Bupivacaine Inhibits Acylcarnitine Exchange in Cardiac Mitochondria

Guy L. Weinberg, M.D., * June W. Palmer, Ph.D., † Timothy R. VadeBoncouer, M.D., ‡ Mikko B. Zuechner, M.D., §
Guy Edelman, M.D., || Charles L. Hoppel, M.D.##

Background: The authors previously reported that secondary carnitine deficiency may sensitize the heart to bupivacaine-induced arrhythmias. In this study, the authors tested whether bupivacaine inhibits carnitine metabolism in cardiac mitochondria.

Methods: Rat cardiac interfibrillar mitochondria were prepared using a differential centrifugation technique. Rates of adenosine diphosphate-stimulated (state III) and adenosine diphosphate-limited (state IV) oxygen consumption were measured using a Clark electrode, using lipid or nonlipid substrates with varying concentrations of a local anesthetic.

Results: State III respiration supported by the nonlipid substrate pyruvate (plus malate) is minimally affected by bupivacaine concentrations up to 2 μM. Lower concentrations of bupivacaine inhibited respiration when the available substrates were palmitoylcarnitine or acetylcarnitine; bupivacaine concentration causing 50% reduction in respiration (IC50 ± SD) was 0.78 ± 0.17 μM and 0.37 ± 0.03 μM for palmitoylcarnitine and acetylcarnitine, respectively. Respiration was equally inhibited by bupivacaine when the substrates were palmitoylcarnitine alone, or palmitoyl-CoA plus carnitine. Bupivacaine (IC50 = 0.26 ± 0.06 μM) and etidocaine (IC50 = 0.30 ± 0.12 μM) inhibit carnitine-stimulated pyruvate oxidation similarly, whereas the lidocaine IC50 is greater by a factor of roughly 5. (IC50 = 1.4 ± 0.26 μM), and ropivacaine is intermediate, IC50 = 0.5 ± 0.28 μM.

Conclusions: Bupivacaine inhibits mitochondrial state III respiration when acylcarnitines are the available substrate. The substrate specificity of this effect rules out bupivacaine inhibition of carnitine palmitoyl transferases I and II, carnitine acyltransferase, and fatty acid β-oxidation. The authors hypothesize that differential inhibition of carnitine-stimulated pyruvate oxidation by various local anesthetics supports the clinical relevance of inhibition of carnitine-acylcarnitine translocase by local anesthetics with a cardiotoxic profile. (Key words: Bupivacaine; cardiotoxicity; carnitine; carnitine-acylcarnitine translocase; local anesthetic; metabolism.)

BUPIVACAINE is a lipophilic local anesthetic widely used for regional anesthesia when prolonged neural blockade is desirable. Its clinical usefulness, however, is limited by its well-known cardiotoxicity. Several mechanisms are proposed to account for the malignant arrhythmias, conduction disturbance, and myocardial depression typical of this phenomenon. Bupivacaine inhibits sodium,1 calcium,2 and potassium5,6 ion channels. It is reported to interfere with β-adrenergic5,6 and lysophosphatidyl signal transduction pathways and can activate the autonomic nervous system.8 At high concentrations bupivacaine also collapses the mitochondrial transmembrane potential8,9 and inhibits electron transport necessary for oxidative phosphorylation.10

A clinical observation by one of the authors suggests another possible mitochondrial site of bupivacaine action.11 We previously reported that a patient with a history of secondary carnitine deficiency experienced severe ventricular dysrhythmias after receiving a small subcutaneous dose of bupivacaine. We hypothesized...
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Fig. 1. Schematic of fatty acid transport into mitochondria. 
FACoA = fatty acyl-CoA; FACn = fatty acylcarnitine; CPT = carnitine palmitoyl transferase; Cn = carnitine; C = cytoplasm; OM = outer mitochondrial membrane; IMS = intermembrane space; IM = inner mitochondrial membrane; M = matrix; TL = carnitine-acylcarnitine translocase.

that bupivacaine inhibition of a carnitine-dependent pathway might explain this event, if carnitine depletion caused the patient’s apparent exaggerated sensitivity to bupivacaine. Carnitine is necessary for transport of fatty acids into the mitochondrial matrix, where they are normally the predominant energy source for the heart.\textsuperscript{12} This is a three-step process: carnitine acylation of the acyl-CoA at the outer mitochondrial membrane, translocation of acylcarnitine across the inner membrane, and regeneration of the acyl-CoA in the mitochondrial matrix (fig. 1). We measured respiration in isolated cardiac mitochondria oxidizing lipid and nonlipid substrates to test the hypothesis that bupivacaine inhibits one or more of these steps.

Materials and Methods

The investigation was approved by the institutional animal care review board of Cleveland Veterans Administration Medical Center and the University of Illinois Animal Care Committee and Biological Resources Laboratory. Experiments were performed at the Cleveland Veterans Administration Medical Center and the University of Illinois College of Medicine in accordance with the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health, Publication No. 85-23, revised, 1985). Cardiac interfibrillar mitochondria were prepared from a homogenate of the rat cardiac ventricles, by differential centrifugation according to the procedure of Palmer et al.\textsuperscript{13} Briefly, after guillotine decapitation, the hearts of two male Sprague-Dawley rats, weighing 250–370 g, were surgically removed; the ventricles were opened; and the atria, connective tissue, and fat were cut away. The remaining tissue was minced with scissors, then homogenized with a pestle in the presence of Nagarse protease (Type XXVII; Sigma #P-4789, St. Louis, MO). The homogenate was centrifuged at 8,000 rpm for 5 min, resuspended, and strained through double-layer gauze. After two more cycles of a 10-min spin at 2,000 rpm and straining the resuspended supernatant, there is a final spin at 5,000 rpm for 10 min. The resulting pellet of interfibrillar mitochondria is resuspended in a potassium chloride buffer containing MOPS, EGTA, and albumin (pH 7.4). Yield is typically on the order of 20 mg mitochondrial protein per heart.

Respiration was measured at 30°C in a 0.5-ml chamber containing mitochondria in a final concentration of 1 mg protein/ml. After equilibration of the Clark oxygen electrode probe, endogenous mitochondrial substrates were depleted by addition of 0.1 mM adenosine diphosphate (ADP). Respiration was then initiated by the addition of substrate to the incubation medium. Mitochondrial oxygen use was monitored during state III (active, ADP-stimulated) and state IV (resting, ADP-limited) respiration by measuring the rate of decrease of oxygen concentration in the chamber. Carnitine-stimulated pyruvate oxidation was taken as the difference in state III respiratory rate before and after addition of carnitine to the reaction chamber containing pyruvate, malonate, and ADP (2 mM). Calculations of respiratory rates, in units of ng atomO·min\textsuperscript{-1}·mg\textsuperscript{-1} protein, were derived from the slope of oxygen concentration in the reaction chamber.\textsuperscript{13}

Statistical Analysis

Anesthetic IC\textsubscript{50} values for inhibition of respiration were determined by fitting the data for each experiment to a second-degree polynomial using SigmaPlot (SPSS, Chicago, IL) for the nonlinear regression; R\textsuperscript{2} values for all curve fits ranged from 0.80 to 0.98. IC\textsubscript{50} values were statistically compared by means of analysis of variance, with the Tukey test used for multiple comparisons. Statistical significance was taken as \(P < 0.05\). Figures 2 and 3 were created by fitting curves to the mean data using SigmaPlot ± SE.

Bupivacaine HCl was purchased from Abbott Laboratories (North Chicago, IL); etidocaine HCl and ropivacaine HCl were purchased from Astra Pharmaceutical (Westborough, MA). All other substrates were purchased from Sigma.
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Fig. 2. Effects of bupivacaine on state III (adenosine diphosphate (ADP)-stimulated) respiration supported by various substrates. Points are mean values expressed as a percent of control ± SE bar. Symbol, substrate, number of experiments, and mean values for control respiration (ngatomo -min -1 · mg -1 protein ± SD) are as follows: circle = pyruvate plus malate, n = 4, 367 ± 29; diamond = palmitoylcarnitine plus malate, n = 4, 310 ± 28.7; square = acetylcarnitine plus malate, n = 3, 199 ± 12; triangle = pyruvate plus malonate and carnitine (carnitine-stimulated pyruvate oxidation), n = 4, 33.1 ± 3.0.

Results

Effect of Bupivacaine on Lipid- and Nonlipid-based Respiration

State III Respiration. Concentration-dependent effects of bupivacaine were measured for state III respiration supported by pyruvate (10 mM), palmitoylcarnitine (10 mM), or acetylcarnitine (5 mM), each in the presence of L-malate (5 mM), which stimulates the tricarboxylic acid cycle. Pyruvate (plus L-malate) oxidation is a measure of respiration not dependent on lipid substrates. Bupivacaine has a negligible effect on pyruvate oxidation, only reducing state III respiration to 96% of control at concentrations up to 2 mM (n = 4). However, bupivacaine at concentrations less than 1 mM strongly inhibits respiration dependent on palmitoylcarnitine and acetylcarnitine; bupivacaine IC 50 ± SD for respiration dependent on palmitoylcarnitine (n = 4) and acetylcarnitine (n = 3) was 0.78 ± 0.17 mM and 0.37 ± 0.05 mM, respectively. The state III data for these substrates, with bupivacaine concentrations ranging from 0 to 1 mM, are shown in figure 2. Control values for respiration are given in the legend.

State IV Respiration. We measured the effects of bupivacaine on rates of state IV respiration supported by pyruvate (plus malate) or acetylcarnitine (plus malate). Rates for bupivacaine 0, 0.5, or 1.0 mM (ngatomo · min -1 · mg -1 protein ± SD; n = 6 for each condition) were as follows: 19.8 ± 2.98, 24.8 ± 4.4, 32.1 ± 7.93 and 16.1 ± 5.17, 20.2 ± 1.91, 23.9 ± 2.05, for pyruvate and acetylcarnitine, respectively. This confirms that bupivacaine accelerates ADP-limited (resting) rates of respiration, consistent with uncoupling of oxidative phosphorylation. The effect was similar for both substrates: 25% increase compared with control rates of respiration at 0.5 mM bupivacaine and a 50–60% increase at 1 mM.

Substrate Specificity. We compared the effects of bupivacaine on respiration supported by either palmitoylcarnitine (40 μM) plus malate (5 mM) or palmitoyl-CoA (40 μM) plus carnitine (2 mM) and malate (5 mM). Control values (n = 3 for all four data sets) were (ngatomo · min -1 · mg -1 protein ± SE): 321 ± 18 for palmitoylcarnitine and 255 ± 43 for palmitoyl-CoA plus carnitine. With addition of bupivacaine (1 mM) the respiratory rates were 219 ± 31 for palmitoylcarnitine and

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173 ± 29 for palmitoyl-CoA plus carnitine. Nearly identical (32%) inhibition of respiration for both conditions indicates that bupivacaine affects a site downstream from these substrates, not the reaction catalyzed by carnitine palmitoyl transferase-I (CPT I), which converts palmitoyl-CoA to palmitoylcarnitine.

Carnitine-stimulated Pyruvate Oxidation
The only shared step in mitochondrial transport of the acylcarnitines examined is catalyzed by carnitine-acylcarnitine translocase. When malate is replaced with malonate, the tricarboxylic acid cycle is inhibited, causing acetyl-CoA to accumulate, and thereby slowing pyruvate oxidation by feedback inhibition of pyruvate dehydrogenase. The addition of carnitine relieves this inhibition by allowing conversion of acetyl-CoA to acetyl-carnitine, which is transported out of the mitochondria via carnitine-acylcarnitine translocase. Thus, in the presence of pyruvate and malonate (10 mM), a carnitine-dependent increase in oxygen consumption is a measure of translocase activity. Bupivacaine strongly inhibited the rate of the carnitine-stimulated pyruvate respiration in the presence of malonate (n = 4; figure 1; IC50 ± SD = 0.26 ± 0.06 mM). We also measured concentration-dependent effects of etidocaine (n = 4), ropivacaine (n = 4), and lidocaine (n = 4) on carnitine-stimulated pyruvate oxidation (fig. 3). Local anesthetic IC50 ± SD were etidocaine: 0.30 ± 0.12 mM; ropivacaine: 0.65 ± 0.28 mM; and lidocaine: 1.41 ± 0.26 mM. Statistical significance was achieved for the differences between the lidocaine IC50 and those of the other three local anesthetics evaluated.

Discussion
Bupivacaine inhibited lipid-based respiration at concentrations that do not appreciably affect pyruvate oxidation in the presence of malate. This observation suggested a bupivacaine action on the carnitine system. Furthermore, equal inhibition of both palmitoyl-CoA and palmitoylcarnitine oxidation implied that CPT-I is not a key site of bupivacaine's effect. Because acetylcarnitine is metabolized by carnitine acetyltransferase, and palmitoylcarnitine is metabolized by CPT-II, an isolated effect on either of these enzymes would not explain the inhibition. Acetylcarnitine goes directly into the TCA cycle, so inhibition of fatty acid β-oxidation is also excluded. These findings are consistent with bupivacaine inhibition of carnitine-acylcarnitine translocase, the only enzyme participating in the mitochondrial transport of both acylcarnitines evaluated.

The translocase mediates the reversible movement of acylcarnitines through the inner mitochondrial membrane in a one-to-one molar exchange (antiport) for free carnitine. Our observation that bupivacaine was a potent inhibitor of the carnitine-dependent oxidation of pyruvate in the presence of malonate is strong evidence for inhibition of carnitine-acylcarnitine translocase. To clarify the relevance of translocase inhibition to clinical cardiotoxicity, we evaluated other local anesthetics with known degrees of cardiotoxicity. We found the etidocaine IC50 for inhibition carnitine-stimulated pyruvate oxidation was nearly the same as that of bupivacaine, whereas the lidocaine IC50 was greater by a factor of 5, and that of ropivacaine was intermediate. Thus, the IC50 of the four anesthetics we measured, paralleled their rank order of cardiotoxicity. The correlation of translocase inhibition with the degree of local anesthetic cardiotoxicity supports the possible clinical relevance of this effect.

Mitochondria oxidize carbon based fuels, either carbohydrate or fatty acid derivatives, creating a chemiosmotic proton gradient across the inner mitochondrial membrane that drives the adenosine triphosphate (ATP) synthetic machinery. State III, or active, respiration measures oxygen consumed in converting ADP to ATP. Local anesthetics slow state III respiration by acting at any of several sites, including electron transport and the ATP synthase. Sztark et al. showed that bupivacaine specifically inhibits complex I of the respiratory chain. Our observations suggest bupivacaine also impairs the mitochondrial transport of lipid fuels necessary for normal aerobic cardiac function.

State IV respiration measures the oxygen consumed in the resting state to maintain the chemiosmotic gradient. A chemical such as dinitrophenol that dissipates this gradient uncouples respiration from ATP synthesis and accelerates state IV respiration. We confirmed the well-known respiratory uncoupling effect of bupivacaine and further showed that acceleration in state IV respiration is similar whether the available substrate is acetyl-carnitine or pyruvate. However, the bupivacaine IC50 for the translocase is lower by nearly an order of magnitude than the concentration (2 mM) that maximizes uncoupling. Nevertheless, the question arises whether the myocardial concentration of bupivacaine during a cardiotoxic reaction is anywhere near the translocase IC50. Covino showed that peak arterial local anesthetic concentrations depend on rate of injection and that after

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rapid intravenous injection, concentrations can reach roughly 100 μM. Furthermore, we previously reported that during continuous bupivacaine intravenous infusion in rats, the average arterial plasma bupivacaine concentration measured at asystole was 290 μM. Thus, accidental intravascular injection of a large bolus of bupivacaine can result in peak arterial bupivacaine concentrations of the same order as the IC₅₀, and bupivacaine concentrations at an experimentally determined cardiootoxic end point slightly exceed the IC₅₀ for translocase activity.

We speculate that inhibition of carnitine–acylcarnitine translocase could explain several clinical features of bupivacaine toxicity. Oxidation of fatty acid derivatives in the mitochondrial matrix normally provides more than 70% of myocardial energy needs. The clinical relevance of this predilection for metabolizing lipid is underscored by the fact that translocase mutations cause severe cardiac dysfunction, including conduction block, that is usually incompatible with life. The combination of translocase inhibition by bupivacaine and the heart’s unique respiratory dependence on fatty acid substrates may account for the relatively low ratio of the toxic bupivacaine dose necessary for cardiovascular collapse to that producing convulsions. Inhibition of carnitine–acylcarnitine translocase could also contribute to bupivacaine-induced cardiac rhythm disturbances because the accumulation of cytoplasmic acylcarnitines, a presumed effect of translocase inhibition, is known to cause arrhythmias. Our hypothesis is consistent with the observation by Heavner et al. and others that hypoxia exacerabtes bupivacaine-induced cardiotoxicity because limiting oxygen could further impair oxidative phosphorylation already inhibited by reduced transport of necessary substrates.

Our findings may provide an additional explanation for the beneficial effect of lipid infusion in treating bupivacaine cardiotoxicity. We previously ascribed this phenomenon to a “lipid sink” effect, with bupivacaine partitioning into the newly formed lipemic phase of plasma. We further hypothesize that infused triglycerides, and subsequently released fatty acids, might also work by mass action to increase the flux of acylcarnitines into the mitochondrial matrix, thereby relieving a block caused by bupivacaine. This hypothesis is limited by our lack of knowledge regarding the precise mechanism (i.e., competitive, noncompetitive, or other) for bupivacaine inhibition of the translocase.

We have shown that bupivacaine inhibits a key step in mitochondrial transport of the dominant fuel source for the heart. Elucidating the underlying mechanisms of local anesthetic cardiotoxicity may help in developing treatment strategies or synthesizing safer long-acting local anesthetics. A focus on lipid metabolic substrates and their use by mitochondria is suggested by the results of this study.

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