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The selective protection afforded by ebselen against lipid peroxidation in an ROS-dependent model of inflammation

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Abstract

The effects of an experimental model of hydrogen-peroxide-induced foot pad oedema on indices of oxidative damage to biomolecules have been investigated. We have demonstrated increased levels of fluorescent protein and lipid peroxides occurring in plasma at 24 and 48 h post-injection. In addition, a decrease in the degree of galactosylation of IgG was observed which kinetically related the degree of inflammation and to the increase in protein autofluorescence (a specific index of oxidative damage).
The effects of ebselen, a novel organoselenium compound which protects against oxidative tissue injury in a glutathione-peroxidase-like manner, have also been examined in this model. Pretreatment of animals with a dose of 50 mg/kg ebselen afforded significant and selective protection against lipid peroxidation only. This effect may contribute to the anti-inflammatory effect of this agent in hydroperoxide-linked tissue damage.

Introduction

The generation of reactive oxygen species (ROS) during the respiratory burst is critical in the hosts defence against micro-organisms. However, in chronic inflammatory lesions such as the rheumatoid joint, ROS are believed to have a destructive role causing tissue injury, lipid peroxidation [1] and inducing autofluorescence (Ex max 360 nm, Em max 454 nm) and aggregation in IgG [2], thereby perpetuating localised inflammation. It has, therefore, been hypothesised that inhibitors or scavengers of ROS may protect directly against tissue injury and biomolecule degradation and, therefore, may be of therapeutic importance in the treatment of inflammatory diseases [3].
Ebselen (2-phenyl-1,2-benzisoselenazole-3(2H)-one) is a novel organoselenium compound which is reported to be efficacious in the treatment of hydroperoxide-linked tissue damage [4]. *In vitro* studies have shown its mechanism of action to be analogous to that of glutathione peroxidase by catalytically inactivating peroxides. In addition, it exerts an inhibitory effect on lipoxygenase, reducing leukotriene B_4 production and thus inhibiting neutrophil recruitment.
An animal model of inflammation has recently been developed which generates hydrogen peroxide *in situ* to mimic the generation of reactive oxygen species (ROS) in the respiratory burst of phagocytes and this causes localised oedema and cellular infiltration at the site of the lesion [5]. We have adapted this model to directly determine the contribution of biomolecular denaturation of lipid, protein and carbohydrate to the generation of an inflammatory lesion.

It has previously been reported that ebselen is an effective anti-inflammatory agent *in vivo*, by reducing oedema and preventing cellular infiltration in this model [6]. Therefore, we have examined whether the anti-inflammatory properties of ebselen *in vivo* are due to its ability to scavenge hydroperoxides such as hydrogen peroxide (H_2O_2) or lipid hydroperoxides, by examining protection against the following biochemical indices of oxidation and inflammation; lipid peroxidation (measured as thiobarbituric acid reactive material), autofluorescence induction and galactosylation status of IgG.

Materials and methods

All materials were from Sigma and buffer reagents from BDH (Analar grade), unless otherwise stated. Foot pad oedema was induced in a series of male Wistar rats by a single subplantar injection of amidated glucose oxidase (GO) (25 μg/50 μl). This enzyme produces H_2O_2 using endogenous glucose as a substrate.

Drug administration

Ebselen (A. Nattermann and Cie/Rhone-Poulenc) was dispersed in polyethylene glycol (PEG) 400 at a dose of 50 mg/kg and administered orally 1 h before challenge. Placebo animals received vehicle alone.

Lipid peroxidation

The extent of lipid peroxidation was assessed by the determination of thiobarbituric acid reactivity (TBAR) in plasma, using an adaptation of the method of Stocks et al. [7]. This reaction measures malondialdehyde released by a variety of peroxides and unsaturated aldehydes under acid conditions, by reaction with TBA to produce a 532 nm chromophore.

Protein autofluorescence

Exposure of IgG and albumin to ROS *in vitro* induces a characteristic autofluorescence spectrum due to oxidation of constituent aromatic amino acids [8]. This has been shown to be a dose-dependent response *in vitro*, and has been confirmed as an index of oxidative damage in proteins isolated from biological fluids. The extent of serum albumin autofluorescence was determined using gel permeation high-performance liquid chromatography with post column UV (280 nm) and fluorescence detection using an orthopthalaldehyde filter (Gilson) according to the method of Lunec et al. [9]. The results are expressed as the fluorescence to UV ratio, thereby correcting for any dilution effects of the inflammatory exudate, and using cytochrome c as an internal standard.

Agalactosylation of IgG

Previously, we have shown that inflammation is associated with a reduction in IgG galactosylation with concomitant exposure of *N*-acetyl glucosamine *in vivo* [10] and that this effect can be mediated by ROS *in vitro* [11]. To determine whether an H_2O_2 model of inflammation could modify the carbohydrate moiety and whether ebselen could protect against this damage during inflammation, IgG was isolated from serum and analysed by a modified enzyme-linked immunosorbent assay procedure using specific lectins (T vulgaris (TV) and E crystagalli (EC) for *N*-acetyl glucosamine and galactose, respectively) [11]. Results are calculated as the ratio of *N*-acetyl glucosamine to galactose as measured by the binding of the lectins TV and EC, respectively.

Statistical analyses

All statistical analyses were made with respect to the control (time 0) using an unpaired "*t*" test.

Results

Groups of rats were given ebselen (50 mg/kg) 1 h before a subplantar injection of amidated GO (25 μg) in one paw, and killed 4, 24 and 48 h later. A steady increase in lipid peroxide levels (assessed by TBA reactive material) was seen over the 24 h period post-challenge which reached a maximum 48 h post-challenge. In contrast, oxidative damage to the protein reached a maximum at 24 h showing a 17% ($p<0.02$) rise, after which levels started to decline. Again a similar response was seen when the degree of agalactosylation was examined; levels rose to a maximum (34% increase over control plasma, $p<0.01$) at 24 h. These data are summarised in Fig. 1.

Pretreatment of animals with ebselen, which inhibited cellular infiltration and oedema formation by

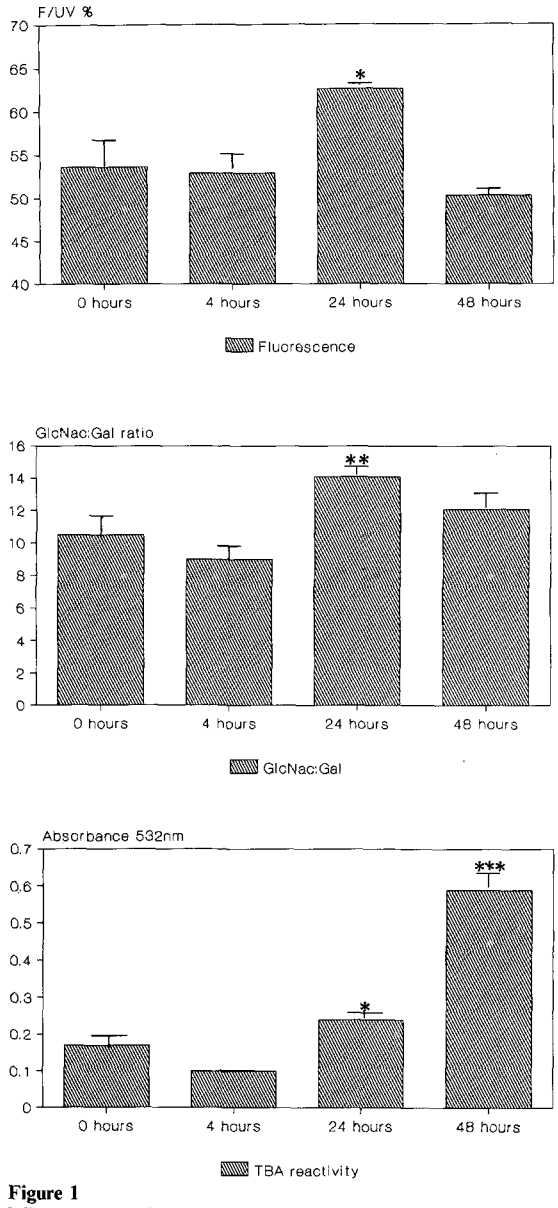

Figure 1
Effects of an ROS model of inflammation on the kinetics of plasma biomolecule degradation. (a) Protein fluorescence was determined by gel permeation chromatography and results are expressed as the mean ± SD of the fluorescence (F) to ultraviolet absorbance (UV) ratio. (b) Galactosylation status of IgG was determined using lectins in a modified ELISA and results are expressed as the mean ratio ± SD of the terminal *N*-acetyl glucosamine (Glc-Nac) to galactose (Gal). (c) Lipid peroxidation was determined by TBA reactivity and results are expressed as the mean absorbance ± SD of the TBA chromophore at 532 nm. For each group, $n=6$ and * $p<0.02$, ** $p<0.01$, *** $p<0.001$.

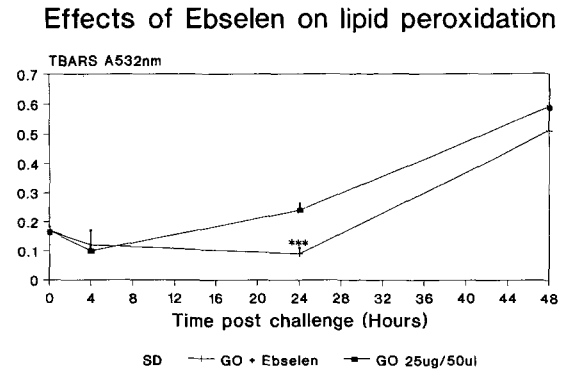

Figure 2
Protective effects of ebselen on induction of plasma lipid peroxidation (measured as TBA reactive substances – TBARS) with time. Animals received a single subplantar 25 µg/50 µl injection of glucose oxidase (GO) or were orally pretreated with 50 mg/kg ebselen 1 h before GO injection. Results are expressed as the mean ± SD of six measurements, where *** $p<0.001$.

50% at 4 h [6] significantly inhibited the formation of lipid peroxides at 24 h (Fig. 2). However, ebselen had no significant effect on oxidative damage to proteins (data not shown).

Discussion

The action of a subplantar injection of glucose oxidase on endogenous glucose to generate H_2O_2 *in situ* causes an oedematous lesion within 15 min of challenge [6]. This is believed to be due to the cytotoxic effect of H_2O_2 on endothelial cells, since uncontrolled production of ROS in close proximity to critical biomolecules can cause biochemical and functional modifications which may contribute to tissue injury and disease [2].

Herein, we have attempted to determine the importance of oxidative damage (induced by GO) to biomolecules such as protein and lipid to the development of an inflammatory lesion, by looking at the putative hydroperoxide scavenger ebselen. The use of heat-inactivated GO induced no acute inflammatory response, indicating that ROS production is central to the generation of inflammation in this model.

In vitro studies have shown that exposure of proteins such as IgG and albumin to ROS induces aggregation and a novel autofluorescence in the visible region (Ex max 360 nm, Em max 454 nm) [8, 12]. Furthermore, IgG from the sera and synovial fluid of patients with the chronic inflammatory

joint disease rheumatoid arthritis have been shown to have elevated levels of autofluorescence, indicative of increased oxidative activity [9]. An abnormality in the glycoform of IgG has also been reported in RA [13] and Axford et al. [14] have postulated that this modification is synthetic in nature, due to decreased activity of B cell galactosyl transferase. However, *in vitro* studies have shown that ROS can destroy the galactose moiety of IgG [11], indicating that agalactosylated IgG may be formed from the post-synthetic action of ROS. In our inflammatory model, we have successfully demonstrated an increase in serum protein visible fluorescence in association with a decrease in galactosylation of IgG. This work is the first reported "*in vivo*" observation of a decrease in galactosylation during an inflammatory episode. It has been suggested that the release of cytokines such as IL1 and IL6 during inflammation may have an indirect effect on the glycoform [15] by, for example, altering the expression of a specific gene such as B cell galactosyl transferase, thereby causing a synthetic defect in IgG galactosylation. However, we have shown that a significant alteration in the glycoform can occur by 6 h post-challenge; thus, it is unlikely that alterations in gene expression of a protein with a 25 day half-life, could explain such a rapid effect. In addition, the induction of visible fluorescence (a specific marker of ROS activity) and increase in agalactosylation occur in parallel, and these findings support the hypothesis that inflammatory mediators such as ROS may damage the carbohydrate moiety directly.

To investigate whether ebselen could protect against direct H_2O_2 injury, protein fluorescence was monitored. No protection was afforded by ebselen to protein oxidation nor was any protection of the carbohydrate moiety seen. These findings indicate either (1) that ebselen is not an efficient *in vivo* scavenger of H_2O_2 and that its breakdown to a more reactive species such as the hydroxyl radical proceeds more rapidly or (2) induction of protein fluorescence *in vivo* proceeds independently of hydroperoxide formation.

Perhaps the most well-documented work on the effects of ROS on biomolecules has been in the study of membranes and free polyunsaturated fatty acids. Peroxidative reactions within membrane lipids proceed very rapidly in the absence of any antioxidant protection; however, there has been some dispute as to whether they represent a burst of oxidative activity after cell death. Herein, we have described an initial increase in TBARs at 24 h, rising to a maximum at 48 h. These observations support the hypothesis that lipid peroxidation is largely an endstage event, occurring after cellular damage (maximal at 2 h [6]) and protein oxidation (maximal at 24 h). When the effects on lipid peroxidation were examined, however, in contrast to the study of protein oxidation, it was found to be completely protective at 24 h. This supports the action of ebselen as a glutathione-peroxidase-like antioxidant, specifically conferring protection against lipid peroxidation. The lack of protection afforded by ebselen at 48 h may reflect a relatively short duration of action of the drug, and we are presently investigating the effects of 12-hourly repeated doses of ebselen.

The data presented in this study have confirmed the importance of ROS as mediators of damage to biomolecules "*in vivo*" and have shown the effect of ebselen in suppressing oedema formation (as previously reported [6]), may be partly due to its capacity to inhibit lipid peroxidation. Finally, these data have provided the first evidence for a direct effect of inflammation on the IgG glycoform, thus showing that its occurrence in chronic inflammatory disease such as rheumatoid arthritis may merely reflect the inflammatory status of the disease.

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