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Determination of ebselen-sensitive reactive oxygen metabolites (ebROM) in human serum based upon N,N′-diethyl-1,4-phenylenediamine oxidation

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ABSTRACT

Background: Oxidative stress occurs through free radical- and non-radical-mediated oxidative mechanisms, but these are poorly discriminated by most assays. A convenient assay for oxidants in human serum is based upon the Fe^{2+}-dependent decomposition of peroxides to oxidize N,N′-diethyl-1,4-phenylenediamine (DEPPD) to a stable radical cation which can be measured spectrophotometrically.
Methods: We investigated modification of the DEPPD oxidation assay to discriminate color formation due to non-radical oxidants, including hydroperoxides and endoperoxides, which are sensitive to ebselen.
Results: Use of serum, which has been pretreated with ebselen as a reference, provides a quantitative assay for non-radical, reactive oxidant species in serum, including hydroperoxides, endoperoxides and epoxides. In a set of 35 human serum samples, non-radical oxidants largely accounted for DEPPD oxidation in 86% of the samples while the remaining 14% had considerable contribution from other redox-active chemicals.
Conclusions: The simple modification in which ebselen-pretreated sample is used as a reference provides means to quantify non-radical oxidants in human serum. Application of this approach could enhance understanding of the contribution of different types of oxidative stress to disease.

1. Introduction

Circumstantial evidence indicates that oxidative stress is a component of many chronic and age-related diseases, yet evidence from large-scale, double-blind interventional trials with the free radical-scavenging antioxidants, vitamins C and E, provides little support for involvement of free radical mechanisms [1]. An alternative hypothesis is that the quantitatively important oxidative reactions contributing to disease involve non-radical oxidant species, such as peroxides, which disrupt redox signaling and control mechanisms [2]. However, distinction between radical and non-radical mechanisms of injury is technically challenging, especially when both free radicals and non-radical oxidants are present in a biologic system.

Another difficulty concerns the nature of oxidants relevant to disease risk. Although much of the oxidative stress literature focuses on highly reactive oxygen species (ROS) which are short-lived in biologic systems, many studies show that there are relatively stable oxidants which persist in human serum even after months of storage at −80 °C. The latter have been termed "reactive oxygen metabolites," for which we use the generic term "ROM" in the present manuscript. ROM in serum can be operationally defined by a reaction with *N,N*'-diethyl-*para*-phenylenediamine (DEPPD) in the presence of Fe^{2+}, which produces a stable radical cation (Fig. 1A) easily measured using a spectrophotometer because it is a red chromophore [3].

This reaction is the basis for the popular d-ROMs assay (Diacron International, Italy; www.diacrom.com) [3] and similar FORT (Free Oxygen Radicals Test; INCOMAT Mediznische, Glashutten, Germany). These tests are simple, relatively inexpensive and have been widely used to measure oxidative stress in human serum [4–11]. Importantly, the results of studies from different research groups suggest that these tests could be useful to assess disease risk. Such use is limited by uncertainty about the chemical nature of ROM.

The ROM test is standardized relative to *tert*-butylhydroperoxide or H_2O_2, giving the impression that the test is measuring H_2O_2 and organic peroxides in serum. However, H_2O_2 and many hydroperoxides are relatively unstable and would not be expected in serum at concentrations indicated by the DEPPD reaction. Moreover, background

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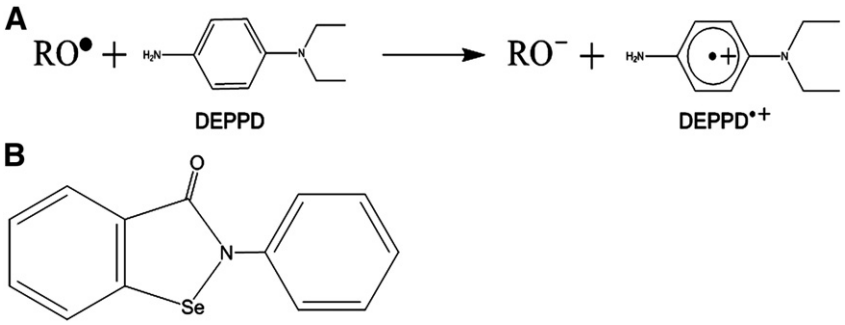

Fig. 1. (A) The reaction between a free radical and DEPPD results in the formation of a stable radical that can be measured at absorbance of 505 nm. (B) The multi ring structure of the antioxidant, ebselen.

chromophores as well as other oxidative reactions, e.g., ceruloplasmin activity [3] can contribute to the spectral change. Use of an EDTA-containing blank can correct for background chromophores; however, EDTA inhibits both peroxide-dependent color formation and ceruloplasmin activity so that an EDTA control does not discriminate factors contributing to the DEPPD oxidation by serum.

Antioxidants, such as butylated hydroxytoluene, added to serum could block DEPPD oxidation, but would not discriminate between the types of oxidants present in the sample. In the present study, we examined whether ebselen, a selenium-containing chemical which reduces peroxides in the presence of thiols [12], could be used to improve the specificity of the DEPPD reaction for peroxides in human serum. Ebselen, 2-phenyl-1,2-benzisoselenazol-3(2 H)one (Fig. 1B), catalyzes thiol-dependent reduction of peroxides by a non-radical mechanism [12,13]. Although earlier literature suggested that ebselen has properties such as free radical quenching [12], this activity was subsequently shown to have little effect [14]. Sequential reduction of ebselen by glutathione produces a selenol which is the direct reductant for the peroxides [13].

The present research shows that ebselen pretreatment of serum largely eliminates the serum-dependent oxidation of DEPPD. Studies with a purified endoperoxide and two epoxides show that, in addition to previously characterized oxidation of DEPPD by hydroperoxides, both of these oxidants support the DEPPD oxidation reaction and are eliminated by pretreatment with ebselen. Use of an imidazole N-oxide to trap radicals provided further evidence that the radical products are derived from non-radical species. Thus, use of an ebselen-pretreated serum sample as a reference for the DEPPD-dependent spectrophotometric assay provides means to improve specificity for non-radical oxidants in serum. This simple, yet novel, approach provides means to discriminate ebselen-dependent and ebselen-independent oxidative mechanisms that contribute to the DEPPD signal and potentially can be useful for population studies to test for distinct associations of disease risk with non-radical and radical mechanisms.

2. Materials and methods

2.1. Materials

N,N-diethyl-1,4-phenylenediamine (DEPPD), *tert*-butylhydroperoxide (tBH), $FeSO_4$, bathocuproine disulfonate (BCDS), glutathione peroxidase (bovine erythrocyte, Gpx), catalase, microsomal epoxide hydrolase (human, EH), rhodococcus epoxide hydrolase (EHr), styrene oxide, and 16,17-epoxy-21-acetoxy-pregnenolone were from Sigma-Aldrich (St Louis, MO). Prostaglandin H_2 (PGH_2) was from Cayman Chemical (Ann Arbor, MI). Peroxiredoxin-1 (human) was purchased from Lab Frontier (Seoul, South Korea). Ebselen [2-phenyl-1,2-benzisoselenazol-3(2 H)one] was purchased from Alexis Biochemicals (San Diego, CA). Other chemicals were of reagent grade and purchased locally.

Human studies were conducted with approval of the Emory University Investigational Review Board. Some serum samples for methods development were obtained commercially.

2.2. Methods

Experimentation was performed in 4 parts, with the goals to 1) adapt the DEPPD assay as developed by Alberti et al. [3] to a 96-well plate format and test it against a commercially available kit, 2) use selective chemical reactivity and enzymatic activity to gain information on the chemical nature of ROM in serum and 3) test model endoperoxide and epoxides to determine whether these classes of chemicals could be components of ROM in plasma and 4) compare the original Alberti assay to those obtained using an ebselen blank to determine whether this modification could improve specificity for the assay.

The final standard protocol adapted from Alberti et al. [3] for a 96-well plate format was as follows: Serum samples were diluted 1:40 in acetate buffer (37.32 mmol/l, pH 4.8). Five microliter aliquots were transferred to adjacent rows in 96-well plates with 100 μl of acetate buffer without or with 5 μl of 1 mmol/l ebselen in DMSO (50 μmol/l final concentration). Control experiments with DMSO without ebselen showed that DMSO had no effect on the color formation at the volume added. After 6 min at room temperature with shaking, 10 μl of 3.9 mmol/l DEPPD with 2.8 mmol/l $FeSO_4$ was added with a multi-channel pipette, and samples were shaken for 6 min prior to reading absorbance at 505 nm on a multiwell platereader. Values are reported as ΔA_{505} at 6 min using as reference wells which were identically treated except for substitution of EDTA for Fe^{2+}. The ΔA_{505} can be readily converted to peroxide equivalents using the tBH standard curve or to Carr units [3], which are arbitrary units corresponding to 0.08 mg H_2O_2 per 100 ml (24 μmolo/l). Where used, the FORT kit (INCOMAT Medizinische, Glashutten, Germany) was performed according to manufacturer's instructions.

For studies of the chemical nature of ROM in plasma, reagents were prepared at pH 7.0 and added to serum without adjustment of pH of the serum. Pretreated serum aliquots were then added to acetate buffer and DEPPD was added. Absorbance was scanned over the range of 450 to 575 nm, with data reported for the scans taken at 6 min after addition of either Fe^{2+} or EDTA. Concentrations of agents used for pretreatments were as follows: bathocuproine disulfonate, 1 mmol/l; Na_2S, 50 μmol/l and 5 mmol/l; GSH 100 μmol/l and 5 mmol/l; Cys, 50 μmol/l and 5 mmol/l; ebselen, 0.03, 0.12, 0.5 and 1 mmol/l; microsomal epoxide hydrolase, 0.2 mg/ml; rhodococcus epoxide hydrolase, 0.2 mg/ml; peroxiredoxin-1, 0.1 mg/ml; glutathione peroxidase-1, 50 units/ml (dialyzed to remove dithiothreitol); catalase, 0.2 mg/ml (dialyzed to remove thymol).

Assays with peroxides and epoxides were performed under identical conditions as above, with respective solutions replacing serum. Concentrations added were as follows: PGH_2, 90 μmol/l; 16,17-epoxy-21-acetoxy-pregnenolone, 1 mmol/l; styrene oxide, 1 mmol/l. For each experiment with purified peroxide or epoxide, a parallel experiment was