Lipid Emulsion Reverses Levobupivacaine-induced Responses in Isolated Rat Aortic Vessels

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ABSTRACT

Background: The goal of this *in vitro* study was to investigate the effects of lipid emulsion (LE) on local anesthetic levobupivacaine-induced responses in isolated rat aorta and to determine whether the effect of LE is related to the lipid solubility of local anesthetics.

Methods: Isolated rat aortic rings were suspended for isometric tension recording. The effects of LE were determined during levobupivacaine-, ropivacaine-, and mepivacaine-induced responses. Endothelial nitric oxide synthase and caveolin-1 phosphorylation was measured in human umbilical vein endothelial cells treated with levobupivacaine alone and with the addition of LE.

Results: Levobupivacaine produced vasoconstriction at lower, and vasodilation at higher, concentrations, and both were significantly reversed by treatment with LE. Levobupivacaine and ropivacaine inhibited the high potassium chloride–mediated contraction, which was restored by LE. The magnitude of LE-mediated reversal was greater with levobupivacaine treatment than with ropivacaine, whereas this reversal was not observed in mepivacaine-induced responses. In LE-pretreated rings, low-dose levobupivacaine- and ropivacaine-induced contraction was attenuated, whereas low-dose mepivacaine-induced contraction was not significantly altered. Treatment with LE also inhibited the phosphorylation of endothelial nitric oxide synthase induced by levobupivacaine in human umbilical vein endothelial cells.

Conclusions: These results indicate that reversal of levobupivacaine-induced vasodilation by LE is mediated mainly through the attenuation of levobupivacaine-mediated inhibition of L-type calcium channel–dependent contraction and, in part, by inhibition of levobupivacaine-induced nitric oxide release. LE-mediated reversal of responses induced by local anesthetics may be related to their lipid solubility.

What We Already Know about This Topic

- Cardiovascular collapse following large circulating concentrations of local anesthetics may reflect, in part, vasodilatation from these drugs
- Lipid emulsions are used to treat local anesthetic cardiotoxicity, but their mechanisms of action are not well understood

What This Article Tells Us That Is New

- In isolated aortic rings, lipid emulsions reversed local anesthetic-induced vasodilatation, in part, by reducing nitric oxide signaling

INTRAVENOUS lipid emulsion (LE) has been reported as rescue therapy for the cardiovascular collapse triggered by toxic doses of local anesthetics, which is largely resistant to standard cardiopulmonary resuscitation.[1] Several animal studies and human case reports showed that LE was effective in treating refractory cardiac arrest resulting from an overdose of local anesthetics, including bupivacaine, levobupivacaine, and ropivacaine.[2–7] Levobupivacaine and ropivacaine

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produce vasoconstriction at low concentrations followed by vasodilation at high concentrations that correspond to toxic plasma levels, suggesting that vasodilation may contribute to the vascular collapse seen in high-dose local anesthetic toxicity.[8–11] It is known that intravenous LE can be used to effectively treat patients with vascular collapse due to an overdose of local anesthetics.[5–7] However, the effect of LE on high-dose levobupivacaine-induced vasodilation remains unknown. One proposed theory for the mechanism of action of intravenous LE in local anesthetic toxicity is that it serves as a lipid sink, providing a large lipid phase that extracts local anesthetics from plasma.[1,2] Therefore, the goals of this study were to investigate the association between the effects of LE and lipid solubility of local anesthetics in the vascular system. Specifically, we tested whether LE reverses vasodilation associated with high-dose levobupivacaine, ropivacaine, and mepivacaine through a mechanism related to their lipid solubility.

Materials and Methods

All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee (Jinju, Gyeongnam, Korea) at Gyeongsang National University and were performed in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences.

Experimental Preparation

Male Sprague-Dawley rats weighing 250–350 g were anesthetized by intraperitoneal administration of sodium thiopental (50 mg/kg). The descending thoracic aorta was dissected free, and surrounding connective tissue and fat were removed under microscopic guidance in a Krebs solution bath (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.4 mM $CaCl_2$, 25 mM $NaHCO_3$, 11 mM glucose, and 0.03 mM EDTA). The aorta was cut into 2.5-mm rings, suspended on Grass isometric transducers (FT-03; Grass Instruments, Quincy, MA) under a 4.0-g resting tension in a 10-ml Krebs bath at 37°C, and aerated continuously with 95% O_2 and 5% CO_2 to maintain pH within the range of 7.35–7.45. The rings were equilibrated for 90 min, changing the bathing solution every 30 min. Endothelium was removed from some aortic rings by inserting a 25-gauge needle tip into the lumen of the rings and gently rubbing for a few seconds. The contractile response in isotonic 60 mM KCl was measured for all aortic rings and defined as the reference value (100%). In experiments with endothelium-intact aorta, endothelial integrity was confirmed by the observation of more than 75% relaxation in the presence of 10^{-5} M acetylcholine after the stabilization of contraction induced by 10^{-7} M phenylephrine.

After washing out the KCl from the organ bath and allowing a return to the baseline resting tension, a cumulative concentration-response curve to local anesthetics (*i.e.*, levobupivacaine, ropivacaine, and mepivacaine) with or without LE (SMOFlipid® 200 mg/ml; Fresenius Kabi Korea, Seoul, Korea) was obtained as described below. In experiments involving endothelium-denuded rings, the Krebs solution also contained the endothelial nitric oxide synthase (eNOS) inhibitor, N_ω-nitro-L-arginine methyl ester (10^{-4} M), to prevent the release of endogenous nitric oxide from any residual endothelium.

Study Design

The first series of experiments assessed the cumulative LE concentration-response curve in vasodilation induced by high-dose levobupivacaine (3×10^{-4} M), ropivacaine (10^{-3} M), and mepivacaine (10^{-2} M) in the aorta with or without an endothelium. After the cumulative concentration-response curves reached a stable, sustained level in the presence of levobupivacaine (10^{-5} to 3×10^{-4} M), ropivacaine (3×10^{-5} to 10^{-3} M), and mepivacaine (10^{-5} to 10^{-2} M), cumulative LE concentration (0.24, 0.48, 0.95, and 1.39%)-response curves were obtained in 3×10^{-4} M levobupivacaine-, 10^{-3} M ropivacaine-, and 10^{-2} M mepivacaine-vasodilated aorta (fig. 1). In addition, dose-response curves to LE alone in the absence of local anesthetics were obtained in the endothelium-intact aorta. Subsequent doses of levobupivacaine, ropivacaine, mepivacaine, and LE were added after the previous dose had elicited a sustained, stable contraction for 5 min (fig. 1). After low-dose levobupivacaine (3×10^{-5} M)-, ropivacaine (1.5×10^{-4} M)-, and mepivacaine (10^{-3} M)-induced contraction reached a stable, sustained level for 10 min, LE (0.95, 1.39, and 1.81%) was cumulatively added to the aortic rings.

A series of experiments was designed to examine the role of voltage-operated calcium channels (VOCCs) in levobupivacaine-mediated vasodilation. After treatment with VOCC antagonist verapamil (10^{-7} M or 5×10^{-7} M), the effect of levobupivacaine was assessed in endothelium-denuded vessels. The LE concentration response was assessed in high-dose local anesthetic (3×10^{-4} M levobupivacaine, 10^{-3} M ropivacaine, or 10^{-2} M mepivacaine)-induced vasodilation of the endothelium-denuded aorta precontracted with 60 mM KCl. After high-dose local anesthetics produced a stable, sustained vasodilation, incremental concentrations of LE (0.24, 0.48, 0.95, 1.39, and 1.81%) were added to generate concentration-response curves. Subsequent doses of LE were added after the previous dose had elicited a sustained, stable response for 5 min.

The effect of LE (0.95% and 1.81%) on cumulative local anesthetic (levobupivacaine: 10^{-6} to 3×10^{-4} M; ropivacaine: 3×10^{-5} to 10^{-3} M; mepivacaine: 10^{-5} to 10^{-2} M) concentration-response curves in the endothelium-denuded aorta was assessed by comparing the local anesthetic-induced contractile response in the presence or absence of LE. LE was directly added to the organ bath 30 min before the addition of local anesthetics.

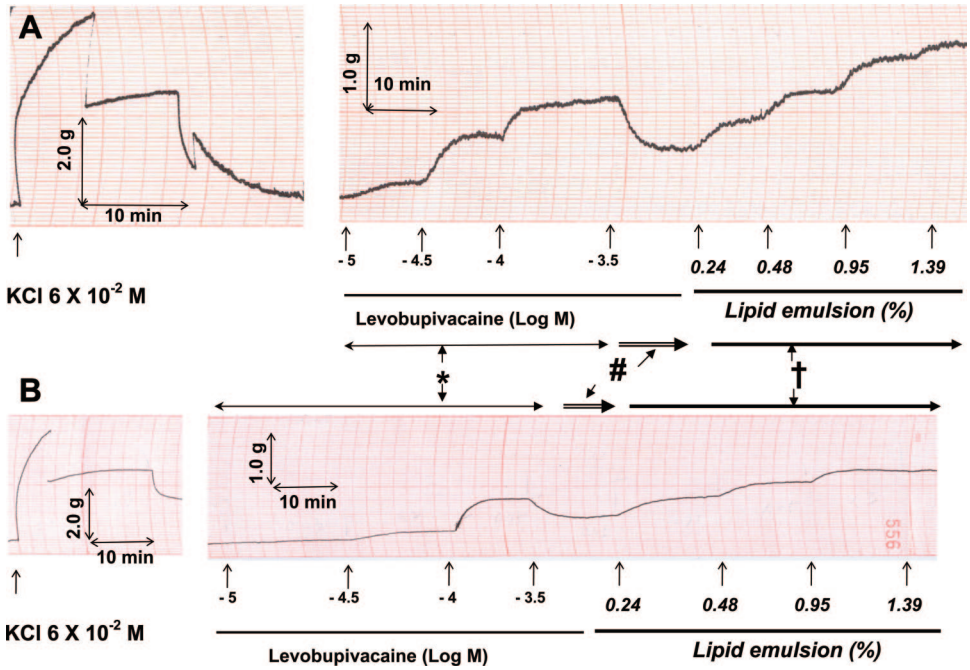

Fig. 1. Traces showing the change in tension in endothelium-denuded (*A*) and endothelium-intact (*B*) aortic rings in response to 60 mM KCl, levobupivacaine and lipid emulsion. Cumulative concentration-response curves to levobupivacaine and lipid emulsion are shown. * Vasoconstriction, # vasodilation, and † reversal of vasodilation.

Cell Culture

Human umbilical vein endothelial cells purchased from BioBud (Seoul, Korea) were seeded in a gelatin-coated, 100-mm tissue-culture dish and grown in M199 (Invitrogen, Carlsbad, CA) culture medium supplemented with 20% fetal bovine serum, 10 U/ml heparin (Sigma-Aldrich, St. Louis, MO), and 20 ng/ml basic fibroblast growth factor (Upstate, Charlottesville, VA). The cells were used between passages 4 and 6 for all experiments.

Western Blot Analysis

After treatment, cells were washed and scraped off the dish with ice-cold phosphate-buffered saline and then sonicated in lysis buffer (pH 7.2) containing 320 mM sucrose, 200 mM HEPES, 1 mM EDTA, phosphatase inhibitor cocktail (PhosSTOP, Roche, Germany), and protease inhibitor cocktail (Sigma-Aldrich). Protein concentrations were determined using the Pierce BCA Protein Assay kit (Pierce, Rockford, IL). Total protein (15 μg per lane) was separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride microporous membrane (Immobilon-P Transfer Membrane; Millipore, Milford, MA) by incubating in transfer buffer (25 mM Tris, 192 mM glycine, and 10% methanol) overnight at 4°C and 30 V. Membranes were blocked for 1 h with 5% bovine serum albumin in Tris-buffered saline-Tween buffer (40 mM Tris-HCl, pH 7.4, 25 mM NaCl, and 0.1% Tween® 20) and incubated with specific primary antibody for eNOS (rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA), phospho-eNOS (Ser1177, rabbit polyclonal; Cell Signaling Technology, Beverly, MA), caveolin-1 (rabbit polyclonal; Santa Cruz Biotechnology), phospho-caveolin-1 (Tyr14, rabbit polyclonal; Cell Signaling Technology), and anti-β-actin (monoclonal; Sigma-Aldrich) for 2 h at room temperature. Secondary antibody conjugated to horseradish peroxidase was visualized with an ECL system (Super Signal® West Pico Chemiluminescent Substrate; Pierce).

Materials

All drugs were of the highest purity available commercially. N_ω-nitro-L-arginine methyl ester, acetylcholine, and verapamil were obtained from Sigma-Aldrich. Ropivacaine and levobupivacaine were donated by AstraZeneca (North Ryde, Australia) and Abbott Laboratories (Elverum, Norway), respectively. Lipid emulsion (SMOFlipid® 20%) was donated by Fresenius Kabi Korea (Seoul, Korea). Mepivacaine was donated by Hana Pharmaceutical Co., Ltd. (Kyonggi-do, Korea). All concentrations are expressed as final molarity for local anesthetics and as final percentage for LE in the organ chamber. All drugs were dissolved and diluted in distilled water.

Data Analysis

Data are expressed as mean ± standard deviation (SD). The logarithm of the drug concentration eliciting 50% of the maximum contractile response (ED_{50}) was calculated using nonlinear regression analysis by fitting the concentration-response relation for local anesthetics to a sigmoidal curve using commercially available software (Prism, version 5.0; GraphPad Software, San Diego, CA). N indicates the number of rats from which descending thoracic aortic rings were derived. The band intensities from Western blot analyses

were analyzed by two-tailed unpaired Student *t* test. Responses to each concentration of levobupivacaine, ropivacaine, mepivacaine, and LE were compared using repeated measures analysis of variance, followed by a Tukey multiple comparison test. The magnitudes of LE-mediated reversal of the vasodilation induced by 3×10^{-4} M levobupivacaine in endothelium-denuded and intact aorta are expressed as a percentage of the absolute value of vasodilation. The effect of endothelial denudation and local anesthetics on the LE-induced reversal was analyzed by two-way analysis of variance with Bonferroni post test (GraphPad Prism version 5.00 for Windows). The effect of verapamil, LE, and local anesthetics on local anesthetics concentration-response curve or LE dose-response curve was analyzed by two-way analysis of variance with Bonferroni post test. *P* values less than 0.05 were considered significant.

Results

Aortic ring contraction in response to varying concentrations of levobupivacaine, ropivacaine, mepivacaine, and LE are shown in figures 1 and 2. Levobupivacaine (3×10^{-5} and 10^{-4} M) increased contraction in a concentration-dependent manner, with a maximal response at 10^{-4} M levobupivacaine. In contrast, the maximal contractions were significantly attenuated at 3×10^{-4} M levobupivacaine (fig. 2A). Similarly, ropivacaine induced vasoconstriction at 10^{-4} and 3×10^{-4} M, followed by vasodilation at 10^{-3} M (fig. 2B). A similar pattern was observed in mepivacaine-treated aorta (fig. 2C).

To determine the effects of LE on responses to local anesthetic, LE was cumulatively added to the vessel under local anesthetic stimulation. LE (0.24–1.39%) significantly reversed the levobupivacaine (3×10^{-4} M)-induced vasodilation of isolated rat aorta in a dose-dependent manner ($P < 0.001$ *vs.* 3×10^{-4} M levobupivacaine; fig. 3A). Vasodilation caused by 10^{-3} M ropivacaine was reversed by treatment with LE at 0.48–1.39% ($P < 0.001$ *vs.* 10^{-3} M ropivacaine; fig. 3B), whereas LE had no effect on the 10^{-2} M mepivacaine-induced vasodilation (fig. 3C). In addition, LE (0.24–1.81%) itself had no effect on the resting tension (n = 6; data not shown).

To determine the role of endothelial cells in LE reversal, the magnitude of reversal in response to LE was compared in vessels with and without endothelium. LE-mediated reversal of the vasodilation induced by levobupivacaine was greater in endothelium-denuded, compared with endothelium-intact, aorta (fig. 3A). In contrast, the reversal kinetics were significantly increased in endothelium-intact aorta ($P < 0.001$ *vs.* endothelium-denuded aorta at 0.48–1.39% LE; fig. 3D). The LE-induced reversal was also greater in the levobupivacaine-induced than in ropivacaine-induced vasodilation of isolated endothelium-denuded rat aorta ($P < 0.001$ *vs.* 10^{-3} M ropivacaine at 0.24–1.39% LE; figs. 3A–B).

The involvement of VOCCs in levobupivacaine-induced vasoconstriction was assessed using verapamil. Verapamil (10^{-7} or 5×10^{-7} M) inhibited levobupivacaine-induced contractions in endothelium-denuded aorta in a dose-dependent manner ($P < 0.001$ for 5×10^{-7} verapamil *vs.* no drug; fig. 4).

Interestingly, the contraction in response to high potassium chloride (60 mM) was inhibited by high-dose local anesthetics ($P < 0.001$; fig. 5). The levobupivacaine- and ropivacaine-mediated inhibition of high potassium-chloride–induced contraction was reversed by the administration of LE, whereas mepivacaine-induced inhibition was not affected (fig. 5). This LE-induced reversal was greater in the

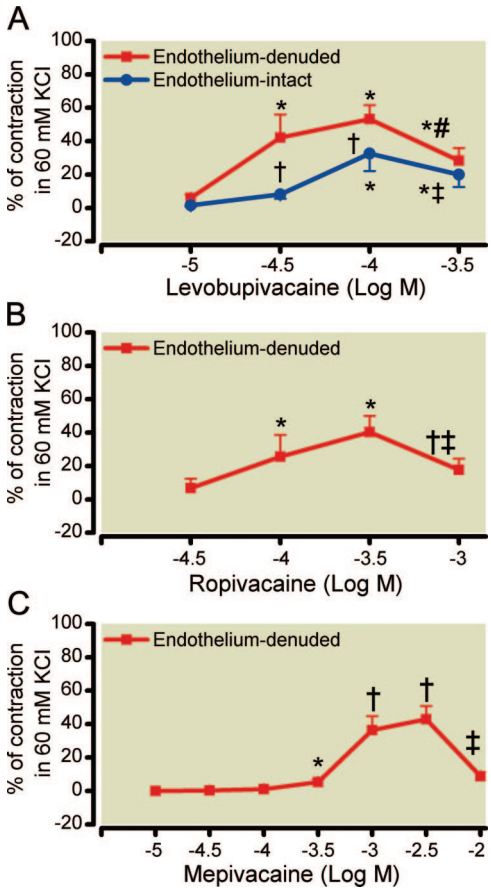

Fig. 2. (*A*) Levobupivacaine (LBV) dose-response curve in isolated endothelium-intact or -denuded aorta. * $P < 0.001$ *versus* 10^{-5} M LBV; † $P < 0.001$ *versus* endothelium-intact; ‡ $P = 0.0048$, # $P < 0.001$ *versus* 10^{-4} M LBV. (*B*) Ropivacaine (RPV) dose-response curve in isolated endothelium-denuded aorta. * $P < 0.001$, † $P = 0.0136$ *versus* 3×10^{-5} M RPV; ‡ $P < 0.001$ *versus* 3×10^{-4} M RPV. (*C*) Mepivacaine (MPV) dose-response curve in isolated endothelium-denuded aorta. * $P = 0.0138$, † $P < 0.001$ *versus* 10^{-5} M MPV; ‡ $P < 0.001$ *versus* 3×10^{-3} M MPV. Data are shown as mean ± SD and are expressed as the percentage of the maximal contraction induced by isotonic 60 mM KCl (100% = 2.41 ± 0.23 g [n = 7] and 100% = 2.79 ± 0.15 g [n = 7] for aortic rings with or without endothelium, respectively in *A*; 100% = 3.16 ± 0.47 g [n = 7] for aortic rings without endothelium in *B*; 100% = 3.18 ± 0.36 g [n = 7] for aortic rings without endothelium in *C*). N indicates the number of rats from which descending thoracic aortic rings were derived.

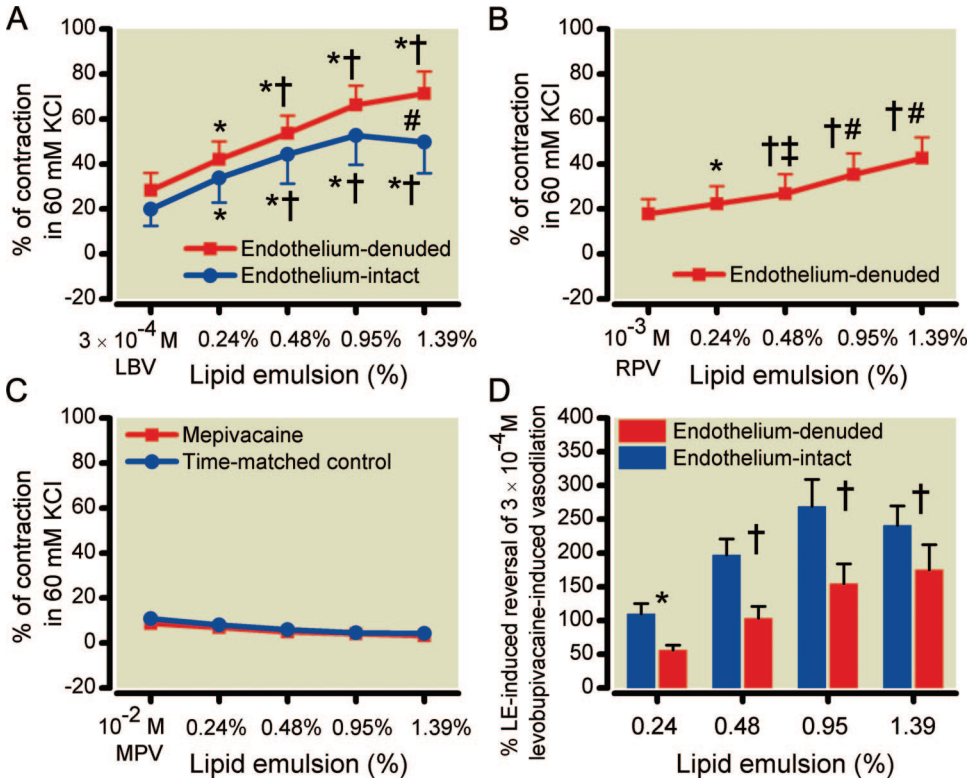

Fig. 3. (*A*) Lipid emulsion dose-response curve in isolated aorta with or without endothelium after 3×10^{-4} M levobupivacaine (LBV)-induced vasodilation. * $P < 0.001$ *versus* 3×10^{-4} M LBV; † $P < 0.001$ *versus* 0.24% lipid emulsion; # $P < 0.001$ *versus* endothelium-intact aorta at 1.39% LE. (*B*) Lipid emulsion dose-response curve in 10^{-3} M ropivacaine (RPV)-induced vasodilation of isolated aorta without endothelium. * $P = 0.0078$, † $P < 0.001$ *versus* 10^{-3} M RPV; ‡ $P = 0.0079$, # $P < 0.001$ *versus* 0.24% lipid emulsion. (*C*) Lipid emulsion dose-response curve in the isolated aorta without an endothelium after 10^{-2} M mepivacaine (MPV)-induced vasodilation. There was no significant difference in lipid emulsion dose-response curves in the endothelium-denuded aorta between 10^{-2} M MPV and the time-matched control treated with MPV, but not lipid emulsion. Data are shown as mean ± SD and are expressed as a percentage of the maximal contraction induced by isotonic 60 mM KCl (100% = 2.41 ± 0.23 g [n = 7] and 100% = 2.79 ± 0.15 g [n = 7] for aortic rings with or without endothelium, respectively in *A*; 100% = 3.16 ± 0.47 g [n = 7] for aortic rings without endothelium in *B*; 100% = 3.18 ± 0.36 g [n = 7] for aortic rings without endothelium in *C*). N indicates the number of rats from which descending thoracic aortic rings were derived. (*D*) The effect of endothelial denudation on the lipid emulsion (LE)-mediated reversal of 3×10^{-4} M levobupivacaine (LBV)-induced vasodilation in the isolated aorta. Data are shown as mean ± SD and are expressed as the percentage of reversal from 3×10^{-4} M LBV-induced vasodilation (3×10^{-4} M LBV-induced vasodilation: 100% = 0.31 ± 0.09 g [n = 7] and 100% = 0.70 ± 0.11 g [n = 7] for aortic rings with and without endothelium, respectively). N indicates the number of rats from which descending thoracic aortic rings were derived. * $P = 0.003$, † $P < 0.001$ *versus* endothelium-denuded aorta.

levobupivacaine-treated than in the ropivacaine-treated group ($P < 0.001$ *vs.* 10^{-3} M ropivacaine at 0.24–1.81% LE; fig. 5).

To determine whether LE would inhibit the vasodilation induced by high-dose local anesthetics or would affect the vasoconstriction at low doses, the effects of LE pretreatment on levobupivacaine-induced responses were analyzed. LE pretreatment for 30 min inhibited both vasoconstriction and vasodilation induced by levobupivacaine (fig. 6). ED_{50} (control = −4.74 ± 0.08 M) of levobupivacaine-induced contraction was significantly increased by LE (−4.44 ± 0.07 and −4.19 ± 0.09 M in 0.95 and 1.81% of LE, respectively; $P < 0.001$; fig. 6). The LE-mediated inhibition of ropivacaine-induced contraction was observed only at 1.81% LE (ED_{50}: −3.86 ± 0.22 M [n = 6] *vs.* −4.12 ± 0.15 M [n = 6] in controls; $P = 0.0288$). However, LE did not significantly alter the ED_{50} of mepivacaine-induced contraction (ED_{50}: control = −3.25 ± 0.07 M [n = 6]; 0.95% LE = −3.29 ± 0.13 M [n = 6]; $P = 0.6927$ *vs.* control; 1.81% LE = −3.20 ± 0.14 M [n = 6]; $P = 0.5399$ *vs.* control). The magnitude of LE-induced reversal was greater in levobupivacaine (3×10^{-5} M)- than in ropivacaine (1.5×10^{-4} M)-induced vasoconstriction of isolated endothelium-denuded aorta ($P < 0.001$ *vs.* 1.5×10^{-4} M ropivacaine at 1.39% LE), whereas LE had no effect on 10^{-3} M mepivacaine-induced vasoconstriction (3×10^{-5} M levobupivacaine and 1.5×10^{-4} M ropivacaine; $P < 0.001$ *vs.* 10^{-3} M mepivacaine).

Treatment of human umbilical vein endothelial cells with low (3×10^{-5} M)- and high (3×10^{-4} M)-dose levobupivacaine significantly increased eNOS phosphorylation at 10 and 1 min, respectively (fig. 7). However, posttreatment with 0.95% LE significantly attenuated the activation of

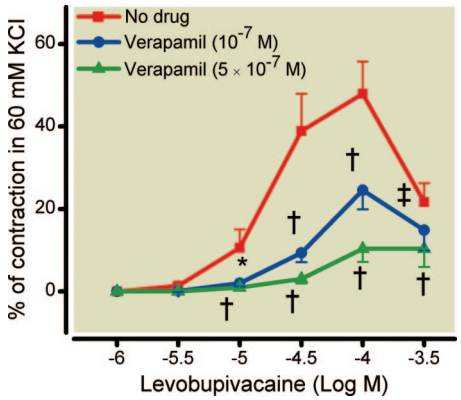

Fig. 4. The effect of verapamil on levobupivacaine (LBV) concentration-response curve in the endothelium-denuded aorta. Data are shown as mean ± SD and are expressed as a percentage of the maximal contraction induced by isotonic 60 mM KCl (100% = 3.61 ± 0.71 g [n = 6], 100 = 3.12 ± 0.49 g [n = 6], and 100% = 3.16 ± 0.39 g [n = 6] for endothelium-denuded aorta with no drug, 10^{-7} M verapamil, and 5×10^{-7} M verapamil, respectively). N indicates the number of rats from which descending thoracic aortic rings were derived. Verapamil (10^{-7} M): * $P = 0.0027$ *versus* no drug at 10^{-5} M LBV; ‡ $P = 0.0108$ *versus* no drug at 3×10^{-4} M LBV. † $P < 0.001$ *versus* no drug.

eNOS by levobupivacaine (fig. 7). In addition, basal levels of caveolin-1 phosphorylation, which are consistent with stimulation of eNOS activity, remained unchanged after levobupivacaine treatment, whereas posttreatment with LE significantly decreased phosphorylation, indicating inhibition of eNOS activity (fig. 8).

Discussion

This is the first study to demonstrate that LE reverses vasodilation caused by local anesthetics in isolated rat aorta through a mechanism that seems to be related to their lipid solubility. In this study, LE significantly reversed vasodilation caused by a high dose of levobupivacaine and ropivacaine. The magnitude of the reversal was greater in the levobupivacaine- than in ropivacaine-induced vasodilation. In contrast, LE did not alter the mepivacaine-induced response, and LE itself had no effect on baseline resting tension. The magnitude of LE-mediated reversal of local anesthetic-induced responses seems greatest for levobupivacaine, followed by ropivacaine, and then mepivacaine. Their lipid solubility (lipid/H_2O partition coefficient) also follows the same order: levobupivacaine (27.5), ropivacaine (2.8), and then mepivacaine (0.8).[12] LE inhibited vasoconstriction caused by low-dose levobupivacaine and ropivacaine, as well as vasodilation at high doses. LE increased the ED_{50} of levobupivacaine- and ropivacaine-induced contractile responses. Taking into consideration the lipid solubility, previous reports[1,2] and the above results suggest that the magnitude of LE-mediated reversal of local anesthetic-induced vasodilation and vasoconstriction may result from differences in their lipid solubility.

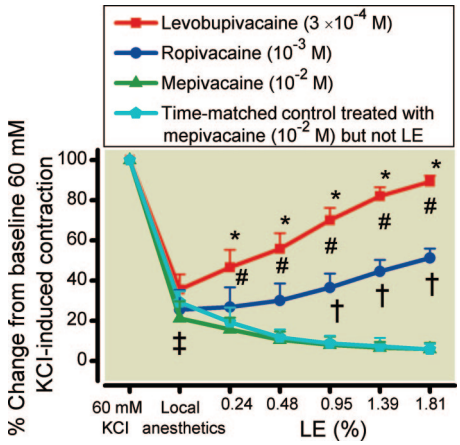

Fig. 5. High-dose local anesthetic (levobupivacaine, ropivacaine, and mepivacaine) and lipid emulsion (LE) dose-response curves in the endothelium-denuded aorta precontracted with 60 mM potassium chloride (KCl). Data are shown as mean ± SD and expressed as the percentage change from maximal baseline precontraction induced by isotonic 60 mM KCl (100% = 2.88 ± 0.67 g [n = 6], 100% = 2.99 ± 0.38 g [n = 6], 100% = 3.21 ± 0.45 g [n = 6], and 100% = 2.83 ± 0.25 g [n = 3] for endothelium-denuded aorta with 3×10^{-4} M levobupivacaine, 10^{-3} M ropivacaine, 10^{-2} M mepivacaine, and time-matched control for 10^{-2} M mepivacaine, respectively). There were no significant differences in LE dose-response curves in endothelium-denuded aorta between 10^{-2} M mepivacaine and the time-matched control treated with mepivacaine, but not LE. N indicates the number of rats from which descending thoracic aortic rings were derived. * $P < 0.001$ *versus* 3×10^{-4} M levobupivacaine; † $P < 0.001$ *versus* 10^{-3} M ropivacaine; ‡ $P < 0.001$ *versus* 60 mM KCl-induced contraction; # $P < 0.001$ *versus* 10^{-3} M ropivacaine at 0.24–1.81% LE.

In agreement with previous reports,[10,11,13] lower concentrations of local anesthetics produced vasoconstriction followed by vasodilation at 3×10^{-4} M levobupivacaine, 10^{-3} M ropivacaine, and 10^{-2} M mepivacaine, which correspond to toxic plasma concentrations.[8,9,14] The effects of local anesthetics on the peripheral vasculature are not uniform and may depend on the method of administration (*i.e.*, intravenous, intraarterial, or local), concentration, target organ, and isomer.[15] The final effect of local anesthetics on vasculature may be vasoconstriction or vasodilation, mainly depending on the local tissue concentration and vascular bed.

The formulation of SMOFlipid® 20% contains 6% soybean oil, 6% medium-chain triglycerides, 5% olive oil, 3% fish oil, 0.02% tocopherol, 1.2% purified egg phospholipids, 2.5% glycerol, and water for injection.[16] In this study, 0.24% LE reversed 3×10^{-4} M levobupivacaine- and 10^{-3} M ropivacaine-induced vasodilation of isolated rat aorta. In addition, levobupivacaine and ropivacaine have a similar clinical and pharmacologic profile.[10,11,17] Various formulations of 20% LE have been suggested for the treatment of local anesthetic toxicity in human cases.[5–7,18] However, the appropriate dosing regimen for LE to reverse local anesthetic-induced systemic toxicity remains to be determined. The clinical relevance of LE-mediated reversal of vasodilation

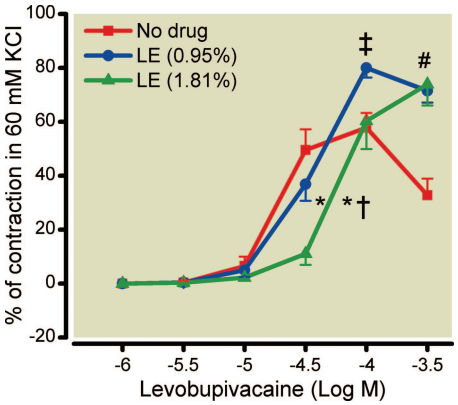

Fig. 6. Effect of lipid emulsion (LE) on levobupivacaine (LBV) dose-response curves in the endothelium-denuded aorta. Data are shown as mean ± SD and are expressed as a percentage of the maximal contraction induced by isotonic 60 mM KCl (100% = 2.95 ± 0.44 g [n = 6], 100% = 2.92 ± 0.45 g [n = 6], and 100% = 3.08 ± 0.53 g [n = 6] for aortic rings with no drug, 0.95% LE, and 1.81% LE, respectively). ED_{50}: * $P < 0.001$ *versus* no drug † $P < 0.001$ *versus* 0.95% LE; 0.95% LE: ‡ $P < 0.001$ *versus* no drug at 10^{-4} M LBV; 0.95 and 1.81% LE: # $P < 0.001$ *versus* no drug at 3×10^{-4} M LBV.

caused by toxic doses of levobupivacaine must be tempered by the fact that isolated aorta was used in this study, whereas organ blood flow is controlled by small resistance arterioles. Even with this limitation however, this study is the first to suggest that LE may contribute to a reversal of standard cardiopulmonary resuscitation-resistant vascular collapse secondary to toxic doses of levobupivacaine, which are encountered after accidental intravascular injection. Conversely, LE-mediated reversal of low-dose levobupivacaine-induced contraction may contribute to an increase in local blood flow, vascular absorption, and elimination of levobupivacaine, leading to a shorter duration of levobupivacaine-induced regional anesthesia. Similarly, a previous study reported that LE does not improve recovery from mepivacaine-induced cardiac arrest.[19]

In vasodilation caused by high-dose levobupivacaine, the magnitude of LE-induced reversal was greater in the endothelium-intact than in the endothelium-denuded aorta (fig. 3D). The results in this study showed that levobupivacaine regulated the activity of eNOS. These results imply that levobupivacaine-mediated response may be related to endothelial activation. Caveolin-1 is known to associate with, and inhibit, eNOS in caveolae of the plasma membrane.[20] It is phosphorylated on Tyr14 by Src kinase,[21] and Kiss *et al.*[22] recently found that caveolin-1 phosphorylation by Src kinase reduced interaction with other signaling enzymes. Thus, increased phosphorylation of caveolin-1 could activate eNOS, particularly through phosphorylation of a typical site, such as Ser1179, as suggested by Maniatis *et al.*[23] Further, Src kinase phosphorylates eNOS at Tyr83,[24] therefore stimulating phosphorylation of both eNOS and caveolin-1. Although we evaluated only one site (Ser1177) of eNOS phosphorylation, our results suggest that the acute attenuation of caveolin-1 phosphorylation on posttreatment with LE could result in a decrease of eNOS activity. The decreased phosphorylation of eNOS and caveolin-1 may additively inhibit levobupivacaine-induced nitric oxide production. In the isometric tension model of this *in vitro* study, the LE-mediated reversal kinetics of vasodilation induced by levobupivacaine were more potent in the endothelium-intact than in the endothelium-denuded aorta (fig. 3D). This result could be ascribed to the LE-mediated inhibition of levobupivacaine-activated eNOS.

Verapamil attenuated contractions in response to levobupivacaine in a dose-dependent manner. In the endothelium-denuded aorta precontracted with 60 mM KCl, high-dose local anesthetics produced vasodilation ($P <$ 0.001; fig. 5). Levobupivacaine (10^{-5} M) had a maximal

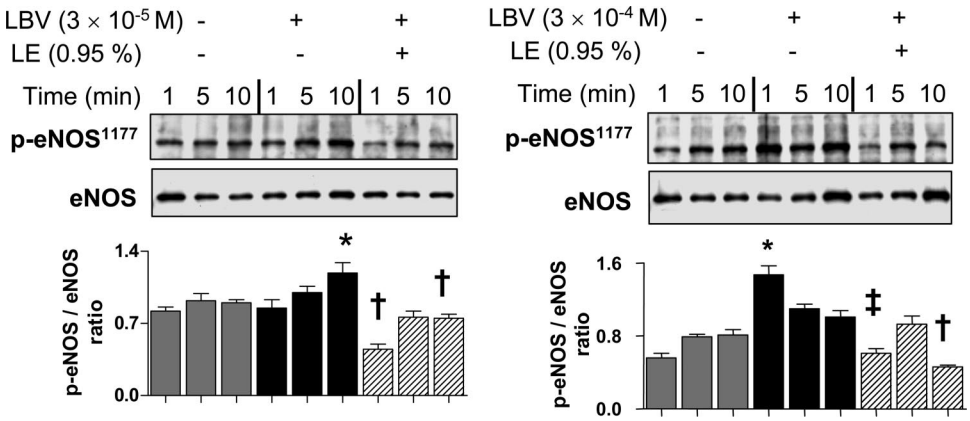

Fig. 7. Acute effect (1, 5, and 10 min) of levobupivacaine (LBV; 3×10^{-5} and 3×10^{-4} M) with or without lipid emulsion (LE; 0.95%) on endothelial nitric oxide synthase (eNOS) activation by phosphorylation at Ser^{1177} in human umbilical vein endothelial cells. LE was added for 1, 5, or 10 min after pretreatment with LBV for 1 min. Data are shown as mean ± SD of three independent experiments. * $P < 0.05$ *versus* each time point of untreated controls. † $P < 0.01$ and ‡ $P < 0.05$ *versus* each time point of LBV-treated samples; $P = 0.032$ *versus* 10-min point of untreated controls; $P = 0.006$ and $P = 0.008$ *versus* the values at 1- and 10-min points of 3×10^{-5} M LBV-treated cells, respectively; $P = 0.017$ *versus* 1-min point of untreated controls; $P =$ 0.041 and $P = 0.002$ *versus* the values at 1- and 10-min points of 3×10^{-4} M LBV-treated cells, respectively.

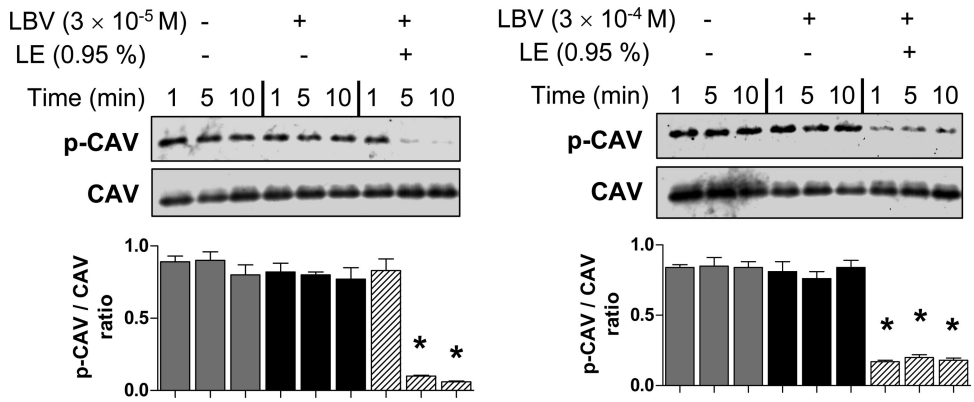

Fig. 8. Acute effect (1, 5, and 10 min) of levobupivacaine (LBV; 3×10^{-5} and 3×10^{-4} M) with or without lipid emulsion (LE; 0.95%) on the caveolin-1 (CAV) activation by phosphorylation at Tyr^{14} in human umbilical vein endothelial cells. LE was added for 1, 5, or 10 min after pretreatment with LBV for 1 min. Data are shown as mean ± SD of three independent experiments. * $P <$ 0.05 *versus* each time point of LBV-treated samples; $P = 0.011$ and $P = 0.014$ *versus* the values at 5- and 10-min points of 3×10^{-5} M LBV-treated cells, respectively; $P = 0.031$, $P = 0.029$, and $P = 0.018$ *versus* the values at 1-, 5-, and 10-min points of 3×10^{-4} M LBV-treated cells, respectively.

inhibitory effect on L-type calcium channels in isolated cardiac myocytes, which may contribute to decreased cardiac output.[25] Levobupivacaine- and ropivacaine-induced contraction of rat aortic smooth muscle is dependent on extracellular calcium influx.[26],‡‡ From these results and other studies,[25,26]‡‡ it can be assumed that high-dose (10^{-4}, 3×10^{-4} M) levobupivacaine has an inhibitory effect on VOCCs in vascular smooth muscle after an initial activation that accounts for levobupivacaine-induced contraction. Long-chain fatty acids induce an increase in voltage-operated calcium current in cardiac myocytes.[27] These results and previous reports[1,12,27] suggest that the magnitude of LE-induced reversal seems to be associated with LE-mediated, lipid solubility–related clearance of toxic doses of levobupivacaine that are involved in both VOCC inhibition and enhanced endothelial nitric oxide release. Further studies are needed to elucidate the detailed cellular mechanisms responsible for the effect of LE on the both VOCC inhibition and enhanced endothelial nitric oxide release induced by toxic levels of levobupivacaine in blood vessels.

In conclusion, this study indicates that LE-induced reversal of toxic dose (3×10^{-4} M) levobupivacaine-induced vasodilation in the isolated rat aorta is mediated mainly by the attenuation of levobupivacaine-mediated inhibition of VOCC-dependent contraction in vascular smooth muscle and, partly, by the inhibition of levobupivacaine-induced endothelial nitric oxide release *via* a mechanism that seems to be related to the lipid solubility of local anesthetics. In addition, the magnitude of LE-mediated reversal of vasodilation and vasoconstriction induced by local anesthetics (*i.e.*, levobupivacaine, ropivacaine, and mepivacaine) may be related to their lipid solubility. This LE-mediated reversal may be the mechanism by which LE rescues the vascular collapse triggered by toxic doses of local anesthetics.

‡‡ Sohn J-T, Baik J: Calcium dependent mechanism in the levobupivacaine-induced contraction of rat aortic smooth muscle. A94. From the Proceedings of the 2009 Annual Meeting of American Society of Anesthesiologists, New Orleans, Louisiana, October 17, 2009.

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