INTRODUCTION

Noxious stimuli by anesthesia induction, operative incision, laryngoscopy, tracheal intubation, and/or extubation excite the sympathetic nervous system, resulting in heart rate increase, arterial blood pressure elevation, and cardiac ischemia occurrence. The perioperative use of β-adrenergic receptor antagonists has been suggested to reduce the risk of such heart events as tachycardia, hypertension, myocardial ischemia and infarction and the surgery-relating cardiac morbidity and mortality during general anesthesia (Devereaux et al., 2005; Wiesbauer et al., 2007; Zangrillo et al., 2009). In addition, β-blockers show the antiinflammatory property to decrease intraoperative analgesic requirements (Davidson et al., 2001) and the blocking effects on voltage-gated sodium channels (Wang et al., 2010).

Propranolol was previously used as a pre-, intra-, and postoperative β-blocker (Ivey et al., 1983; Wiesbauer et al., 2007), followed by espropranolol, labetalol, nadolol, timolol, and alprenolol (Burns et al., 1988; Fleisher et al., 2009). However, these conventional drugs have the possibility to cause long-lasting cardiac failures and respiratory side-effects due to their concomitant β2-blocking effects. Although cardioselective β-blockers such as atenolol and metoprolol were alternatively used, their duration and intensity of action were problematic for the perioperative use, leading to the development of short-acting β1-selective esmolol with the selectivity of β1/β2 = 33 and the half-life (t1/2) = 9.19 min (Sum et al., 1983). The subsequent studies produced ultra-short-acting highly β1-selective landiolol with the selectivity of β1/β2 = 255 and the half-life (t1/2) = 3.96 min (Iguchi et al., 1992). Landiolol...
and esmolol show Ki values of 62/1890 nM and 125/2620 nM in human β1, β2-adrenergic receptors and 993/14216 nM and 1054/5900 nM in dog β1, β2-adrenergic receptors, indicating that the β1-selectivity relative to propranolol is 78–380 for landiolol and 39–263 for esmolol (Japan Pharmaceutical Information Center [JAPIC], 2012). These sophisticated β1-blockers have been evaluated as an agent suitable for perioperative tachycardia and hypertension without the risk of prolonged cardiac depression but with the benefit to decrease anesthetic requirements (Saito et al., 2005; Tanabe et al., 2009).

The selectivity of antagonists is exclusively attributed to their structure-specific binding to receptors embedded in biomembranes. Besides receptor proteins, however, β-blockers also act on membrane lipids to modify the physicochemical properties of biomembranes such as fluidity (Varga et al., 1999; Lombardi et al., 2009). Because lipid bilayers provide transmembrane receptors with the surrounding environments optimal for their activity, changes in membrane fluidity influence the β-adrenergic receptor signaling (Ma et al., 1997). The property to change membrane fluidity has been suggested for several drugs acting on β-adrenergic receptors (Butler et al., 2006; Lombardi et al., 2009). Conventional β1-blockers possess the ability to interact with lipid bilayer membranes (Varga et al., 1999; Pereira-Leite et al., 2013). The membrane-interacting characteristics including potency and selectivity were recently reported to be useful for differentiating between non-selective β1-blockers (including propranolol, atenolol, and oxprenolol) and selective β1-blockers (including atenolol, metoprolol, and esmolol; Mizogami et al., 2010).

Although both landiolol and esmolol are classified as a short-acting β1-selective blocker, they are different in pharmacological features (Iguchi et al., 1992; Saito et al., 2005). However, there have been no investigations on the membrane effects to characterize landiolol despite that its structurally relating or structurally same structural moiety of compound acts on lipid membranes (Tian et al., 2011). In order to provide a novel pharmacological insight into landiolol, we studied its interactivity with different kinds of biomimetic membranes by comparing with β1-selective esmolol and non-selective propranolol and alprenolol.

**MATERIALS AND METHODS**

**REAGENTS**

Landiolol [(−)-1-[2,2-dimethyl-1,3-dioxolan-4-yl]methyl 3-[4-[[([S]-2-hydroxy-3-([2S][2S]-2-morpholinocarbonyl)amino)ethylamino-propanyl]phenylpropionate) and its metabolite [3-[4-[[([S]-2-hydroxy-3-([2S]-2-morpholinocarbonyl)amino)ethylamino]propanyl]phenylpropionic acid] were supplied by Otsu Pharmaceuticals (Osaka, Japan), and esmolol by Marushi Pharmaceuticals (Osaka, Japan). Propranolol and alprenolol were purchased from Sigma-Aldrich (St. Louis, MO, USA), and 4-ethylmorpholine (EM) and 2,2-dimethyl-1,3-dioxan-4-ol (DMD) from Tokyo Chemical Industries (Tokyo, Japan). Their chemical structures are shown in Figure 1. DiPalmitylphosphatidylcholine (DPPC), 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), 1,2-dioleoylphosphatidylcholine (DOPC), 1-palmitoyl-2-oleoylphosphatidylethanolamine (POPE), 1-palmitoyl-2-oleoylphosphatidylserine (POPS), bovine heart cardiolipin (CL), porcine brain phosphatidylinositol (PI), porcine brain sphingomyelin (SM), and porcine brain cerebrosides (CB) were purchased from Avanti Polar Lipids (Alabaster, AL, USA), and cholesterol and α-tocopherol from Wako Pure Chemicals (Osaka, Japan). 1,6-Diphenyl-1,3,5-hexatriene (DPH) was obtained from Molecular Probes (Eugene, OR, USA), and diphenyl-1-pyrenylphosphine (DPPP) and peroxynitrite from Dojindo (Kumamoto, Japan). Dimethyl sulfoxide (DMSO) of spectroscopic grade (Kishida, Osaka, Japan) was used for preparing reagent solutions.

**MEMBRANE PREPARATION**

Biomimetic membranes labeled with DPH were prepared with phospholipids and cholesterol to be unilamellar vesicles suspended in a buffer as reported previously (Tsuchiya and Mizogami, 2008). In brief, an aliquot (250 μl) of the ethanol solution of phospholipids and cholesterol (total lipids of 10 μM) and DPH (50 μM) was injected four times into 199 ml of 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer of pH 7.4 containing 125 mM NaCl and 25 mM KCl under stirring above the phase transition temperatures of phospholipids. The membrane lipid compositions were as follows: (1) 100 mol% DPPC for DPPC liposomal membranes which have been most frequently used in membrane interaction experiments (Mizogami et al., 2010; Pereira-Leite et al., 2013), (2) 25 mol% POPC, 20 mol% POPE, 5 mol% POPS, 5 mol% PI, 5 mol% SM, and 40 mol% cholesterol for cardiomycocyte-mimetic membranes (Wheelhorn et al., 1965), (3) 16.7 mol% DOPC, 16.7 mol% POPE, 16.7 mol% SM, 16.7 mol% CB, and 33.3 mol% cholesterol for lipid raft model membranes (Schröder et al., 1994) and (4) 25 mol% POPC, 16 mol% POPE, 3 mol% POPS, 10 mol% CL, 3 mol% PI, 3 mol% SM, and 40 mol% cholesterol for mitochondria-mimetic membranes (Tsuchiya et al., 2010a).

**MEMBRANE INTERACTIVITY**

The membrane interactivity was determined by analyzing the drug-induced changes in membrane fluidity as reported previously (Tsuchiya et al., 2011). In brief, landiolol, its metabolite, its structurally relating compounds (EM and DMD), esmolol, propranolol, and alprenolol were dissolved in DMSO. The resulting solutions were applied to the membrane preparations so that a final concentration of drugs was 0.5–200 μM. These drug concentrations were chosen because the tested β1-blockers were reported to show blood concentrations of a micromolar level in their pharmacokinetic studies (de Brujin et al., 1987; Murakami et al., 2005). The concentration of DMSO was adjusted to be 0.25% (v/v) of the total volume so as not to affect the fluidity of intact membranes. Beta1-selective esmolol and non-selective propranolol and alprenolol were used for the comparisons because they have the structurally same substituent (2-hydroxy-3-isopropylamino)propyl group) attached to aromatic rings (see Figure 1). Control experiments were conducted with the application of an equivalent volume of DMSO vehicle. 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 excitation 360 nm and at emission 430 nm as...
reported previously (Mizogami et al., 2010). Polarization values were calculated by the formula $I_{VV} - G I_{VH} (I_{VV} + G I_{VH})$ according to the method of Ushijima et al. (2005), in which $I$ is the fluorescence intensity and the subscripts V and H refer to the vertical and horizontal orientation of excitation and emission polarizer, respectively. The grating correction factor ($G = I_{HV}/I_{HH}$) is the ratio of the detection system sensitivity for vertically and horizontally polarized light, which was used to correct the polarizing effects of a monochromator. Decreasing and increasing polarization changes from controls mean an increase (membrane fluidization) and a decrease of membrane fluidity (membrane rigidification), respectively.

**ANTIOXIDANT ACTIVITY**

The antioxidant activity to inhibit membrane lipid peroxidation was determined by the liposomal system as reported previously (Tsuchiya et al., 2010b). In brief, DPPP-incorporated membranes with the molar ratio of DPPP to total membrane lipids of being 1:100 were prepared to be liposomes suspended in Dulbecco’s phosphate-buffered saline of pH 7.4 (Dainippon Pharmaceuticals, Osaka, Japan). Their membrane lipid compositions were (1) 100 mol% DOPC for unsaturated phospholipid membranes and (2) 25 mol% POPC, 16 mol% POPE, 3 mol% POPS, 10 mol% CL, 3 mol% PI, 3 mol% SM, and 40 mol% cholesterol for mitochondria-mimetic membranes (Tsuchiya et al., 2010a). Liposome suspensions of 3.97 ml were pre-incubated at 37°C for 30 min with each 10 μl of selective and non-selective β1-blockers or α-tocopherol solutions in DMSO (a final concentration of 100 μM for each drug) or the α-tocopherol solution in DMSO (2.5 μM) as a reference antioxidant. A corresponding volume (0.25%, v/v) of DMSO vehicle was added to controls. Lipid peroxidation was induced by adding 20 μl of the peroxynitrite solution in 0.1 M NaOH (a final concentration of 20 μM) and then incubating at 37°C for 10 min. Since membrane-incorporated DPPP quantitatively reacted with a lipid hydroperoxide to produce a fluorescent phosphine oxide, the liposome suspensions were fluorometrically analyzed at excitation 355 nm and at emission 382 nm. When the peroxynitrite-induced increase in fluorescence intensity reached a plateau, membrane lipid peroxidation was defined as completed (100%). The lipid peroxidation-inhibiting percentages were determined by comparing the fluorescence intensity with controls. Because DMSO has the antioxidant property to potentially inhibit lipid peroxidation (Sanmartín-Suárez et al., 2011), it may cooperatively increase the lipid peroxidation-inhibitory effects of the tested drugs. In the present study, the fluorescence intensity of liposomes treated with DMSO alone was subtracted from that of liposomes treated with drugs plus DMSO so that the determined activity was not influenced by DMSO.
STATISTICAL ANALYSIS
All results are expressed as means ± SEM (n = 8 for membrane interactivity experiments and n = 5 for antioxidant activity 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). A p value of being < 0.05 was taken as significant.

RESULTS
INTERACTION WITH BIOMIMETIC MEMBRANES
Propranolol and alprenolol interacted with different membrane preparations to increase the fluidity of all of them as shown by polarization decreases in Figure 2. These non-selective β₁-blockers fluidized DPPC liposomal membranes (Figure 2A), cardiomyocyte-mimetic membranes (Figure 2B) and lipid raft model membranes (Figure 2C) at 20–200 μM and mitochondria-mimetic membranes (Figure 2D) at lower concentrations of 0.5–20 μM. In contrast, selective β₁-blockers so differently acted on DPPC liposomal membranes that landiolol decreased the membrane fluidity at 20–200 μM as shown by polarization increases, but not esmolol (Figure 2A). Landiolol and esmolol-induced much less fluidization in cardiomyocyte-mimetic membranes (Figure 2B) and no fluidization in lipid raft model membranes (Figure 2C) even at 200 μM. However, both selective β₁-blockers interacted with mitochondria-mimetic membranes to fluidize them at 20–200 μM as well as non-selective propranolol and alprenolol (Figure 2D).

MEMBRANE EFFECTS OF LANDIOLOL AND RELATED COMPOUNDS
Not only landiolol but its metabolite and a hydrolysis fragment analog EM rigidified DPPC liposomal membranes (Figure 3). However, another hydrolysis fragment DMD was not effective in rigidifying the membranes or reversely fluidized the membranes at a relatively high concentration.

ANTIOXIDANT EFFECTS ON BIOMIMETIC MEMBRANES
Both selective and non-selective β₁-blockers inhibited the peroxynitrite-induced peroxidation of DOPC liposomal membranes and mitochondria-mimetic membranes as well as antioxidant α-tocopherol (Figure 4). Propranolol was greatest in antioxidant activity on biomimetic membranes, followed by alprenolol, landiolol, and esmolol in the decreasing order of potency.

FIGURE 2 | Interaction of selective and non-selective β₁-blockers with different kinds of biomimetic membranes. All drugs were reacted at the indicated concentrations with 100 mol% DPPC liposomal membranes (A), cardiomyocyte-mimetic membranes (B), lipid raft model membranes (C), and mitochondria-mimetic membranes (D), followed by measuring DPH fluorescence polarization. Values represent means ± SEM (n = 8). *p < 0.05 and **p < 0.01 vs. control.
Tsuchiya and Mizogami Biomimetic membrane interactivity of landiolol and reference drugs. Our main findings are as follows:

**DISCUSSION**

Pereira-Leite et al. (2013) used different fluorescence probes DPH and 1-[4-(trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) for determining the membrane interactivity of landiolol. Both non-selective and selective β₁-blockers exhibit different effects on the membrane fluidity in biomimetic membranes. Landiolol is a rigidifying agent on DPPC liposomal membranes, whereas propranolol and alprenolol are membrane-fluidizing agents. These findings suggest that the morpholine moiety provides landiolol with a rigidifying effect on DPPC membranes. Landiolol characteristically acted on DPPC liposomal membranes to fluidize all of them at sub-μM concentrations, although landiolol is metabolically hydrolyzed by plasma and liver esterases, and the resulting metabolite is pharmacologically inactive. Biological membranes are not a simple bilayer structure of uniformly distributed lipids, but contain the microdomain lipid rafts biophysically different from bulk membranes (Simons and Toomre, 2000). Highly ordered membrane microdomains encompass β₁-adrenergic receptors and provide them with the platform to regulate their functions (Lanoul et al., 2005). Lipid rafts form caveolae by polymerizing with caveolins which bind to cholesterol. The localization in caveolae/lipid rafts is prerequisite to β₁-adrenergic receptors for physiological signaling, but not to β₁-adrenergic receptors (Xiang et al., 2002). Propranolol and alprenolol act on lipid raft model membranes and fluidize them. Membrane fluidization is associated with the decreased function of β₁-adrenergic receptors (Lombardi et al., 2009). Non-selective

[Image 90x692 to 324x853]

**FIGURE 3** Effects of landiolol, its hydrolysis metabolite and structural fragments (40 and 200 μM for each) on 100 mol% DPPC liposomal membranes. Values represent means ± SEM (n = 5). *p < 0.05 vs. control.

**FIGURE 4** Inhibitory effects of selective and non-selective β₁-blockers (100 μM for each) and antioxidant 2-isoceposphen (2.5 μM) on peroxynitrite-induced lipid peroxidation of 100 mol% DOPC liposomal membranes and mitochondria-mimetic membranes. Values represent means ± SEM (n = 5). *p < 0.05 and **p < 0.01 vs. control.

(1) propranolol and alprenolol interact with DPPC liposomal, cardiomyocyte-mimetic, lipid raft model, and mitochondria-mimetic membranes to fluidize all of them at sub-μM or μM concentrations, although landiolol and esmolol are not so interactive with cardiomyocyte-mimetic and lipid raft model membranes, (2) only landiolol rigidifies DPPC liposomal membranes in contrast to membrane-fluidizing propranolol and alprenolol or membrane-inactive esmolol, and (3) both non-selective and selective β₁-blockers interact with cardiomyocyte-mimetic membranes to increase their fluidity together with inhibiting the peroxynitrite-induced lipid peroxidation of biomimetic membranes.

β₁-blockers are structurally composed of an aromatic ring and a 2-hydroxy-3-(isopropylamino)propanoyl group or its structural analog. Alprenolol is the phenyl derivative with a 2-hydroxy-3-(isopropylamino)propanoyl group and a 2-propenyl group at the ortho-position and propranolol has a bulky α-naphthalene nucleus with a 2-hydroxy-3-(isopropylamino)propanoyl group. Such molecular structures of non-selective β₁-blockers occupy more space in membrane lipid bilayers with the resultant perturbation of the alignment of phospholipid acyl chains, thereby inducing fluidity changes in biomimetic membranes. On the other hand, landiolol and esmolol have two side chains in the para-positions. Therefore, they show an almost linear configuration in membrane lipid bilayers which allows drug molecules to align approximately parallel to phospholipid acyl chains. Due to such an alignment, these selective β₁-blockers could not induce significant changes in membrane fluidity even if penetrating into cardiomyocyte-mimetic and lipid raft model membranes (Mizogami et al., 2010).

Landiolol characteristically acted on DPPC liposomal membranes to rigidify them. Its metabolite lacking a DMD substructure and its hydrolysis fragment analog EM also rigidified DPPC liposomal membranes, but not landiolol hydrolysis fragment DMD, suggesting that the morpholine moiety provides landiolol with a rigidifying effect on DPPC membranes. Landiolol is metabolically hydrolyzed by esterase in plasma and liver and the resulting metabolite is pharmacologically inactive. Biological membranes are composed of different phospholipids and cholesterol, not of DPPC alone. Although the action on DPPC membranes is of much interest as a unique physicochemical property of landiolol, it is unlikely to clinically contribute to blocking β₁-adrenergic receptors.

A recent concept on biomembranes has indicated that they are not a simple bilayer structure of uniformly distributed lipids but contain the microdomain lipid rafts biophysically different from bulk membranes (Simons and Toomre, 2000). Highly ordered membrane microdomains encompass β₁-adrenergic receptors and provide them with the platform to regulate their functions (Lanoul et al., 2005). Lipid rafts form caveolae by polymerizing with caveolins which bind to cholesterol. The localization in caveolae/lipid rafts is prerequisite to β₁-adrenergic receptors for physiological signaling, but not to β₁-adrenergic receptors (Xiang et al., 2002). Propranolol and alprenolol act on lipid raft model membranes and fluidize them. Membrane fluidization is associated with the decreased function of β₁-adrenergic receptors (Lombardi et al., 2009). Non-selective
β1-blockers would reduce the β1-adrenergic receptor activity by interacting with membrane lipid rafts together with antagonizing β1-adrenergic receptors by binding to β1-receptor proteins, thereby producing the non-selective blockade. Their effects on cardiomyocyte membranes may also contribute to blocking β2-adrenergic receptors. Because neither landiolol nor esmolol interact with lipid raft model membranes, these selective β1-blockers could not influence the β2-adrenergic receptor activity through membrane fluidization, enhancing the selectivity to β1-adrenergic receptor blockade. The differentiation between selectivity and non-selectivity to β1-adrenergic receptors is compatible with that between non-interactivity and interactivity with biomimetic membranes, which is consistent with the previous comparisons between selective (atenolol, metoprolol, esmolol) and non-selective β1-blockers (alprenolol, oxprenolol, propranolol; Mizogami et al., 2010). A correlation between membrane interaction and low β1-specificity is likely to apply to most non-selective drugs. Unlike β1-non-selective propranolol, β1-selective landiolol and esmolol show no interactions with lipid raft model membranes or much less interactivity with cardiomyocyte-mimetic membranes. The β1-selectivity associated with the membrane non-interactivity is consistent with the relative β1-specificity of landiolol (74–88%), esmolol (33–263%), and propranolol (1) reported previously (Sum et al., 1983; Iiguchi et al., 1992; Japan Pharmaceutical Information Center [JAPIC], 2012).

Both non-selective and selective β1-blockers not only interact with mitochondria-mimetic membranes to increase their fluidity but also inhibit lipid peroxidation of DOPC liposomal membranes and mitochondria-mimetic membranes. In this study, mitochondria-mimetic membranes were prepared to contain 10 mol% CL. CL is preferentially located in cardiac mitochondrial membranes to play an important role in cardiac mitochondrial membranes to play an important role in heart functions and it comprises 8–20% of total mitochondrial membranes to increase the membrane fluidity as well as propranolol, alprenolol, and esmolol. Its lipid peroxidation-inhibitory effect associated with membrane fluidization would produce the clinical benefit of cardioprotection common to non-selective and selective β1-blockers by the mechanism independent of blocking β1-adrenergic receptors.

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AUTHOR CONTRIBUTIONS

Hironori Tsuchiya: Designed the study, conducted the study, and wrote the manuscript. Maki Mizogami: Performed the experiments, analyzed the data, and wrote the manuscript.

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CONCLUSION

To our knowledge, this is the first study to determine the membrane interactivity of landiolol depending on the lipid composition of biomimetic membranes. Landiolol is characterized by the non-interactivity with membrane lipid rafts which enhances its selectivity to β1-adrenergic receptor blockade. On the other hand, landiolol is able to interact with CL-containing mitochondrial membranes to increase the membrane fluidity as well as propranolol, alprenolol, and esmolol. Its lipid peroxidation-inhibitory effect associated with membrane fluidization would produce the clinical benefit of cardioprotection common to non-selective and selective β1-blockers by the mechanism independent of blocking β1-adrenergic receptors.

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The clinical implications of the membrane interaction of β1-blockers may be argued about their relevant concentrations to modify membrane fluidity. The concentrations of landiolol, esmolol, and propranolol to inhibit membrane lipid peroxidation almost correspond to those to protect from the ischemia-reperfusion injury (Kurosawa et al., 2003). Hydrophobic β1-blockers are concentrated in membrane lipid bilayers and intracellularly accumulated over 1000 times higher than their incubation medium concentrations (Butler et al., 2006; Kramer et al., 2006).

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