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Peroxynitrite affects lidocaine by acting on membrane-constituting lipids

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Abstract
Inflammation frequently decreases local anesthetic effects, especially in dental anesthesia in patients with pulpitis and periodontitis. The pharmacokinetics and the mode of action of local anesthetics are closely associated with the hydrophobic interactions between these drugs and lipid bilayers that change the membrane physicochemical property, fluidity. A lipid oxidant, peroxynitrite, is produced by inflammatory cells, and it may act on nerve cell membranes and affect anesthetic efficacy. With respect to this speculated action, we addressed whether peroxynitrite acted on membrane-constituting lipids to decrease the membrane interactivity of lidocaine. Membrane fluidity changes were determined by measuring the fluorescence polarization of liposomes prepared with different phospholipids. Peroxynitrite (0.1–50 μM) rigidified nerve-cell model membranes consisting of unsaturated phospholipids, as well as liposomal membranes consisting of 1,2-dioleoylphosphatidylcholine and 1-stearoyl-2-arachidonyl-phosphatidylcholine, but peroxynitrite did not rigidify 1, 2-dipalmitoylphosphatidylcholine liposomal membranes. The pretreatment of nerve-cell model membranes with peroxynitrite (0.1–50 μM) decreased the membrane-fluidizing effects of lidocaine (5.0 mg·ml^{-1}) to 63%–86% of the control (not treated with peroxynitrite) depending on the peroxynitrite concentration. As one of the mechanisms of the local anesthetic failure associated with inflammation, inflammatory peroxynitrite may affect local anesthesia by acting on membrane-constituting unsaturated phospholipids.

Key words Peroxynitrite · Lidocaine · Membrane lipids · Fluidity change · Anesthetic failure

To reach nerve fibers, it is essential for local anesthetics to permeate hydrophobic (lipoid) barriers such as nerve sheaths or perineuria. Subsequently, local anesthetics must penetrate into or across the lipid bilayers of nerve-cell membranes to bind to their receptors at the cytoplasmic portions of transmembrane ion channels. While the blockade of voltage-gated sodium channels embedded in plasma membranes is the primary mode of action of local anesthesia, it is also caused by the interaction between drugs and membrane lipids. Local anesthetics induce changes in physicochemical properties, such as the fluidization or disordering of the lipid bilayers surrounding receptors and ion channels, resulting in the modification of their activities through the conformational alteration of membrane proteins [1]. These cellular events are determined by the hydrophobic interactions of local anesthetics with lipid components. A relationship has been found between local anesthetic potency and hydrophobicity [2].

The failure of local anesthesia, or poor analgesia, frequently occurs in the presence of inflammation, especially when performing dental anesthesia in patients with pulpitis and periodontitis [3,4]. Such clinical phenomena have been conventionally explained by the metabolic acidosis of inflamed tissues, because the numbers of uncharged drug molecules with membrane permeability and hydrophobic interactivity decrease with a lowering of the pH [5,6]. However, experimental verifications are challenging this mechanistic theory [7,8]. One alternative mechanism was proposed by focusing on peroxynitrite [9], which is produced by inflammatory cells and is relevant to various diseases [10].

Peroxynitrite was recently found to decrease the inhibitory effect of lidocaine on trigeminal nerve response [11]. Such a change is caused by the interaction of peroxynitrite with drugs or by the action of peroxynitrite on nerve-cell membranes, or both. The former was experimentally proven for lidocaine [9], whereas the latter has not yet been proven. Membrane fluidizer anesthetics were previously hypothesized to interact with any compounds with the opposite membrane activ-

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ity [12]. Cigarette smoke, containing nitric oxide and superoxide anions, which combine to form peroxynitrite, reacts with brain synaptosomes to decrease their membrane fluidity [13]. Peroxynitrite potentially rigidifies platelet membranes [14] and liver plasma membranes [15], together with inhibiting membrane enzymes. While lipid peroxidation is closely associated with the decrease of membrane fluidity, peroxynitrite is capable of oxidizing lipid membranes [16]. Therefore, we carried out this study to verify the possibility that inflammatory peroxynitrite may modify the fluidity of nerve-cell membranes and counteract the membrane-fluidizing effects of local anesthetics. We addressed whether the interactivity of lidocaine with membrane lipid bilayers was influenced by treating liposomal membranes with peroxynitrite.

Liposomes of the lipid bilayer structure (lipid concentration of 0.14 mM) labeled with 1,6-diphenyl-1,3,5-hexatriene (DPH; Molecular Probes, Eugene, OR, USA) were prepared as reported previously [8]. Their lipid composition was 100 mol% 1,2-dipalmitoylphosphatidylcholine (DPPC; Avanti Polar Lipids, Alabaster, AL, USA), 100 mol% 1,2-dioleoylphosphatidylcholine (DOPC), or 100 mol% 1-stearoyl-2-arachidonylphosphatidylcholine (SAPC). Nerve-cell model membranes were prepared with SAPC, 1-palmitoyl-2-oleoylphosphatidylethanolamine, 1-stearoyl-2-oleoylphosphatidylserine, sphingomyelin, and cholesterol (11:16.5:11:16.5:45; mol%). Peroxynitrite in 0.1 M NaOH was added to liposome suspensions in 16 mM sodium phosphate buffer (pH 7.4, containing 100 mM KCl) with vortex-mixing for 5 s, followed by incubation for 5 min at 37°C. The final concentration of peroxynitrite was 0.1–50 μM. To determine membrane fluidity changes, DPH fluorescence polarization was measured by a previous method [17]. Compared with control, decreased and increased polarization values mean the enhancement (membrane fluidization) and the reduction of membrane fluidity (membrane rigidification), respectively. After peroxynitrite pretreatment, nerve-cell model membranes were reacted with 5.0 $mg \cdot ml^{-1}$ lidocaine (Sigma, St. Louis, MO, USA) for 15 min at 37°C. Lidocaine-induced changes in membrane fluidity were determined as described above. Values for results are expressed as means ± SEM (n = 5–7). Data were statistically analyzed by one-way analysis of variance (ANOVA), followed by post-hoc Fisher's protected least significant difference (PLSD) test (StatView 5.0; SAS Institute, Cary, NC, USA); P values < 0.01 were considered significant.

Peroxynitrite acted on liposomes to induce membrane rigidification, as shown by polarization increases (Table 1). In membrane peroxidation, unsaturated lipid components are the primary target of oxidants. DOPC and SAPC liposomal membranes prepared with unsaturated phospholipids were susceptible to peroxynitrite, whereas DPPC liposomal membranes prepared with saturated phospholipids were not susceptible. The potency of peroxynitrite to rigidify membranes was different between DOPC and SAPC liposomes. This difference is accounted for by the degree of unsaturation of membrane-constituting phospholipids [18]. Lipid oxidizability increases with the number of *bis*-allylic methylene positions in phospholipids [19]. Because nerve-cell model membranes consist of unsaturated phospholipids, including SAPC, they are able to undergo the reaction with peroxynitrite, which changes the membrane physicochemical property. In vivo, the concentration and production rate of nitric oxide are estimated to be as high as 3 μM [20] and 8 $\mu M \cdot min^{-1}$ [21]. Although peroxynitrite has been reported to decrease the inhibitory effect of lidocaine on trigeminal nerve response, lidocaine in that study was pretreated with 1 mM peroxynitrite [11], which appears to be too high compared with its pathophysiological concentrations. In the present study, the membrane preparations were reacted with 50 μM peroxynitrite for 5 min at 37°C. These experimental conditions are not in conflict with the peroxynitrite exposure caused by activated inflammatory cells [22].

The broad pharmacological spectra and pharmacokinetics of local anesthetics are interpretable by their action on membrane lipids and by their traverse of lipoid barriers. Their hydrophobic interactions with

Table 1. Effects of peroxynitrite on the fluidity of liposomal membranes with different lipid compositions

Peroxynitrite (μM)	DPH polarization change in			
	DPPC membrane	DOPC membrane	SAPC membrane	Nerve-cell model membrane
50	0.0012 ± 0.0007	0.0042 ± 0.0004**	0.0069 ± 0.0011**	0.0054 ± 0.0004**
10	0.0008 ± 0.0005	0.0041 ± 0.0004**	0.0065 ± 0.0011**	0.0052 ± 0.0009**
2	0.0003 ± 0.0005	0.0036 ± 0.0004**	0.0062 ± 0.0013**	0.0050 ± 0.0005**
0.4		0.0035 ± 0.0007**	0.0059 ± 0.0010**	0.0041 ± 0.0008**
0.1		0.0033 ± 0.0007**	0.0037 ± 0.0007**	0.0040 ± 0.0005**

** $P < 0.01$ vs control
Data values are expressed as means ±SEM (n = 5–7)

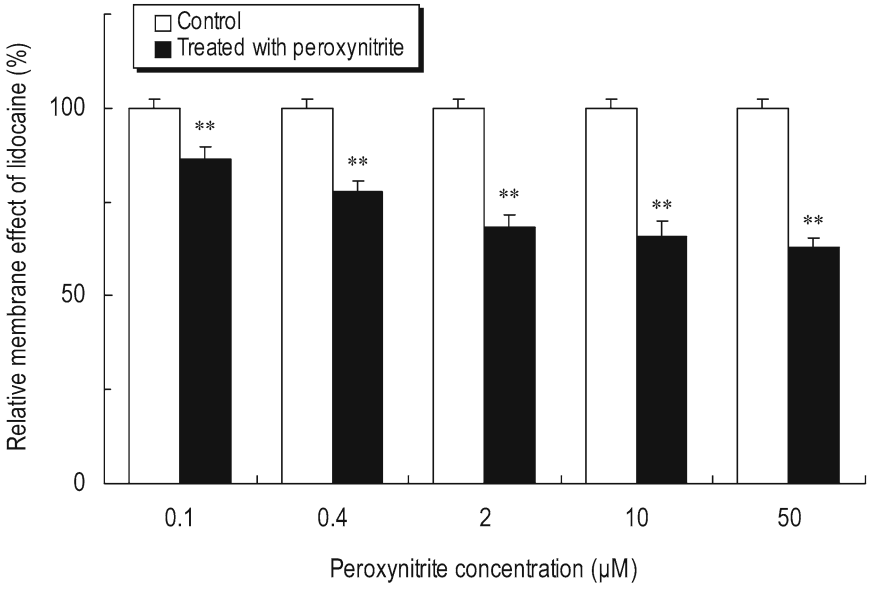

Fig. 1. Membrane-fluidizing effects of lidocaine inhibited by peroxynitrite. After pretreatment with 0.1–50 μM peroxynitrite for 5 min at 37°C, nerve-cell model membranes were reacted with lidocaine (5.0 mg·ml^{-1}) for 15 min. The membrane-fluidizing effects of lidocaine were compared with control values (not treated with peroxynitrite) on the basis of 1,6-diphenyl-1,3,5-hexatriene (DPH) polarization decreases. *Each bar* represents mean ± SEM (n = 7). $^{**}P < 0.01$ vs control

lipid bilayers induce membrane fluidity changes. Therefore, the question is raised as to whether the membrane effects of local anesthetics are altered in nerve-cell membranes rigidified by peroxynitrite. Lidocaine fluidized nerve-cell model membranes at a clinically injectable concentration [23], resulting in the decrease of DPH polarization. However, when pretreating nerve-cell model membranes with 0.1–50 μM peroxynitrite, we found that the membrane fluidization induced by lidocaine was decreased to 63%–86% of the control (not treated with peroxynitrite) in a manner depending on the peroxynitrite concentration (Fig. 1). The effect of a membrane-fluidizer such as lidocaine on membrane lipids is very likely to be counteracted by the action of a membrane-rigidifier such as peroxynitrite.

The present results suggest that inflammatory peroxynitrite affects local anesthesia not only directly by interacting with lidocaine [9] but also indirectly by acting on nerve-cell membranes. Both the drug interaction and the membrane action would decrease the efficacies of topical and infiltration anesthesia, in which local anesthetics are applied into or close to inflamed tissues. Nitrotyrosine, a footprint marker of peroxynitrite, shows widespread tissue distribution [24,25]. Because anesthetic drug solutions are injected relatively near acute inflammatory lesions in dental anesthesia [23], it is possible that peroxynitrite may influence maxillary and mandibular nerve block through the action on nerve-cell membranes, in addition to causing myelin damage [24]. Inflammatory peroxynitrite seems to contribute to the decreased effects of local anesthetics, together with other pathophysiological features of inflammation such as the vasodilatation promoting the absorption and removal of drugs from administered sites [7,23] and the modification of sensory nerves and ion channels that induces hyperexcitability or hyperalgesia [26,27].

In conclusion, as a novel hypothetical mechanism for the local anesthetic failure associated with inflammation, peroxynitrite acts on membrane-constituting unsaturated phospholipids and decreases the membrane interactivity of lidocaine.

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