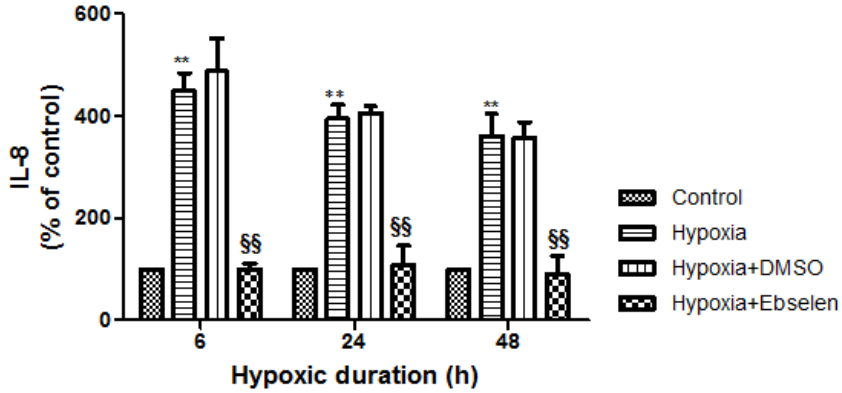

Fig. 4. Effect of hypoxia and ebselen on cytokines level in A549 cells

*A549 cells were exposed to 0.5 % hypoxia for 0, 6, 24 and 48h. Cytokine levels were measured using ELISA in supernatant of hypoxia exposed cells either with or without ebselen treamtent. The data is expressed as relative percent to non-treated control cells. *P < 0.05, ** P < 0.01 compared with control, §§ P < 0.01 hypoxia compared to hypoxia+ebselen*

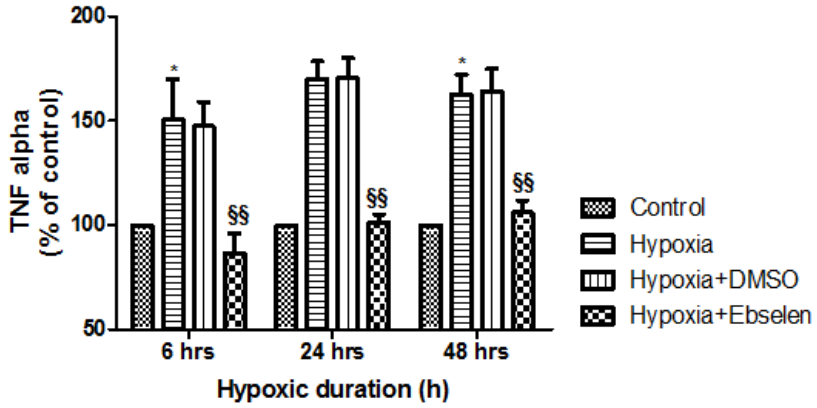

Fig. 5. Effect of hypoxia and ebselen on TNFα level in A549 cells

*A549 cells were exposed to 0.5 % hypoxia for 0, 6, 24 and 48h. TNF alpha levels was measured using ELISA in supernatant of hypoxia exposed cells either with or without ebselen treatment. The data is expressed as relative percent to non-treated control cells. *P < 0.05 compared with control, §§ P < 0.01 hypoxia compared to hypoxia+ebselen*

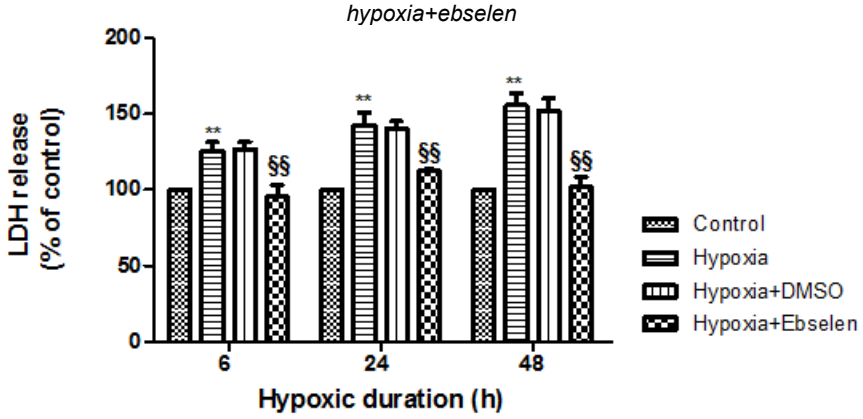

Fig. 6. Effect of hypoxia and ebselen on LDH release by A549 cells

*A549 cells were exposed to 0.5% hypoxia for 0, 6, 24 and 48h with or without ebselen. LDH level was measured immediately after hypoxic exposure. Values are expressed as percent of control levels from sister cultures maintained in normoxia for the same durations. Values represent mean±SD (n=6). ** P < 0.01 compared with normoxic control, * P < 0.05 compared with control, §§ P < 0.01 hypoxia compared to hypoxia+ebselen.*

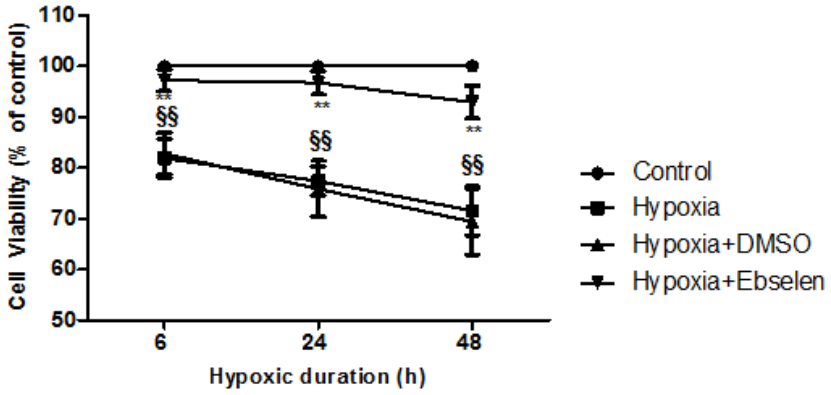

Fig. 7. Effect of hypoxia and ebselen on MTT assay in A549 cells

*A549 cells were exposed to 0.5% hypoxia for 0, 6, 24 and 48h with or without ebselen. Values are expressed as percent of control levels from sister cultures maintained in normoxia for the same durations. Values represent mean±SD (n=6). ** P < 0.01 compared with normoxic control, * P < 0.05 compared with control, §§ P < 0.01 hypoxia compared to hypoxia+ebselen*

Reactive oxygen species oxidizes polyunsaturated lipids and forms malondialdehyde (MDA). This reactive aldehyde reacts with many proteins and form adducts and this adductation could result in loss of function of target proteins. Not only this aldehyde can induce adduct in protein but can also react with deoxyadenosine and deoxyguanosine for adduct. This adduct would lead to mutagenesis in DNA. The hypoxic condition generated in lung carcinogenesis could result in secondary mutations in epithelial cells that could lead to progression of the disease [11]. We did not observe any further increase in the levels of MDA at 24 or 48 h after hypoxia exposure. This could be due to MDA is reacting with protein and DNA and thus did not increase the levels of free MDA. The ebselen treatment completely attenuated the generation of MDA by the cells. The Data correlate well with our observation that ebselen attenuates the increased levels of ROS generation in lung epithelial cell A549. Therefore, ebselen is clearly proven to be a useful chemopreventive agent.

It has been shown that hypoxia causes decrease in the levels of SOD mRNA expression in alveolar type II epithelial (ATII) cells, bronchiolar epithelial cells [34,35]. Levels of SOD protein in lung adenocarcinoma are decreased than in non-malignant lung tissue samples. The increased levels of ROS during hypoxia exposure could be due to decrease in the activity of SOD. Once ebselen increased the activity of SOD to the levels of control, we observed a concomitant decrease in the levels of ROS as well as MDA. This highlights the effectiveness of this compound as chemo preventive in lung carcinogenesis.

The presence of multiple areas of hypoxia (low oxygen tension) is a hallmark feature of human and experimental tumors [11,30,36]. Monocytes are continually recruited into tumors, differentiate into tumor-associated macrophages (TAMs), and then accumulate in these hypoxic areas [8,9]. It has been shown that hypoxia increases levels of chemokine IL-8 in variety of cells [36]. Chemokine IL-8 is known to recruit the monocytes. TAMs have been implicated in progression of the lung carcinoma. ROS has been also shown to regulate the levels of IL-8. We sought to determine if hypoxic exposure because this stimulus induces ROS production that could also increase the levels of IL-8 in lung cancer cell line A549. Exposure to hypoxia in A549 cells increased the amount of IL-8 production (Fig. 4).

This increase was significantly reduced with pretreatment of ebselen. In lung carcinoma the tumor area has hypoxia as well as substantial population of macrophages [8,9]. The recruitment of monocytes and then differentiation to tumor-associated macrophages could be a critical step for lung carcinogenesis. Our data demonstrating the inhibition of IL-8 production by ebselen during hypoxic condition suggest that this molecule can downregulate a critical step of carcinogenesis and could be used as chemo-preventive agent to halt the progression of the disease [10]. The underlying mechanism could be that ebselen is inhibiting hypoxia induced ROS mediated NFkB activation by quenching the ROS.

Hypoxia and ROS both has been shown to induce TNF-α production in variety of the cells [37,38] in epithelial cells and specifically in lung cancer cell lines the effect of hypoxia and ROS on TNF-α is not known. TNF-α, a proinflammatory cytokines plays a key role in tumour development. TNF has been shown to transactivate epidermal growth factor receptor (EGFR) as well as TNF Receptor (TNFR1/2). Activation of both pathways leads to either apoptosis or proliferation of the epithelial cell [6, 7]. This indicates that ebselen can modulate the TNF-α production and could be able to modulate the apoptosis/proliferation homeostasis. Acute hypoxic conditions in lungs may augment NF-kB activation in alveolar macrophages, resulting in enhanced release of inflammatory cytokines such as TNFα. The elevated levels of IL-6 and IL-8 and decreased level of IL-10 have already been reported under hypoxia; our study also demonstrated increased expression of IL-8 and TNFα, which was reversed by ebselen.

Normal physiological levels of apoptosis and proliferation in epithelial cells are critical for the function of epithelial cells. Any cell cycle imbalance could lead to cancer [3,4]. As we observed increased levels of TNF-α during hypoxia and subsequent inhibition with ebselen during hypoxia so we sought to determine the levels of apoptosis and proliferation. Our data with LDH release showed that hypoxic exposure resulted in an increase in cell death rate that was significantly inhibited by ebselen. The pre-treatment of ebselen inhibited the hypoxia effect on proliferation. As excessive apoptosis has been shown to be involved in process of carcinogenesis. In this case it is possible that

hypoxic condition increase apoptosis in lung epithelial cells and this nonphysiological apoptosis led to compensatory proliferation in epithelial cells. This cycle of apoptosis and proliferation may ultimately lead to survival and proliferation of cells with molecular aberrations and that these cells may transform eventually to become neoplastic cells. Since ebselen is inhibiting both apoptosis and proliferation, this may be able to restore the normal pathological levels of apoptosis and proliferation in cell under hypoxic condition. It is evident that extensive preclinical animal trials are required before ebselen can be used as a chemopreventive drug for lung carcinoma in humans.

5. CONCLUSION

In conclusion current study for the first time demonstrates that the addition of ebselen to cultured lung epithelial cells results in a reduction in ROS production, lipid peroxidation, cytokine production, a possible mechanism for antioxidative effect of ebselen in vivo. Considering the importance of oxidative damage in carcinogenesis, the antioxidant effect of ebselen, especially against hypoxia induced free radical generation and proinflammatory cytokine production could potentially be used for a cancer chemopreventive agent development.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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