Scientific
Research

Comparative Interactions of Anesthetic Alkylphenols with Lipid Membranes

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Abstract

Objective: While substituted phenols have a variety of pharmacological activity, the mechanism underlying their anesthetic effects remains uncertain especially about the critical target. We characterized the lipid membrane-interacting properties of different phenols by comparing with general anesthetic propofol and local anesthetics. Based on the results, we also studied the pharmacological effects possibly associated with their membrane interactivities. Methods: 1,6-Diphenyl-1,3,5-hexatriene-labeled lipid bilayer membranes were prepared with 1,2-dipalmitoylphosphatidylcholine as model membranes and with different phospholipids and cholesterol to mimic neuronal membranes. These membrane preparations were treated with phenols and anesthetics at 1 - 200 μM, followed by measuring the fluorescence polarization to determine the membrane interactivities to change membrane fluidity. Antioxidant effects were fluorometrically determined using diphenyl-1-pyrenylphosphine-incorporated liposomes which were treated with 10 - 100 μM phenols, and then peroxidized with 10 μM peroxynitrite. Results: Several phenols interacted with the model membranes and the neuronal mimetic membranes to increase their fluidity at 1 - 10 μM as well as lidocaine and bupivacaine did at 50 - 200 μM. Their comparative potencies were propofol > thymol > isothymol > guaiacol > phenol > eugenol, and bupivacaine > lidocaine, consistent with the rank order of neuro-activity. These phenols inhibited membrane lipid peroxidation at 10 and 100 μM with the potencies correlating to their membrane interactivities. Conclusion: The structure-specific membrane interaction is at least in part responsible for the pharmacology of anesthetic alkylphenols. Membrane-interacting antioxidant alkylphenols may be protective against the peroxynitrite-relating ischemia/reperfusion injury.

Keywords

Alkylphenol, Anesthetic Mechanism, Lipid Membrane, Interaction, Antioxidant

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1. Introduction

Since phenol was introduced as a surgical disinfectant in the 1860s, substituted phenols or phenol derivatives have been applied as antiseptic, antibacterial, analgesic and anti-inflammatory agents. In addition, many of them possess the local anesthetic activity, making them very useful in dentistry when the relevant pain is associated with infection and inflammation. Phenolic agents are extensively used to allay the pain of dental pulps or sedate the inflamed pulps and to disinfect root canals and dentins. Orally and intravenously administered phenols are also effective for inflammatory and peripheral/central nervous pains [1] [2]. In the anesthesiology field, propofol is certainly best known of medicinal phenols. Intravenous anesthetic propofol has been widely used to induce and maintain general anesthesia, as a part of total intravenous anesthesia and for procedural sedation. The other alkylphenols also show the neuro-activity through the affinity for GABA$_A$ receptors similarly to propofol [3] [4].

As suggested by Haeseler and Leuwer [5], a benzene ring with some substituents is the structural characteristics for pharmacologically active agents such as pulp-sedative thymol, eugenol and guaiacol, general anesthetic propofol and local anesthetic lidocaine (see **Figure 1** for their structures). In addition to analgesic and anesthetic effects, alkylphenols have antimicrobial, anti-inflammatory, antithrombotic and anticancer effects [6]-[8]. However, the molecular mechanisms to explain such diversity in pharmacological activity have not necessarily been elucidated especially about the critical target.

Drugs and chemicals mechanistically act not only on receptor, channel and enzyme proteins but also on membrane lipids and their constituting lipid bilayers, thereby inhibiting or reducing bacterial growth, inflammatory reaction, platelet aggregation and tumor cell proliferation. Antimicrobial [9], non-steroidal anti-inflammatory [10], antiplatelet [11] and antiproliferative drugs [12] change the physicochemical properties such as fluidity, order and elasticity of biological and artificial lipid membranes by interacting with membrane lipids. Since antimicrobial, anti-inflammatory, antiplatelet and anticancer phenols and their structurally-relating compounds act on biomembranes [13] [14], the interactivity with lipid bilayers is presumed to be one of principal characteristics in common with phenolic agents.

Both local anesthetics and general anesthetic propofol block voltage-operated ion channels, but they differently act on GABA$_A$ receptors. Lidocaine, bupivacaine and procaine inhibit GABA-induced currents, possibly
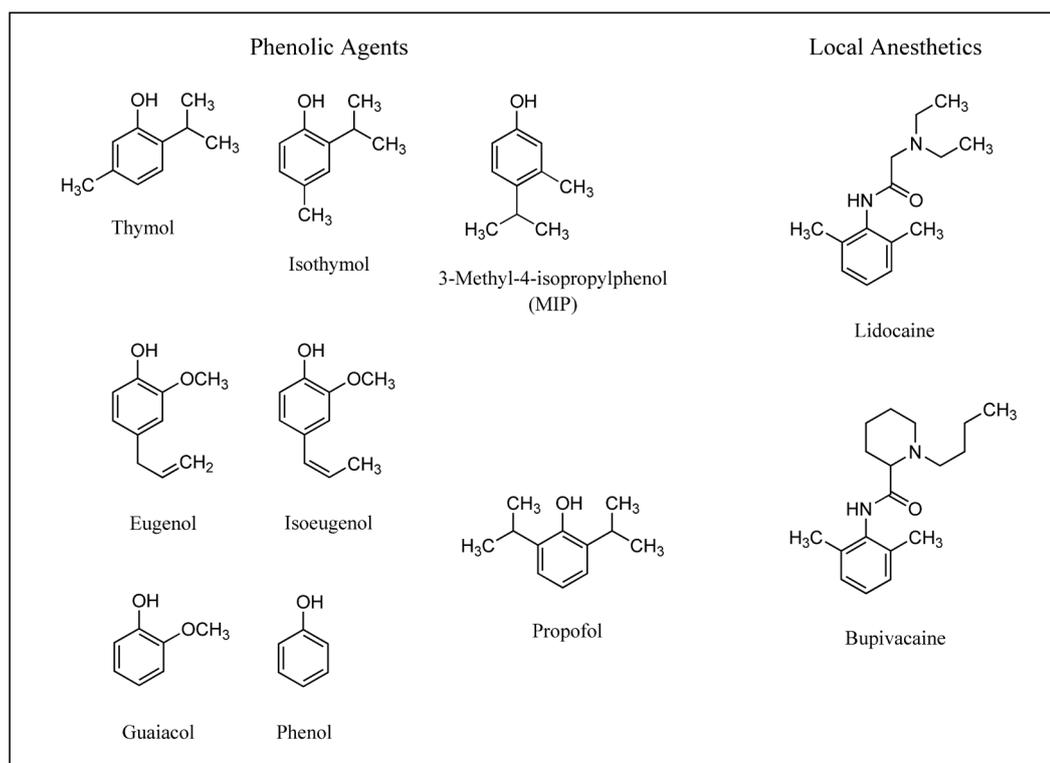
Figure 1. Phenols and anesthetics tested in this study.

producing their CNS toxicity [15], whereas propofol potentiates GABA-evoked currents and directly activates GABA$_A$ receptors [5]. Thymol, eugenol and guaiacol share the effects on peripheral and central nerves with lidocaine and propofol, respectively. These dental phenols block voltage-operated ion channels and inhibit sodium currents as well as local anesthetics [16] [17], although they allosterically positively modulate GABA$_A$ receptors similarly to propofol [3] [4].

The physicochemical properties of lipid bilayer membranes regulate voltage-dependent sodium channel and GABA$_A$ receptor functions [18] [19]. Besides membrane-embedded channels and receptors, local and general anesthetics possibly interact with membrane lipids to modify the lipid environments surrounding functional proteins as the primary mode of action. In this study, we characterized the lipid membrane-interacting properties of structurally-different anesthetic phenols (Figure 1) by comparing with general and local anesthetics. We also studied the pharmacological effects which could be associated with their membrane interactivities.

2. Methods

2.1. Chemicals

Phenolic thymol (5-methyl-2-isopropylphenol), isothymol (4-methyl-2-isopropylphenol), 3-methyl-4-isopropylphenol (MIP), eugenol (2-methoxy-4-(2-propenyl)phenol), isoeugenol (2-methoxy-4-(1-propenyl)phenol), guaiacol (2-methoxyphenol), phenol and propofol (2,6-diisopropylphenol), local anesthetic lidocaine and bupivacaine, and antioxidant α-tocopherol were supplied by Aldrich (Milwaukee, WI), Sigma (St. Louis, MO), Wako Pure Chemicals (Osaka, Japan) or Tokyo Chemical Industrials (Tokyo, Japan). Phospholipids: 1,2-dipalmitoylphosphatidylcholine (DPPC),1-palmitoyl-2-oleoylphosphatidylcholine (POPC), 1-palmitoyl-2-oleoylphosphatidylethanolamine (POPE), 1-palmitoyl-2-oleoylphosphatidylserine (POPS) and porcine brain sphingomyelin (SM) were purchased from Avanti Polar Lipids (Alabaster, AL), and cholesterol from Wako Pure Chemicals. 1,6-Diphenyl-1,3,5-hexatriene (DPH) was obtained from Molecular Probes (Eugene, OR), and diphenyl-1-pyrenylphosphine (DPPP) and peroxynitrite from Dojindo (Kumamoto, Japan). Dimethyl sulfoxide (DMSO) and ethanol of spectroscopic grade (Kishida; Osaka, Japan) and water of liquid chromatographic grade (Kishida) were used for preparing reagent solutions. All other chemicals were of the highest analytical grade available commercially.

2.2. Membrane Preparation

Lipid bilayer membranes were prepared as reported previously [20] with some modifications as follows. An aliquot (250 µl) of the ethanol solution of phospholipids and cholesterol (total lipids of 10 mM) and DPH (50 µM) was repeatedly injected four times into 199 ml of 20 mM sodium phosphate buffer (pH 7.4, containing 100 mM KCl) under stirring above the phase transition temperatures of phospholipids to prepare DPH-labeled unilamellar vesicles suspended in the buffer. Their lipid compositions were (1) 100 mol% DPPC for DPPC model membranes which have been most frequently used for studying membrane-active drugs and (2) 36 mol% POPC, 22 mol% POPE, 3.5 mol% POPS, 3.5 mol% SM and 35 mol% cholesterol to mimic neuronal membranes.

2.3. Interaction with Lipid Membranes

The DMSO solutions of thymol, isothymol, MIP, eugenol, isoeugenol, guaiacol, phenol, propofol, lidocaine and bupivacaine were applied to the membrane preparations so that final concentrations were 1 - 200 µM. The concentration of DMSO vehicle was adjusted to be 0.5% (v/v) of the total volume so as not to affect the fluidity of intact membranes. DMSO of the corresponding volume was added to controls. After the reaction at 37°C for 15 min, DPH fluorescence polarization was measured by an RF-540 spectrofluorometer (Shimadzu; Kyoto, Japan) equipped with a polarizer and a cuvette thermo-controller as reported previously [20]. 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. Compared with controls, a decrease of fluorescence polarization means an increase of membrane fluidity (membrane fluidization). Because the DPH polarization values of control membranes vary depending on membrane lipid components and their compositions, the polarization changes (%) relative to control polarization values were used for comparing the fluidity-modifying potencies between different membranes.

2.4. Inhibition of Membrane Lipid Peroxidation

The antioxidant activity was determined by the liposomal system reported previously with some modifications [21]. An aliquot (250 µl) of the ethanol solution of 10 mM total lipids and 40 µM DPPP was repeatedly injected four times into 199 ml of Dulbecco's phosphate-buffered saline of pH 7.4 (Dainippon Pharmaceuticals; Osaka, Japan) to prepare DPPP-incorporated liposomes suspended in the buffer. The lipid composition of liposomal membranes was the same as that of neuronal mimetic membranes described in Section 2.2. To the liposome suspensions of 3.97 ml, 10 µl of phenols and reference antioxidant α-tocopherol solutions in DMSO were added to be final concentrations of 10 and 100 µM for phenols and 1 µM for α-tocopherol, followed by incubation at 37°C for 30 min. A corresponding volume (0.25%, v/v) of DMSO vehicle was added for controls. The resulting suspensions were incubated with 20 µl of a peroxynitrite solution in 0.1 M NaOH to peroxidize membrane lipids with 10 µM peroxynitrite. A membrane-incorporated DPPP quantitatively reacts with a lipid hydroperoxide to produce a fluorescent phosphine oxide in lipid bilayers. 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 peroxynitrite-induced increase of fluorescence intensity reached a plateau, the lipid peroxidation was defined as completed (100%). The lipid peroxidation inhibitions (%) were determined by comparing the fluorescence intensity with controls. The fluorescence intensity of liposome suspensions treated with DMSO alone was subtracted from that of liposome suspensions treated with phenols plus DMSO so that the determined effects were not affected by vehicles.

2.5. Statistical Analysis

All results are expressed as means ± SEM ($n = 6$ for membrane interaction experiments and $n = 5$ for lipid peroxidation experiments). Data were statistically analyzed by a one-way analysis of variance (ANOVA), followed by a *post hoc* Fisher's protected least significant difference (PLSD) test using StatView 5.0 (SAS Software; Cary, NC). *P* values of 0.05 or less were regarded as statistically significant.

3. Results

3.1. Effects on DPPC Membranes

In pilot studies using DPPC model membranes, the membrane-interacting potentials of phenols and anesthetics were confirmed (**Figure 2**). Except for MIP and isoeugenol, phenols increased the DPPC membrane fluidity at
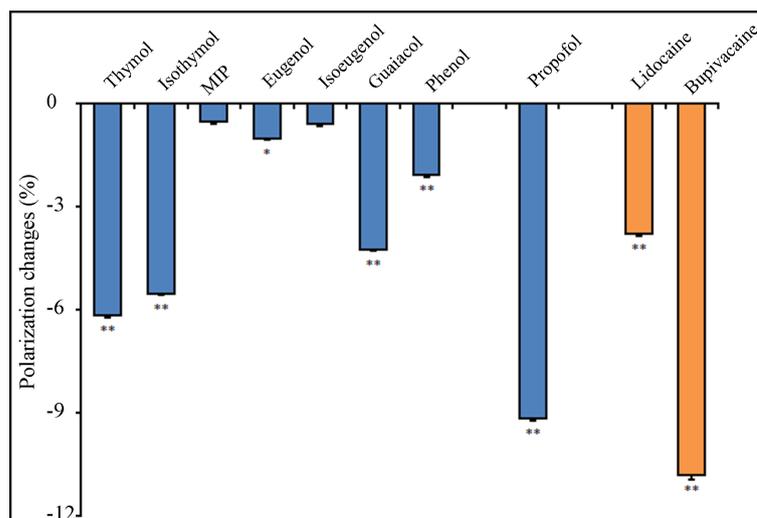
Figure 2. Effects of phenols and local anesthetics on DPPC model membranes. DPPC membranes were treated with phenols (10 µM for each) and local anesthetics (200 µM for each), followed by measuring DPH fluorescence polarization. The polarization changes (%) relative to control values are shown as means ± SEM ($n = 6$). *$P < 0.05$ and **$P < 0.01$ compared with control.

10 μM as well as lidocaine and bupivacaine did at 200 μM. Their comparative potencies were propofol > thymol > isothymol > guaiacol > phenol > eugenol, and bupivacaine > lidocaine. At concentrations higher than 200 μM, alkylphenols such as propofol and thymol induced the aggregation of DPPC unilamellar vesicles as previously reported for polyphenols that aggregated phosphatidylcholine liposomes [22].

3.2. Effects on Neuronal Mimetic Membranes

Phenols other than isoeugenol interacted with neuronal mimetic membranes to increase their fluidity at 10 μM (**Figure 3**). Propofol was most potent of phenols as shown by the significant DPH polarization decrease at 1 μM, followed by thymol, isothymol, guaiacol, phenol, eugenol and MIP. Local anesthetics also increased the fluidity of neuronal mimetic membranes at 50 - 200 μM with the comparative potency being bupivacaine > lidocaine. The membrane effects of both local anesthetics were weaker than those of membrane-interacting phenols. Compared with DPPC model membranes, the membrane fluidity decreases by phenols and local anesthetics were smaller.

3.3. Effects on Membrane Lipid Peroxidation

While being not as potent as antioxidant α-tocopherol, all the tested phenols inhibited peroxynitrite-induced membrane lipid peroxidation at 10 and 100 μM (**Figure 4**). When the lipid peroxidation inhibitions (%) at 10 μM for each were plotted against the induced polarization changes (%) of neuronal mimetic membranes, the linear regression analysis provided $y = -0.214x - 0.789$, $R^2 = 0.918$. The relative antioxidant activities of phenols correlated to their membrane interactivities.

4. Discussion

The presence of a certain amount of cholesterol in lipid bilayers decreases the fluidity of membranes [23]. Because neuronal mimetic membranes were prepared with phospholipids plus cholesterol, they are more rigid than the model membranes consisting of DPPC alone. The different membrane-fluidizing potencies of phenols and local anesthetics between neuronal mimetic and DPPC membranes are attributed to such a difference of membrane property due to lipid compositions.

Lidocaine and bupivacaine interacted with neuronal mimetic membranes at 20 - 200 μM, although their effects were not as great as phenols interacting with the same membranes at 1 - 10 μM. The relative potencies of lidocaine and bupivacaine to fluidize the membranes correlate to those of local anesthesia induced by them. A
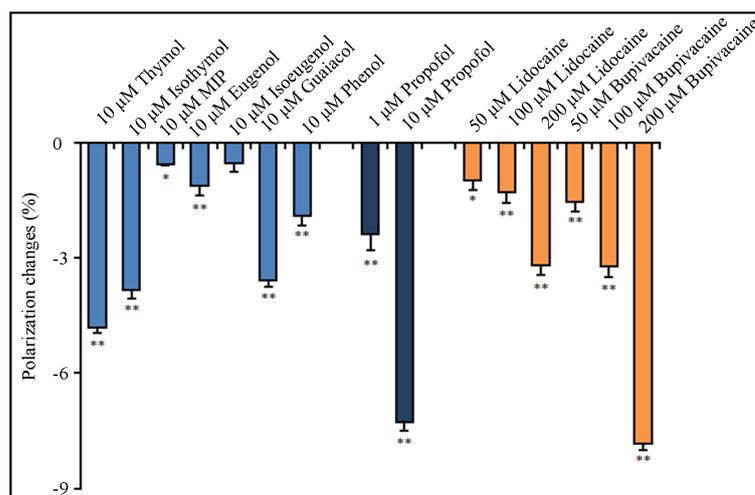
Figure 3. Effects of phenols and local anesthetics on neuronal mimetic membranes. Membranes prepared with phospholipids and cholesterol to mimic neuronal membranes were treated with phenols and local anesthetics at the indicated concentrations, followed by measuring DPH fluorescence polarization. The polarization changes (%) relative to control values are shown as means ± SEM ($n = 6$). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with control.
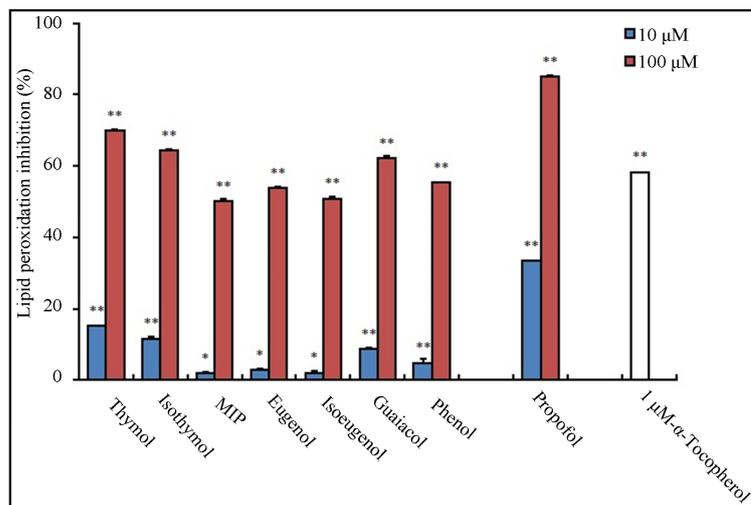
Figure 4. Effects of membrane-interacting phenols on membrane lipid peroxidation. DPPP-incorporated membranes consisting of unsaturated phospholipids and cholesterol were treated with 10 or 100 μM phenols and 1 μM α-tocopherol as a reference antioxidant, and then with 10 μM peroxynitrite, followed by the fluorometric analysis. The lipid peroxidation inhibitions (%) were determined by comparing the fluorescence intensity with controls. The results are shown as means ± SEM (n = 5). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with control.

relatively large volume of high concentration drugs is injected to ensure adequate local anesthesia for surgery, as 20 - 30 ml of 20 mg/ml lidocaine are typically injected to block large nerves in humans [24]. Nociceptive C-fibers of rat sciatic nerves are blocked by continuously superfusing lidocaine of 0.33 mg/ml [25]. Considering clinically/experimentally-relevant and membrane-fluidizing concentrations, the membrane interaction is related to local anesthetic effects.

Even if local anesthetics are concentrated in lipid bilayers so that their intra-membrane concentrations are higher than their concentrations in the bulk aqueous phase [26], they could not achieve high micromolar concentrations in the CNS. The membrane interactions of lidocaine and bupivacaine are unlikely to contribute to the inhibition of GABA-induced currents at membrane-embedded GABA$_A$ receptors.

Thymol, eugenol and propofol penetrate into lipid bilayers to locate in the region between the polar group, the glycerol moiety and the acyl chains, with the preference of more lipophilic phenols to the deeper hydrophobic region. The structure and membrane activity relation indicated that lipophilicity and 2,6-dialkylphenol structure impart greater membrane interactivity to alkylphenols, which is consistent with previous reports [27] [28]. When comparing thymol isomers, the position of an isopropyl group is important for membrane interaction as significantly different between thymol and MIP, but not that of a methyl group as slightly different between thymol and isothymol. Additional substituents at the *para*-position decreases the membrane interactivity as shown by the comparisons of guaiacol, eugenol and phenol, and a 2-propenyl group is preferable to a 1-propenyl group as seen in a difference between eugenol and isoeugenol.

When orally and intravenously administered, alkylphenols show the effects on central and peripheral nerves [1] [2]. In specimens from a forensic autopsy case of a man intravenously misinjected with 500 mg propofol, its determined concentrations were 26.8 ng/ml in blood and 129.6 ng/g in brain [29]. Higher lipophilic propofol and thymol are able to cross the blood-brain barrier and penetrate into neuronal membranes where they are concentrated. Alkylphenols concentrated in brains and nerve cells should be low micromolar levels or less, at which they could interact with membrane lipids [28] [30]. Lipid bilayer membranes mediate the effects of drugs through their induced perturbation of membrane structures, physicochemical properties or dynamics [14]. Membrane fluidity, curvature, elasticity and dipolar arrangement modulate the receptor-mediated interactions between drugs and membranes [31]. The anesthetic effects of alkylphenols are referred to as the combined results of their interactions with receptor or channel proteins and those with membrane lipids to modify the lipid environments surrounding such functional proteins [32].

Phenols like eugenol applied to pulp cavities achieve the concentrations in dental pulps and dentins adjacent to the pulp space to be 10 - 100 μM, but their concentrations are unlikely to be in excess of 0.1 mM in pulp tissues [33] [34]. Dental phenols show the concentration-dependent effects: pulp-sedative at relatively low concentrations but irritant or cytotoxic at relatively high concentrations [35]. Membrane-active phenols would reversibly exert anesthetic and analgesic effects by acting on neuronal membranes at low micromolar concentrations which cover their membrane-interacting concentrations. At high micromolar or millimolar concentrations, however, they are assumed to irreversibly damage neural and pulp tissues by the lysis of lipid membranes as alkylphenols adversely acted on DPPC model membranes.

Compounds to positively modulate GABA$_A$ receptors possess the antioxidant activity, which may lead to the neuro-protection [27]. Antioxidant dugs are expected to reduce oxidative stress in the perioperative period and ischemia/reperfusion injury [36]. While the antioxidant activity of drugs has been frequently evaluated by the inhibition of lipid peroxidation, lipid samples are treated with hydroxyl, ferryl, *tert*-butyl hydroperoxide and 2,2-diphenyl-1-picrylhydrazyl radicals in a homogenous or aqueous phase. These experimental conditions do not necessarily reflect the *in vivo* lipid peroxidation. Peroxynitrite produced from nitric oxide and superoxide anion plays a crucial role for the membrane lipid peroxidation in myocardial ischemia/reperfusion [37], and its peroxidation ability is modulated by the antioxidant mechanism [38]. According to the *in vivo* pathogenic oxidation, the antioxidant effects of phenols were determined by the phospholipid bilayer membranous system using peroxynitrite. Consequently, several phenols have been revealed to show promising effects with the rank order of lipid peroxidation inhibition being propofol > thymol > isothymol > guaiacol > phenol > eugenol, consistent with that of neuronal mimetic membrane fluidization. The basic structure of phenol and substituents at the *ortho*-position appear to potentiate both membrane interaction and lipid peroxidation inhibition, but substituents at the *para*-position reduce [21] [27].

The peroxidation rate of membrane lipids depends on the propagation of oxidant and antioxidant molecules in lipid bilayers which is determined by membrane physicochemical properties such as fluidity [39]. The tested phenols are considered to interact more efficiently with radicals in more fluid lipid bilayers [40]. Their membrane-fluidizing effects are preferable to make the interaction between antioxidant molecules and radicals more efficient [41].

5. Conclusion

Phenols interact with the membranes consisting of unsaturated phospholipids and cholesterol to modify their fluidity with the potency correlating to anesthetic effects. The structure and membrane activity relation indicates that branched-chain alkyl groups in the *ortho*-position relative to a hydroxyl group increase the membrane interactivity, but additional substituents at the *para*-position decrease. Thymol and propofol meeting the structural requirements show the greatest membrane interaction at clinically-relevant concentrations. The structure-specific membrane interaction likely underlies the pharmacology of anesthetic phenols. Since the interaction with unsaturated phospholipid membranes could be associated with the inhibition of membrane lipid peroxidation, membrane-interacting antioxidant alkylphenols may be effective in protecting against the peroxynitrite-relating ischemia/reperfusion injury.

Conflict of Interest

The authors declare there are no conflicts of interest.

Acknowledgements

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Abbreviation List

3-Methyl-4-isopropylphenol (MIP);
1,2-Dipalmitoylphosphatidylcholine (DPPC);
1-Palmitoyl-2-oleoylphosphatidylcholine (POPC);
1-Palmitoyl-2-oleoylphosphatidylethanolamine (POPE);
1-Palmitoyl-2-oleoylphosphatidylserine (POPS);
Sphingomyelin (SM);
1,6-Diphenyl-1,3,5-hexatriene (DPH);
Diphenyl-1-pyrenylphosphine (DPPP);
Dimethyl sulfoxide (DMSO).

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