

ORIGINAL ARTICLE

Lipid peroxidation-inhibitory effects of perioperatively used drugs associated with their membrane interactions

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Key Words

Biomimetic membrane;
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Membrane interaction;
Nitrate stress;
Oxidative stress;
Perioperative drug

Abstract

Objective: Oxidative/nitrate stress, an imbalance between oxidant production and antioxidant defense in the biological system, is induced not only by various diseases but also by anesthesia and surgical trauma. Since the choice of drugs is expected to reduce oxidative/nitrate stress in the perioperative period, the lipid peroxidation inhibition by different drugs associated with surgery was studied together with investigating one of their possible mechanisms.

Methods: Lipid peroxidation-inhibitory effects were fluorometrically determined using the liposomes of diphenyl-1-pyrenylphosphine-incorporated lipid bilayers which were treated with 10-200 μ M drugs and reference antioxidants, and then peroxidized with 20 μ M peroxynitrite. Membrane interactions were evaluated by the drug- and antioxidant-induced changes in membrane fluidity which were determined by measuring the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene-labeled biomimetic membranes after treating with drugs and antioxidants at 1-200 μ M.

Results: All of the tested drugs concentration-dependently inhibited peroxynitrite-induced lipid peroxidation as well as antioxidant α -tocopherol, quercetin and (–)-epigallocatechin-3-gallate. The inhibition at 10 μ M was greatest in propofol, followed by guaiacol, thiopental, thymol, phenol, midazolam, diazepam, lidocaine, eugenol, procaine, bupivacaine, ropivacaine, sevoflurane, ketamine, mepivacaine and prilocaine. Antioxidant drugs including propofol, local anesthetics and phenol derivatives, and reference antioxidants interacted with biomimetic membranes consisting of phospholipids and cholesterol to modify the membrane fluidity, while their membrane-interacting potencies did not necessarily correlate to their lipid peroxidation-inhibiting ones, suggesting that the interaction between drugs and membrane lipid bilayers, at least in part, underlies the lipid peroxidation inhibition.

Conclusion: In addition to their inherent effects, propofol and other drugs to inhibit lipid peroxidation may be protective against perioperative oxidative/nitrate stress. The membrane interaction could be a guide for discovering novel antioxidant drugs.

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INTRODUCTION

The cause and consequences of a wide range of diseases are closely related to oxidative/nitrate stress, an imbalance between oxidant production and antioxidant defense in the biological system. Moreover, anesthesia and surgical trauma have been recognized to induce oxidative/nitrate stress [1-3]. Surgery is a pathological condition which enhances the generation of oxygen-derived reactive species and weakens the *in vivo* defense against their attacks. Oxidative/nitrate stress is implicated in ischemia-reperfusion injury and also referred to as one of major factors for postoperative morbidity and mortality. The antioxidant activity of perioperatively used drugs is of clinical interest because such activity is presumed to play a protective role against the pathological states associated with oxidative/nitrate stress [4].

Reactive oxygen/nitrogen species consisting of radicals and non-radicals are responsible for oxidative/nitrate stress. These reactive species damage membrane lipids, proteins, DNA and other biomolecules [5]. Lipid

peroxidation of biological membranes is the primary event and its degree is used as an indicator of oxidative/nitrate stress. Oxidative/nitrate stress in the conditions of myocardial ischemia/reperfusion produces lipid peroxidation products [6]. Therefore, the choice of drugs with the antioxidant activity or the lipid peroxidation-inhibiting ability is expected to reduce oxidative/nitrate stress in the perioperative period [4, 7]. Since the antioxidant activity of drugs is commonly evaluated on the basis of lipid peroxidation inhibition by them, lipids have been frequently peroxidized in experiments with hydroxyl, ferryl, *tert*-butyl hydroperoxide and 2,2'-azo-bis(2-amidinopropane) dihydrochloride radicals [8, 9]. However, such experimental conditions do not reflect the *in vivo* lipid peroxidation. Biological oxidation situations produce nitric oxide and superoxide anion, both of which readily react to form a stronger oxidant peroxynitrite. Peroxynitrite is able to peroxidize membrane lipids and its ability is modulated by the antioxidant mechanism [10, 11]. Oxidative/nitrate stress induces the myocardial production of peroxynitrite to initiate the

cytotoxic processes like lipid peroxidation [12]. Oxidative/nitrative stress also affects biological membranes and the *in vivo* reaction of oxidants occurs at a lipid membrane level [13]. Peroxynitrite plays a crucial role in the membrane lipid peroxidation in myocardial ischemia/reperfusion [14].

The aim of this study was to verify the antioxidant activity of different drugs associated with surgery, anesthesia and perioperative use (structures of tested drugs and antioxidants are shown in Fig.1). Their inhibitory effects on peroxynitrite-induced lipid peroxidation were determined together with investigating their interactions with lipid bilayer membranes as one of possible antioxidant mechanisms.

MATERIALS AND METHODS

Reagents

General anesthetics: propofol, thiopental sodium, diazepam, midazolam, ketamine and sevoflurane; local anesthetics: lidocaine, prilocaine, bupivacaine, ropivacaine, mepivacaine and procaine; phenol derivatives: phenol, guaiacol, eugenol and thymol; and reference antioxidants: α -tocopherol, quercetin and (-)-epigallocatechin-3-gallate (EGCG) were supplied by Aldrich (Milwaukee, WI, USA), Sigma (St. Louis,

MO, USA), Wako Pure Chemicals (Osaka, Japan) or Tokyo Chemicals (Tokyo, Japan). Phospholipids: 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), 1-palmitoyl-2-oleoylphosphatidylserine (POPS), 1-palmitoyl-2-oleoylphosphatidylethanolamine (POPE) and porcine brain sphingomyelin (SM) were purchased from Avanti Polar Lipids (Alabaster, AL, USA), and cholesterol from Wako Pure Chemicals. Diphenyl-1-pyrenylphosphine (DPPP) and peroxynitrite were obtained from Dojindo (Kumamoto, Japan), and 1,6-diphenyl-1,3,5-hexatriene (DPH) from Molecular Probes (Eugene, OR, USA). Dimethyl sulfoxide (DMSO) of spectroscopic grade (Kishida; Osaka, Japan) was used for preparing reagent solutions. All other reagents were of the highest analytical grade available commercially.

Lipid peroxidation inhibition

The effects to inhibit lipid peroxidation were determined by the liposome system as reported previously [15]. In brief, an aliquot (0.25 ml) of ethanol solutions of 10 mM total lipids and 40 μ M DPPP was repeatedly (four times) injected into 199 ml of Dulbecco's phosphate-buffered saline of pH 7.4 (Dainippon Pharmaceuticals; Osaka, Japan) to prepare DPPP-incorporated lipid bilayer membranes suspended in the buffer. The membrane lipid composition of

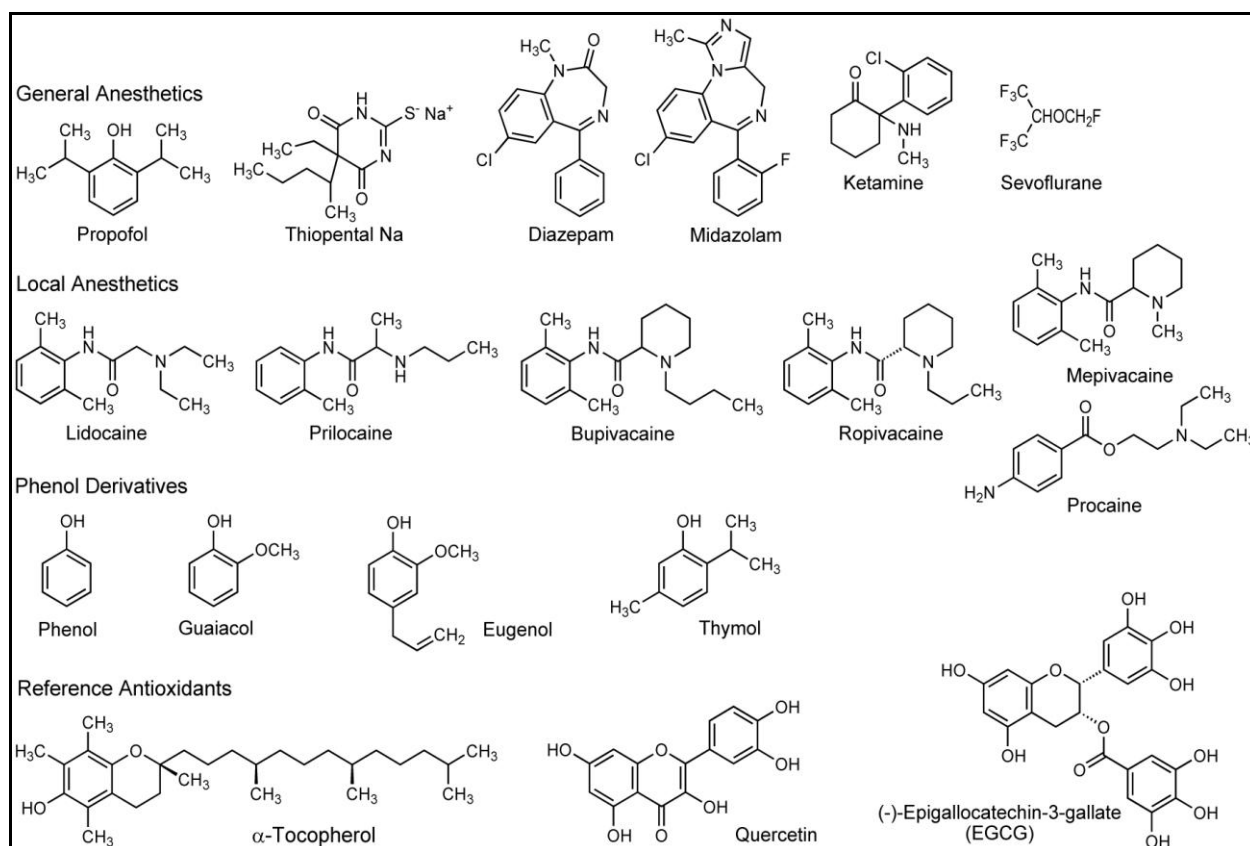


Figure 1. Chemical structures of drugs and reference antioxidants used in this study.

liposomes was 11 mol% POPC, 11 mol% POPS, 16.5 mol% POPE, 16.5 mol% SM and 45 mol% cholesterol. Liposome suspensions of 3.97 ml were incubated at 37°C for 30 min with 10 µl of DMSO solutions of drugs and reference antioxidants (to be a final concentration of 10-200 µM). The reagent solution of volatile sevoflurane was prepared according to its quantitative chromatographic method reported by Burrows [16]. Just before analysis, sevoflurane chilled at 4°C in an ice bath was added to a volumetric flask, and then the flask was diluted to volume with DMSO chilled to 20°C. An aliquot (10 µl) of the prepared dilute solution was subjected to the incubation with liposome suspensions. A corresponding volume (0.25%, v/v) of DMSO vehicle was added for controls. The resulting suspensions were incubated with 20 µl of a peroxyinitrite solution in 0.1 M NaOH (to be a final concentration of 20 µM) to peroxidize membrane lipids. DPPP is non-fluorescent, but membrane-incorporated DPPP quantitatively reacts with a lipid hydroperoxide to produce a fluorescent phosphine oxide in lipid bilayer membranes [17]. After the incubation at 37°C for 10 min, the liposome suspensions were fluorometrically analyzed at 355 nm for excitation and at 382 nm for emission. When the peroxyinitrite-induced increases of fluorescence intensity reached a plateau, the lipid peroxidation was defined as completed (100%). The lipid peroxidation-inhibition percentages were determined by comparing the fluorescence intensity with controls. DMSO used as a vehicle might additionally increase the lipid peroxidation-inhibitory effects of the tested drugs because it has the antioxidant property to reduce lipid peroxidation [18]. Therefore, the fluorescence intensity of liposome suspensions treated with DMSO alone was subtracted from that of liposome suspensions treated with drugs plus DMSO so that the determined effects were not affected by DMSO.

Membrane interaction

DPH-labeled biomimetic membranes with the lipid bilayer structure were prepared with phospholipids and cholesterol (10 mM for total lipids) to be unilamellar vesicles suspended in Dulbecco's phosphate-buffered saline of pH 7.4 as reported previously [19]. The molar ratio of DPH to total lipids was 1:200 and the membrane lipid composition was the same as that of liposomes described above. The potencies to interact with biomimetic membranes and change the membrane fluidity were determined as reported previously [20]. In brief, drugs and reference antioxidants dissolved in DMSO were applied to the membrane preparations so that a final concentration was 1-200 µM. The concentration of DMSO vehicle was adjusted to be 0.25% (v/v) of the total volume so as not to affect the fluidity of intact membranes because DMSO alters the

biophysical property of biomembranes [21]. Control experiments were conducted with an equivalent volume of DMSO. After the reaction at 37°C for 30 min, DPH fluorescence polarization was measured by an RF-540 spectrofluorometer (Shimadzu; Kyoto, Japan) equipped with a polarizer at 360 nm for excitation and at 430 nm for emission. Polarization values were calculated by the formula " $(I_{VV} - GI_{VH}) / (I_{VV} + GI_{VH})$ " in which I is the fluorescence intensity and the subscripts V and H refer to the vertical and horizontal orientation of the excitation and emission polarizer, respectively. The grating correction factor ($G = I_{HV}/I_{HH}$) was used to correct the polarizing effects of a monochromator. Decreasing and increasing polarization changes from controls mean an increase of membrane fluidity (membrane fluidization) and a decrease of membrane fluidity (membrane rigidification), respectively.

Statistical analysis

All results are expressed as mean \pm SEM ($n = 5$ for lipid peroxidation inhibition experiments and $n = 7$ for membrane interaction experiments). Data were analyzed by a one-way analysis of variance (ANOVA) followed by a post hoc Fisher's protected least significant difference (PLSD) test using StatView version 5.0 (SAS Institute; Cary, NC, USA). $P < 0.05$ was considered as significant for all comparisons.

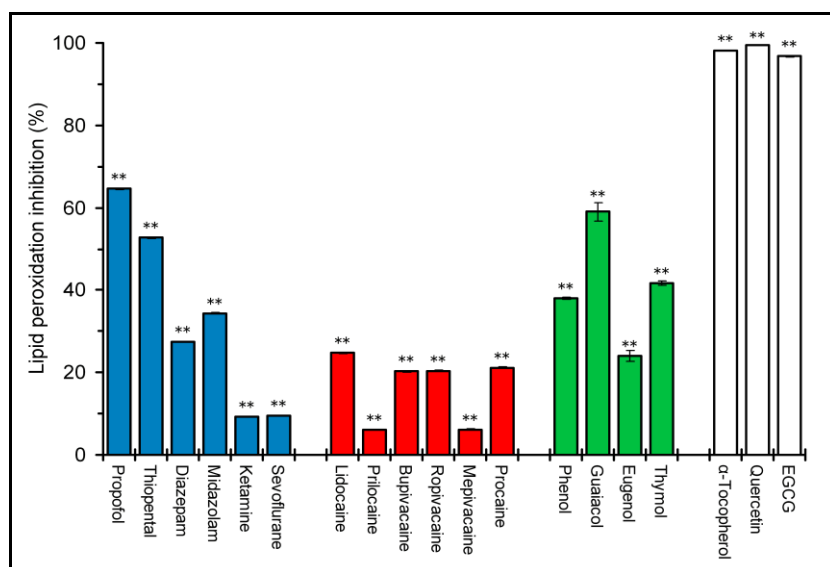
RESULTS

Lipid peroxidation inhibition

All the tested general anesthetics, local anesthetics and phenol derivatives concentration-dependently inhibited the peroxyinitrite-induced peroxidation of membrane lipids as well as antioxidant α -tocopherol, quercetin and EGCG (Fig.2). Inhibitory effects varied among drugs, showing that the inhibition at 10 µM was greatest in propofol, followed by guaiaicol, thiopental, thymol, phenol, midazolam, diazepam, lidocaine, eugenol, procaine, bupivacaine, ropivacaine, sevoflurane, ketamine, mepivacaine and prilocaine. They showed greater inhibition at higher concentrations (data not shown).

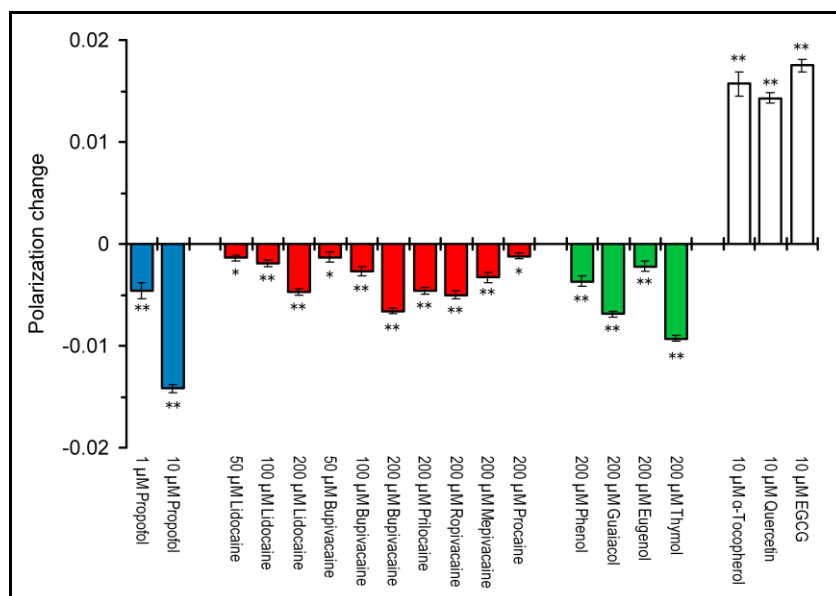
Membrane interaction

Lipid peroxidation-inhibiting propofol, local anesthetics and phenol derivatives interacted with biomimetic membranes to increase the membrane fluidity at 1-200 µM as shown by polarization decreases (Fig.3). Representative antioxidant drugs such as propofol, lidocaine and bupivacaine fluidized biomimetic membranes in a concentration-dependent manner. Reference antioxidants also interacted with biomimetic membranes at 10 µM, although they decreased the membrane fluidity. The membrane effects of flavonoid antioxidants were not determined

**Figure 2.**

Inhibitory effects of general anesthetics, local anesthetics, phenol derivatives and reference antioxidants on membrane lipid peroxidation. The liposomes of DPPC-incorporated lipid bilayers were treated with 10 μ M drugs and antioxidants, and then peroxidized with 20 μ M peroxynitrite, followed by the fluorometric analysis to determine the lipid peroxidation inhibition. Values represent mean \pm SEM (n = 5). **P < 0.01 vs control.

Figure 3. Membrane interactions of propofol, local anesthetics, phenol derivatives and reference antioxidants. Drugs and antioxidants were reacted at the indicated concentrations with DPH-labeled biomimetic membranes and their induced changes in membrane fluidity were determined by measuring fluorescence polarization. Values represent mean \pm SEM (n = 7). *P < 0.05 and **P < 0.01 vs control.



at higher concentrations because they have the property to quench the fluorescence of a membrane probe DPH at high micromolar concentrations [22].

Antioxidant and membrane activity relation

The lipid peroxidation-inhibitory effects of local anesthetics and phenol derivatives were plotted against their induced polarization changes indicating the membrane fluidization (Fig.4). Although all the tested antioxidant drugs interacted with biomimetic membranes, the linear regression analysis showed that the antioxidant activity did not necessarily correlate to the membrane-interacting potency. Reference antioxidants interacted with biomimetic membranes, but the resultant fluidity modification (membrane rigidification) was qualitatively different from that of antioxidant drugs (membrane fluidization).

DISCUSSION

Main findings of this study are: (1) different drugs such as general anesthetics, local anesthetics and phenol derivatives inhibit the peroxynitrite-induced membrane lipid peroxidation with varying inhibition potencies; (2) lipid peroxidation-inhibiting drugs commonly interact with biomimetic membranes to increase the membrane fluidity, while their membrane-interacting potencies do not necessarily agree with their lipid peroxidation-inhibiting ones; and (3) all the reference antioxidants interact with the membranes, but they decrease the membrane fluidity in contrast to the membrane-fluidizing effects of antioxidant drugs.

Since surgery is referred to as a pathological condition to enhance the generation of reactive oxygen/nitrogen species, one of important subjects in the perioperative

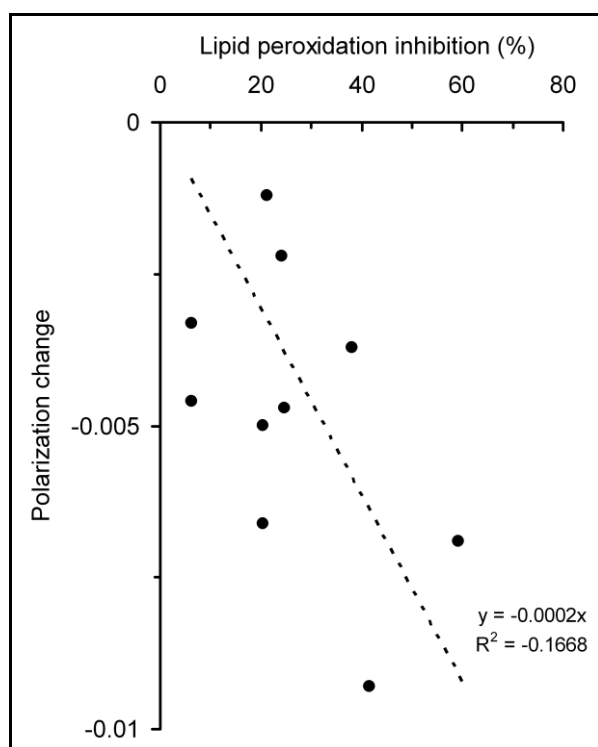


Figure 4. Lipid peroxidation-inhibiting and membrane-interacting potencies. The inhibitions (%) by local anesthetics and phenol derivatives were plotted against their induced DPH polarization changes.

period is to modulate or reduce the oxidative/nitrative stress of patients undergoing surgery and anesthesia. Peroxynitrite mediates oxidative/nitrative stress, ischemia-reperfusion injury, surgery-relating complication and cardiovascular damage through the lipid peroxidation of biomembranes [14, 23, 24]. Drugs with the anti-lipid-peroxidative activity would be effective in reducing the biological damages by reactive oxygen/nitrogen species. When treating the membrane preparations with peroxynitrite of a pathologically relevant concentration, the induced lipid peroxidation was inhibited by various anesthetic drugs and phenol derivatives. The latter antioxidants, including phenol, guaiacol, eugenol and thymol, are commonly used for the purpose of disinfection and analgesia. General anesthetic, local anesthetic and disinfectant/analgesic drugs, all of which are able to inhibit lipid peroxidation, may be protective against oxidative/nitrative stress as previously suggested for anesthesia-related drugs to modify oxidative/nitrative stress and ischemia-reperfusion injury [4, 7, 25].

Propofol inhibits lipid peroxidation by two mechanisms in which it reacts with lipid peroxy radicals to form the relatively stable phenoxyl radicals and scavenges peroxynitrite to experimentally inhibit peroxynitrite-induced chemiluminescence [26]. Its relating phenols with the hydrogen-donating property similarly show the

antioxidant ability to inhibit lipid peroxidation [27]. Lidocaine and procaine potentially scavenge hydroxyl radicals or hydrogen peroxide [28]. However, different types of drugs: general anesthetics, local anesthetics and disinfectant/analgesic phenols showed lipid peroxidation-inhibitory effects in common with them, suggesting the possibility that the mechanism other than scavenging radicals and depressing reactive species production may additionally contribute to their antioxidant activity.

The lipid peroxidation inhibited by antioxidants is associated with their properties not only to scavenge radicals but also to act on membrane lipid molecules [29]. The lipid peroxidation inhibition in membrane systems is more suitable for evaluating the antioxidant potency than the radical scavenging in homogeneous non-membrane systems [27]. The susceptibility to reactive oxygen/nitrogen species relates to the fluidity of lipid membranes [30]. Since unsaturated lipid components in membranes are the primary target of oxidants, lipid oxidizability is enhanced with an increase in the unsaturation degree of membrane-constituting phospholipids which makes membrane lipid bilayers more fluid. While thiopental, diazepam, midazolam, ketamine and volatile anesthetics have been proved to inhibit lipid peroxidation in this study, all of them were previously reported to interact with biological and artificial membranes to increase their fluidity [31-35]. With respect to other drugs tested for the antioxidant activity, propofol, local anesthetics with a substituted benzene ring like lidocaine and phenol derivatives have a close analogy in structure, pharmacological feature and the mode of membrane action as reported previously [36, 37]. Therefore, the present membrane interaction study focused on antioxidant propofol, local anesthetics, disinfectant/analgesic phenols. Consequently, they were characterized to interact with lipid bilayer membranes and change their fluidity. The perioperatively used drugs like β_1 -blockers also show both lipid peroxidation-inhibitory and membrane-fluidizing effects [38]. The degree of lipid peroxidation correlates to that of membrane fluidity change [39]. Peroxynitrite was reported to peroxidize membrane lipids and decrease the fluidity of liposomal phospholipid membranes concentration-dependently at 0.1-50 μ M [40]. Radicals and antioxidant molecules are likely to interact more efficiently in the membrane lipid environments fluidized by propofol and phenolic compounds [13, 15, 41]. The activity to interact with lipid bilayers and increase the membrane fluidity would produce the inhibition of membrane lipid peroxidation cooperatively with the radical scavenging activity.

In comparisons of structurally-related drugs, the rank order of lipid peroxidation-inhibitory effects is propofol > guaiacol > thymol > phenol > eugenol and that of

membrane-fluidizing effects is propofol > thymol > guaiacol > phenol > eugenol. The basic structure of phenol and certain substituents at the *ortho*-position (an isopropyl group in propofol and thymol, and a methoxyl group in guaiacol) appear to potentiate lipid peroxidation inhibition and membrane interaction, but a substituent at the *para*-position (an allyl group in eugenol) reduces both. Such relations are almost consistent with the previous report [15].

The propagation of oxidant and antioxidant molecules in lipid bilayers is a rate- or efficiency-limiting step of lipid peroxidation. Membrane fluidity is a determinant of lipid peroxidation rates and the antioxidant potency of radical scavengers requires the membrane environment [42]. General anesthetic, local anesthetic and disinfectant/analgesic drugs, all of which share both effects to inhibit lipid peroxidation and fluidize membranes, are speculated to interact more efficiently with radicals in more fluid membrane lipid bilayers [43]. Their membrane-fluidizing effects could favor the antioxidant capability by making the interaction between antioxidant molecules and radicals more efficient [13].

Antioxidant α -tocopherol, quercetin and EGCG, which scavenge radicals and inhibit lipid peroxidation, interact with biomimetic membranes but rigidify them in contrast to anesthetics and phenol derivatives. These α -tocopherol and flavonoids preferentially partition into the internal hydrophobic membrane regions and decrease the fluidity referring to the motional freedom of molecules in lipid bilayers as indicated by the polarization increase of DPH to localize in the hydrocarbon core of membranes. Propofol, local anesthetics and disinfectant/analgesic phenols are also considered to affect the same membrane regions because DPH was used for analyzing the membrane interactions of these drugs. Unlike such membrane-fluidizing drugs, however, the membrane rigidification by reference antioxidants could lead to the inhibition of lipid peroxidation by hampering the diffusion of radicals and thereby decreasing the kinetics of radical reactions in membrane lipid environments [44]. Anyway, the modification of membrane fluidity is presumed to be, at least in part, responsible for the inhibitory effects of perioperatively used drugs on membrane lipid peroxidation.

The clinical implications of lipid peroxidation-inhibiting drugs may be argued about their *in vivo* effects. General anesthetics, local anesthetics and disinfectant/analgesic phenols not only inhibit lipid peroxidation but also interact with lipid membranes at clinically relevant concentrations [15, 45]. Amphiphilic or hydrophobic drugs like anesthetics and analgesics are concentrated in lipid bilayers to show their intra-membrane concentrations much higher than their

concentrations in the bulk aqueous phase [46, 47]. The potential effects of antioxidant drugs on lipid peroxidation would lead to the protection against the pathological membrane states associated with oxidative/nitrative stress.

In addition to their inherent effects, propofol and other drugs to inhibit lipid peroxidation may be effective in reducing oxidative/nitrative stress in the perioperative period. The membrane interaction possibly underlying lipid peroxidation-inhibitory effects could be used as a guide for discovering novel antioxidant drugs.

ABBREVIATIONS

- DMSO, dimethyl sulfoxide
- DPH, 1,6-diphenyl-1,3,5-hexatriene
- DPPP, diphenyl-1-pyrenylphosphine
- EGCG, (–)-epigallocatechin-3-gallate
- POPC, 1-palmitoyl-2-oleoylphosphatidylcholine
- POPE, 1-palmitoyl-2-oleoylphosphatidylethanolamine
- POPS, 1-palmitoyl-2-oleoylphosphatidylserine
- SM, sphingomyelin

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COMPETING INTERESTS

The author declares there are no conflicts of interest for this study. The author is solely responsible for the contents and writing of the paper.

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