Effect of Bupivacaine and Levobupivacaine on Exocytotic Norepinephrine Release from Rat Atria

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Background: The cardiotoxic mechanism of local anesthetics may include interruption of cardiac sympathetic reflexes. The authors undertook this investigation to determine if clinically relevant concentrations of bupivacaine and levobupivacaine interfere with exocytotic norepinephrine release from cardiac sympathetic nerve endings.

Methods: Rat atria were prepared for measurements of twitch contractile force and [3H]-norepinephrine release. After nerve endings were loaded with [3H]-norepinephrine, the tissue was electrically stimulated in 5-min episodes during 10 10-min sampling periods. After each period, a sample of bath fluid was analyzed for radioactivity and [3H]-norepinephrine release was expressed as a fraction of tissue counts. Atria were exposed to buffer alone during sampling periods 1 and 2 (S1 and S2). Control atria received saline (100 μl each, n = 6 atria) in S3-S10. Experimental groups (n = 6 per group) received either bupivacaine or levobupivacaine at concentrations (in μM) of 5 (S3-S4), 10 (S5-S6), 30 (S7-S8), and 100 (S9-S10).

Results: Bupivacaine and levobupivacaine decreased stimulation-evoked fractional [3H]-norepinephrine release with inhibitory concentration 50% values of 5.1 ± 0.5 and 6.1 ± 1.3 μM. The inhibitory effect of both local anesthetics (~70%) approached that of tetrodotoxin. Local anesthetics abolished the twitch contractions of atria with inhibitory concentration 50% values of 12.6 ± 5.0 μM (bupivacaine) and 15.7 ± 3.9 μM (levobupivacaine). In separate experiments, tetrodotoxin inhibited twitch contractile force by only 30%.

Conclusions: The results indicate that clinically relevant cardiotoxic concentrations of bupivacaine and levobupivacaine markedly depress cardiac sympathetic neurotransmission. A possible mechanism of local anesthetics in reducing evoked norepinephrine release from sympathetic endings is blockade of tetrodotoxin-sensitive fast sodium channels.

Sudden cardiovascular collapse can occur after the accidental intravascular injection of potent local anesthetics such as bupivacaine. The intravascular free concentration of bupivacaine measured during clinical cardiotoxicity is in the range of 5–15 μM. These anesthetic concentrations cause pronounced negative inotropic and dromotropic effects together with cardiac arrhythmias. The cardiac action potential in the cardioactive concentration range of bupivacaine has reduced maximal upstroke velocity, decreased conduction velocity, and abnormal postrepolarization refractoriness. Bupivacaine and its congeners partially inhibit myocardial fast sodium channels (in isolated cardiomyocytes), primarily by stabilizing the inactivated state of the channel. Electromechanical dysfunction is generally viewed as a direct effect of bupivacaine on cardiomyocytes.

One would expect the sympathetic nervous system to provide reflex compensation for cardiovascular depression caused by any directly acting cardiac depressant, assuming that the baroreceptor mechanism is intact. However, the validity of such an assumption is unknown in the case of bupivacaine. The presence of β-adrenergic receptor blockers tends to aggravate bupivacaine cardiotoxicity.

As there are no published data to our knowledge establishing the effect of bupivacaine on adrenergic neurotransmission in the heart, the current study was undertaken to determine whether bupivacaine or levobupivacaine affects evoked norepinephrine release from sympathetic nerve endings present in rat left atrium. We decided to conduct the study in atrial rather than ventricular tissue because atria have a denser sympathetic innervation than the ventricles. Our working hypothesis was that bupivacaine or levobupivacaine in the concentration range observed in clinical cardiotoxicity can block the evoked release of norepinephrine from cardiac sympathetic nerve endings.

Materials and Methods

According to a protocol approved by our Institutional Animal Care and Use Committee (Hektoen Institute for Medical Research, John H. Stroger, Jr. Hospital of Cook County, Chicago, IL) isolated rat left atrium was prepared for simultaneous measurement of twitch contractile force and [3H]-norepinephrine release.

Measurement of Twitch Contractile Force in Isolated Heart Muscle

Adult Sprague-Dawley rats (250–300 g) were anesthetized with sevoflurane. Each heart was removed and immediately perfused to purge the blood. The left atrium was excised and attached between a force-displacement transducer (Grass FT 03; Grass Instruments; Quincy, MA) and a fixed point using a pair of stainless-steel hooks (fig. 1). The transducer was connected to a strain gauge amplifier (Hewlett-Packard Model 78201B; Hewlett-Packard Co; Palo Alto, CA) that produces a calibrated voltage.
To label sympathetic nerve endings, atrial preparations were paced at mechanical threshold (10 V, 0.4 Hz) and bathed for 60 min in Krebs solution supplemented with 27 μCi of DL norepinephrine hydrochloride [7-3H(N)]. To remove excess tracer, the bath liquid was replaced 12 times at 5-min intervals. At the end of each period, a 500-μl sample was withdrawn from the bath to be used for determination of tracer washout kinetics.

Protocol for Stimulation-evoked Release of 3[H]-norepinephrine

Preparations were bathed in Krebs solution supplemented with 0.3 μM desipramine (to inhibit norepinephrine uptake 1), 15,14 10 μM metanephrine (to inhibit norepinephrine uptake 2), 15,16 and 10 μM 3',4'-dihydroxy-2-methylpropiophenone (U-0521, to inhibit catechol-O-methyltransferase) 17 for the remainder of the experiment. This portion of the experiment was divided into twelve 10-min sampling periods (S), i.e., S0 (no stimulation), priming (10 min of suprathreshold stimulation) and S1-S10. For S1-S10, the preparations received, during the first 5 min only, field stimulation (100 V, 3 Hz) well above the neural threshold to evoke exocytotic norepinephrine release. 18 We chose a stimulation frequency of 3 Hz for two reasons: 1) to ensure a measurable release of transmitter during sampling periods and 2) because isolated atrial muscle can tolerate this frequency of stimulation, so that simultaneous measurements of cardiac contractile force and neurotransmitter release are possible. At the end of each 10-min period, a 500-μl sample of the bath liquid was withdrawn for later determination of tritium, and the bath liquid was replaced with Krebs solution containing fresh inhibitors.

Atria were exposed to buffer alone during S1-S2. Control atria received saline (100 μl each, n = 6 atria) in periods S3-S10. Experimental groups (n = 6 per group) received either bupivacaine or levobupivacaine at concentrations (in μM) of 5 (S3-S4), 10 (S5-S6), 30 (S7-S8), and 100 (S9-S10). Each dose of local anesthetic was added to the bath at the beginning of the corresponding sampling period. In separate experiments, the effects of tetrodotoxin (0.1 μM, n = 6) 19,20 were tested (S3-S10). To determine the effects of local anesthetic agents on unstimulated release of 3[H]-norepinephrine we used a protocol similar to that described above except that the preparations received no electrical stimulation during periods S1-S10.

At the conclusion of each experiment, the atrial preparation was blotted, weighed, and placed overnight in a glass vial containing 1 ml of 2% (v/v) perchloric acid. Radioactivity in tissue and collected samples of the bath fluid was determined by mixing them with a biodegradable counting cocktail (Econo-Safe; Mount Prospect, IL) followed by liquid scintillation counting (Packard 2000 Tri-Carb; Packard Instrument Co., Meriden, CT). 3[H]-norepinephrine release (overflow) in periods S1-S10 was expressed as a fraction of tissue radioactivity with cor-

Measurement of Exocytotic 3[H]-norepinephrine Release

To label sympathetic nerve endings, atrial preparations were paced at mechanical threshold (10 V, 0.4 Hz) and heated (33°C), oxygenated (100% oxygen) Krebs-Henseleit solution. Oxygen was provided at constant flow into a chamber port containing a sintered glass fixture to generate small oxygen bubbles surrounding the tissue. The drain port allows fluid to be removed from the chamber as needed. The tissue is mounted between a fixed point on the tissue support rod and a stainless steel wire attached to the force-displacement transducer. The atrial preparation is located between two parallel stimulating electrodes connected to a stimulator. Force of contraction, after being detected by the force-displacement transducer, is amplified and displayed on the video monitor of a computer and a penwriter. 3[H]-norepinephrine tracer is added to the chamber liquid to label sympathetic nerve endings; tritium released by electrical stimulation is sampled using a suitable pipette.

output proportional to the force generated by the muscle preparation. The atrial preparation was immersed in a water-jacketed glass chamber (10 ml capacity) containing heated (33°C), oxygenated (100% oxygen) Krebs-Henseleit solution. Oxygen was provided at constant flow into a chamber port via a gas flow meter. After a 30-min equilibration period, the resting tension of the tissue was adjusted to give half-maximal twitch developed force. Electrical stimuli were delivered via a pair of platinum electrodes on either side of the preparation. Twitch contractions were recorded on a penwriter (IITC flatbed recorder, model 45; Woodland Hills, CA) and simultaneously displayed on the video monitor of a computer (Compaq Presario 850; Hewlett-Packard) after being digitized (Labmaster Board; Tecmar, Inc., Solon, OH). Online automated measurements of the peak amplitude of twitch contractions were made and stored in a file for later analysis.

Anesthesiology, V 102, No 5, May 2005

JOSEPH ET AL.
resection for background (unstimulated) release of tritium determined in period 50.

Tissue Uptake of $^3$H-norepinephrine

Using the tracer washout samples, the number of $^3$H-norepinephrine counts remaining in unstimulated tissue was plotted against time. A double-exponential function was fitted to the data as described. The fast component (time constant of $\sim 10$ min) represents interstitial tracer washout. The slow component ($\sim 300$ min) accounts for $\sim 90\%$ of tissue uptake; extrapolation of this exponential component to 0 min gives an estimate of initial tissue uptake of tracer.

Drugs and Solutions

Drugs. Atropine, ascorbic acid, metanephrine, desipramine, and all salts used in the Krebs-Henseleit solution were obtained from Sigma Chemical Co. (St. Louis, MO). Sevoflurane and bupivacaine were obtained from Abbott Laboratories (North Chicago, IL). 3',4'-dihydroxy-2-methylpropionophenone (U-0521) was acquired from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). Levobupivacaine was a gift from Purdue Pharma L.P. (Norwalk, CT). Tetrodotoxin was purchased from Alomone Labs (Jerusalem, Israel).

Solutions. The composition of the Krebs-Henseleit solution (in mM) is: NaCl, 118; KCl, 4.7; CaCl$_2$, 2H$_2$O, 1; HEPES (acid), 5.55; HEPES (sodium salt), 4.45; MgCl$_2$, 0.25; glucose, 10; and Na$_2$EDTA, 0.025. This buffer was supplemented with atropine (1 $\mu$M/l), ascorbic acid (10 $\mu$M/l), metanephrine (10 $\mu$M/l), U-0521 (10 $\mu$M/l), and desipramine 300 $\mu$M/l. The stock solution for U-0521 was prepared in ethanol. The final concentration of ethanol (0.005%) had no effect on $^3$H-norepinephrine release or atrial contractile force on its own. Stock solutions of atropine and desipramine were prepared in Krebs-Henseleit solution. All stock solutions were aliquoted and stored at $-20^\circ$C. Final dilutions of drugs were made into Krebs-Henseleit solution immediately before use. Ascobic acid and metanephrine were weighed and added to the working buffer before each experiment. The working buffer in the tissue chamber had a temperature of 32.9–33.0°C and a pH of 7.36–7.40 throughout the experiments.

Statistical Analysis

Each dose-response curve from an individual experiment was analyzed using the power equation of AV Hill to obtain an inhibitory concentration 50% value for the local anesthetic agent used. For contractile dose-response data, the function utilized was $Basal - [(E_{max} - Basal) \times C/(C + IC_{50})]$, where $Basal$ is fractional norepinephrine release before any drug, $E_{max}$ is the effect of tetrodotoxin, $C$ is the concentration of either anesthetic agent, and $IC_{50}$ is the local anesthetic concentration yielding 50% inhibition; the Hill coefficient used was $N = 1$.

All values are expressed as mean ± SD. Statistical comparisons among group means was made by one-way or two-way analysis of variance as appropriate, and the Newman-Keuls test was used for post hoc comparisons. The unpaired Student $t$-test was utilized in the comparison of mean values for the inhibitory concentration 50%. Differences were considered statistically significant at $P < 0.05$.

Results

We used intense field stimulation to excite labeled sympathetic nerve endings in atria. In the absence of drugs, the fractional release of $^3$H-norepinephrine from tissue (above the background level) varied between 0.07 and 0.08 (fig. 2A) through the ten sampling periods. Bupivacaine (fig. 2B) and levobupivacaine (fig. 2C) were added at concentrations of 5, 10, 30, and 100 $\mu$M in periods S3-S4, S5-S6, S7-S8, and S9–10, respectively. Bupivacaine and levobupivacaine markedly decreased $^3$H-norepinephrine release from sympathetic nerve endings to levels between 0.02 and 0.03. There was no significant difference in the effects of the two anesthetic agents. Anesthetic concentrations greater than 5 $\mu$M significantly decreased evoked transmitter release. Tetrodotoxin (0.1 $\mu$M), a selective inhibitor of nerve sodium channels, blocked $\sim 80\%$ of the evoked release of $^3$H-norepinephrine (fig. 2D).

The data in figure 3 show that bupivacaine and levobupivacaine had no effect on the unstimulated release of $^3$H-norepinephrine. Thus, it is concluded that either anesthetic agent blocked only evoked release. The data shown in Table 1 indicate that in the saline, local anesthetic, and tetrodotoxin groups, loading of nerve endings was statistically identical. The labeled norepinephrine retained by the nerve endings at the end of the experiments was statistically the same in the tetrodotoxin, bupivacaine, and levobupivacaine groups. The value was significantly lower in the saline group compared with all others.

Bupivacaine and levobupivacaine did not differ significantly in the concentration dependency of their effects (fig. 4). The inhibitory concentration 50% values with respect to $^3$H-norepinephrine overflow were 5.1 ± 0.5 and 6.1 ± 1.3 $\mu$M in the presence of bupivacaine and levobupivacaine, respectively, and these values did not differ statistically. The concentration-effect curve pla-
The level of inhibition achieved at 100 μM of either anesthetic agent was close to that produced by tetrodotoxin at a concentration (0.1 μM) known to block Na+ action potentials in nerves (fig. 4, dotted line).

Bupivacaine and levobupivacaine also blocked the twitch contractions generated by the atrial preparations during the 10 sampling periods (fig. 5). The concentration dependency of the two local anesthetics tested was again quite similar. Inhibitory concentration 50% values were 12.6 ± 5.0 μM for bupivacaine and 15.7 ± 3.9 μM for levobupivacaine, and the two values did not differ significantly (fig. 6). We used tetrodotoxin to determine the contribution of sympathetic neurotransmitter release to twitch contractile force (fig. 7). Tetrodotoxin reduced the mean amplitude of twitch contractile force by 30%.

Discussion

We compared clinically relevant concentrations of bupivacaine and its enantiomer levobupivacaine for their effects on 3[H]-norepinephrine “overflow” evoked by electrical stimulation of cardiac sympathetic nerve endings in isolated rat left atria. The results indicate that cardiotoxic concentrations of bupivacaine severely impair sympathetic neurotransmission in the heart. Bupivacaine and levobupivacaine significantly decreased the evoked release of norepinephrine in a concentration-dependent fashion. The inhibitory concentration 50% values obtained were well within the cardiotoxic range of these substances (5–15 μM free plasma concentration) observed after accidental intravascular injection in humans. These observations lead one to expect that the inhibition of sympathetic neurotransmitter release by local anesthetics contributes to the mechanism of their cardiotoxicity.

Normally the direct depressant effect of an anesthetic agent on the myocardium would elicit a sympathetic reflex compensating for reduced cardiac output and central arterial pressure. Our current data imply that the sympathetic reflex is impaired at local anesthetic concentrations comparable to those seen in patients experiencing bupivacaine cardiotoxicity. We are aware of no previous report of a high sensitivity of cardiac sympathetic neurons to local anesthetic agents in the micromolar range.

There are numerous conceivable mechanisms that could explain the effects of bupivacaine and levobupivacaine on evoked norepinephrine release. For example, bupivacaine shows a similar concentration dependency
in blocking norepinephrine release (in the current study) and in inhibiting directly measured fast sodium ionic currents. Valenzuela et al. reported inhibitory concentration 50% values for S(11002) and R(11001) bupivacaine of 4.8 and 2.9 μM with respect to the inactivated sodium channel and 4.3 and 3.3 μM with respect to the open (activated) channel. Similarly Nau et al. found inhibitory concentration 50% values of 4.4 μM S(11002) and 3.8 μM R(11001) with respect to inactivated sodium channels. These inhibitory concentration 50% values are quite similar to our inhibitory concentration 50% values of 5.1 μM (bupivacaine) and 6.1 μM (levobupivacaine).

Some local anesthetics block Na+ channels that are resistant to tetrodotoxin. The concentration of bupivacaine required to reduce the amplitude of tetrodotoxin-resistant action potentials by half was 110 μM. The concentrations of the local anesthetics needed to produce inhibition of KCl (60 mM)-induced norepinephrine release from sympathetic neurons in the rat tail artery were very high (EC50 of 4.1 × 10⁻⁵ M for bupivacaine and 22.6 × 10⁻⁵ M for lidocaine) compared to those needed to inhibit contraction elicited by electrical stimulation in the same preparation (EC50 of 3.7 × 10⁻⁶ M for bupivacaine and 60.8 × 10⁻⁶ M for lidocaine). In the condition of K⁺-induced membrane depolarization, the fast sodium channels should be voltage-inactivated. It has been reported that bupivacaine at a concentration of 0.125% blocks norepinephrine release from cultured sympathetic neurons induced by eclamptic plasma. This study did not establish a mechanism for the effect of plasma on norepinephrine release and the concentration of bupivacaine used, 4 mM so one order of magnitude higher than the highest concentration of bupivacaine used in our current study and produced only a partial inhibitory effect.

Local anesthetics could affect a variety of ionic channels involved in norepinephrine release besides the fast

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**Table 1. Loading of Nerve Endings and Tissue Counts at the End of Experiments in Control (Saline) and Experimental Groups**

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Bupivacaine</th>
<th>Levobupivacaine</th>
<th>Tetrodotoxin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loading of nerve endings (cpm/mg)</td>
<td>27,977 ± 2032</td>
<td>26,205 ± 1391</td>
<td>28,934 ± 3930</td>
<td>26,501 ± 3712</td>
</tr>
<tr>
<td>Tissue counts at end of experiment (cpm/mg)</td>
<td>7892 ± 650</td>
<td>9393 ± 789*</td>
<td>10,343 ± 1742*</td>
<td>9907 ± 1931*</td>
</tr>
</tbody>
</table>

Loading was determined by extrapolation of tracer washout curves to zero time, as described (21). Atrial preparations were removed from the apparatus at the end of sampling period S10 and counted for residual radioactivity remaining in nerve endings. Values are given as mean ± SD.

*Statistical significance (P < 0.05) from the saline control.
Control

Bupivacaine

Levobupivacaine

Fig. 5. Original penwriter records of twitch contractile force during the 10 sampling periods. Pulse trains of 100 V, 3 Hz, and 2 ms were delivered through platinum field electrodes for 5 min during each stimulation period. Note blocking effect of the two anesthetic agents. S1-S10 denote the sampling period. Bupivacaine or levobupivacaine was applied at concentrations (in µM) of 5 (S3-S4), 10 (S5-S6), 30 (S7-S8), and 100 (S9-S10).

Fig. 6. Concentration-effect curves for bupivacaine (solid squares) and levobupivacaine (triangles) with respect to peak twitch contractile force. Functions of best fit by least squares method are drawn through the data points. Error bars = SD. Six atria were tested in each group.

Na⁺ channel. However, in many instances, the concentration dependency for these effects is much higher than that found in our study. Hirota et al.31 found that bupivacaine displaced a radiolabeled dihydropyridine L-channel antagonist from rat cerebrocortical membranes with a \( K_{50} \) of 0.48 mm; this concentration is nearly fivefold higher than our highest concentration effective in inhibiting evoked norepinephrine release from cardiac sympathetic nerve endings. Regarding the \( K_{ATP} \) channel, this channel is activated under ischemic conditions32 not utilized in our study and blocking of this \( K^+ \) conductance predicts an augmentation of norepinephrine release (because of membrane depolarization or prolongation of the nerve action potential) rather than an inhibition, as observed with bupivacaine in our study.33

With regard to the possibility that bupivacaine blocks reverse-mode operation of the uptake-1 mechanism for norepinephrine, our experimental conditions using desipramine to block this carrier renders such a mechanism unlikely. In our experimental conditions this carrier operates in the reuptake mode (not reverse mode) and is not directly responsive to electrical stimulation. To our knowledge, reverse mode operation is documented for this carrier under adenosine triphosphate depleting conditions such as ischemia.34–36 Sugiyama et al.37 showed that bupivacaine can inhibit the \( \omega \)-conotoxin (GVIA)-sensitive N-type calcium channel in rat DRG neurons \textit{in vitro}. However their \( K_d \) value of 156 µm is still some 30 times greater than our inhibitory concentration 50% value.

In the current study, the values in Table 1 further indicated that the loading of nerve endings by tritiated norepinephrine was similar in all groups. Likewise the tissue counts at the end of experiments were similar to or slightly higher than the control and did not differ significantly among the experimental groups. One can conclude from these observations that the effect of local
The racemic form, our results do not allow a conclusion over the racemic form, bupivacaine. Because we used a selective inhibitor of neuronal fast Na⁺ channels, was provided in periods S3-S10. Note the partial inhibitory action of tetrodotoxin (TTX; 0.1 mM), a selective inhibitor of neuronal fast Na⁺ channels, was provided in periods S3-S10. Note the partial inhibitory action of tetrodotoxin. Values in the graphs are given as mean ± SD. *Statistical significance versus saline control in the corresponding stimulation period (P < 0.05). Six atria were tested in each group.

Anesthetics in decreasing neurotransmitter release cannot be attributed to depletion of norepinephrine from sympathetic nerve endings. In addition, local anesthetics did not make sympathetic nerve endings leaky to [3H]-norepinephrine in the absence of electrical stimulation, as indicated by the data in figure 3.

Although there are several reports in the literature proposing the use of levobupivacaine as a less cardiotoxic form of bupivacaine, our results suggest that with respect to sympathetic blockade the pure S(-) form, levobupivacaine, does not offer a significant advantage over the racemic form, bupivacaine. Because we used the racemic form, our results do not allow a conclusion about advantages of the S(-) form over the R(+) form.

Both local anesthetics that we tested abolished contractile force in the atrial preparations. The inhibitory concentration 50% values for blockade of contractility were about twofold higher than the corresponding inhibitory concentration 50% values for sympathetic nerve blockade. The fact that the component of contractility dependent on sympathetic nerve activity was only 30% implies that the local anesthetics had a direct effect on cardiomyocytes at somewhat higher concentrations. These two mechanisms, a direct effect on sympathetic
cardiac nerves and a direct effect on cardiomyocytes, would both result in reduced cardiac output. One would predict that during cardiac depression resulting from local anesthetics, compensatory sympathetic reflexes would be impaired. If this is correct, then patients receiving beta blockers may be more susceptible to cardiac depression by accidental intravascular injection of local anesthetic agents.

In conclusion, our results indicate that at cardioactive local anesthetic concentrations reported in cases of intravascular accumulation of local anesthetics, a profound blockade of cardiac sympathetic nerve endings takes place. These observations add another mechanism to explain the cardiotonic effects of local anesthetics.

References
37. Sugiyama K, Muteki T: Local anesthetics depress the calcium current of rat sensory neurons in culture. Anesthesiology 1994; 80:1569–78