Anesthesiology 2007; 106:1026–34

Effects of Intermittent Femoral Nerve Injections of Bupivacaine, Levobupivacaine, and Ropivacaine on Mitochondrial Energy Metabolism and Intracellular Calcium Homeostasis in Rat Psoas Muscle

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Background: **Long-acting local anesthetics cause muscle damage. Moreover, long-acting local anesthetics act as uncoupler of oxidative phosphorylation in isolated mitochondria and enhance sarcoplasmic reticulum Ca^{2+} release. The aim of the study was to evaluate effects of perineural injections of local anesthetics on mitochondrial energetic metabolism and intracellular calcium homeostasis *in vivo*.**

Methods: **Femoral nerve block catheters were inserted in adult male Wistar rats. Rats were randomized and received seven injections (1 ml/kg) of bupivacaine, levobupivacaine, ropivacaine, or isotonic saline at 8-h intervals. Rats were killed 8 h after the last injection. Psoas muscle was quickly dissected from next to the femoral nerve. Local anesthetic concentrations in muscle were determined. Oxidative capacity was measured in saponin-skinned fibers. Oxygen consumption rates were measured, and mitochondrial adenosine triphosphate synthesis rate was determined. Enzymatic activities of mitochondrial respiratory chain complexes were evaluated. Local calcium release events (calcium sparks) were analyzed as well as sarcoplasmic reticulum calcium content in saponin-skinned fibers.**

Results: **Eight hours after the last injection, psoas muscle concentration of local anesthetics was less than 0.3 μg/g tissue. Adenosine triphosphate synthesis and adenosine triphosphate–to–oxygen ratio were significantly decreased in the muscle of rats treated with local anesthetics. A global decrease (around 50%) in all of the enzyme activities of the respiratory chain was observed. Levobupivacaine increased the amplitude and frequency of the calcium sparks, whereas lower sarcoplasmic reticulum calcium content was shown.**

Conclusion: **Bupivacaine, levobupivacaine, and ropivacaine injected *via* femoral nerve block catheters induce a deleterious effect in mitochondrial energy, whereas only levobupivacaine disturbs calcium homeostasis.**

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Received from INSERM ERI 25, Hôpital Arnaud de Villeneuve, Montpellier, France. Submitted for publication July 17, 2006. Accepted for publication January 10, 2007. Supported by Université Montpellier 1, Montpellier, France; Université Victor Ségalen Bordeaux 2, Bordeaux, France; Ministère de l'Education nationale, de l'Enseignement supérieur et de la Recherche, Paris, France; INSERM, Paris, France; and Conseil Régional d'Aquitaine, Bordeaux, France. Presented in part at the Annual Meeting of the American Society of Anesthesiologists, Atlanta, Georgia, October 22–26, 2005, and the Annual Meeting of the American Society of Anesthesiologists, Chicago, Illinois, October 14–18, 2006.

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CONTINUOUS infusions of long-acting local anesthetics (LA) in femoral nerve block offer the benefits of prolonged analgesia with fewer side effects, greater patient satisfaction, and faster rehabilitation after knee surgery than with intravenous morphine.[1–3] A survey of the American Society of Anesthesiologists and the American Society of Regional Anesthesia and Pain Medicine members reported that anesthesiologists anticipated a significant increase in number and duration of continuous peripheral nerve blocks in their practice.[4] In clinical practice, high doses of LA might be infused and might represent a potential risk of toxic local concentrations with muscle damage.[5,6] This phenomenon is underestimated partially because of the scattered reports of clinically obvious muscular impairment.[5,7,8] Nevertheless, in clinical concentrations, LA might induce myotoxicity in isolated muscle fibers or in human.[8,9]

In vitro, bupivacaine myotoxicity is mediated by mitochondria.[9] In heart mitochondria, highly lipophilic LA interfere with mitochondrial energy metabolism and induce an inhibition of complex I or an uncoupling of oxidative phosphorylation.[10,11] On rat skeletal muscle mitochondria and isolated myofibers from soleus, bupivacaine causes a mitochondrial membrane depolarization with an opening of the permeability transition pore, which plays a key role in many forms of cell death.[9] This dysfunction is associated with Ca^{2+} dysregulation with Ca^{2+} release from the sarcoplasmic reticulum (SR) and inhibition of Ca^{2+} uptake by the SR.[12] Whereas liposolubility might explain the difference between ropivacaine and bupivacaine on mitochondrial metabolism, stereoselectivity might be involved in alterations of Ca^{2+} intracellular regulation by LA.[10,13,14]

In vivo, when continuous femoral nerve blocks were performed in porcine models using equipotent doses of ropivacaine or bupivacaine injected for as few as 6 h, both LA caused acute effects with severe tissue damage.[15] The lower liposolubility of ropivacaine could limit the extent of histopathologic lesions. However, in rat muscle, this protective ropivacaine property remains to be confirmed on mitochondrial energy metabolism and on calcium homeostasis, both of which are usually described as targets for drug-induced toxicity.[16] Damaged

mitochondria are described *in vivo* after injection of ropivacaine in rats.[10,17] A drug dose–dependent effect has been highlighted, but neither the liposolubility nor the stereoselectivity-dependent effect have been investigated yet in these conditions. The biochemical consequences of the long-acting LA properties, which are well known on isolated mitochondria or on saponin-skinned muscle fibers, remain to be confirmed when intermittent injections are used.[10,14] To investigate possible rat psoas myotoxicity of long-acting LA, we evaluated the effects on mitochondrial energy metabolism and Ca^{2+} signaling of seven intermittent injections of bupivacaine, levobupivacaine, or ropivacaine injected *via* a femoral nerve catheter.

Materials and Methods

Intermittent Long-acting LA Injections and Muscle Isolation

Care of the animals conformed to the recommendations of the Helsinki Declaration, and the study was performed in accordance with the regulations of the official edict of the French Ministry of Agriculture. These experiments were conducted in an authorized laboratory under the supervision of authorized researchers (K.N.G and A.L.). Experiments were conducted in adult male Wistar rats, 10–12 weeks old, weighing 220–280 g, obtained from Janvier Laboratories (Le Genest Saint Isle, France). After anesthesia with intraperitoneal pentobarbital sodium (40 mg/kg) and subcutaneous injection of lidocaine (10 mg) for skin incision and dissection, a 20-gauge, 0.9-mm-OD plexus catheter (Pajunk, Geisingen, Germany) was inserted under the inguinal ligament near the left femoral nerve sheath using a surgical method. Having confirmed the correct position, the catheter was fixed by single stitches on the quadriceps muscle, was passed under the skin, exited at the neck, and was fixed on the skin. Subsequently, incisions were closed by suturing.

At the end of the surgical procedure, animals were randomized to four different groups: 0.25% bupivacaine group, 0.28% levobupivacaine group, 0.375% ropivacaine group, or saline group. Local anesthetic dilutions for clinical practice as 0.5% bupivacaine hydrochloride and 0.75% ropivacaine hydrochloride from AstraZeneca (Rueil-Malmaison, France) and 0.56% levobupivacaine hydrochloride from Abbott (Rungis, France) were used. Rats received seven injections (1 ml/kg) 8 h apart, which induced a decrease in pinprick sensation in the cutaneous distribution of femoral nerve but no complete motor blockade the first hour after each LA injection. Rats were killed by cervical dislocation 8 h after the last injection. Psoas muscle was quickly dissected from next to the femoral nerve, with the former tip region of each catheter located in the middle of the tissue block.

Long-acting LA Muscle Concentrations

Residual bupivacaine or ropivacaine concentrations in psoas muscle 1 and 8 h after the last injection were determined using a high-performance liquid chromatography method. Tissue standard samples containing known amounts of ropivacaine and bupivacaine were prepared by spiking homogenized tissues to yield concentrations between 50 and 1,000 ng/ml for ropivacaine and 100 and 1,000 ng/ml for bupivacaine, from 1 mg/ml methanol stock solutions of bupivacaine and ropivacaine. Tissue samples were washed in cold isotonic buffer and then blotted, weighted, and quickly frozen at −80°C. Before analysis, tissue samples were diluted, (1/2, wt/vol) and homogenized in physiologic buffer with a mixer. To 500 μl homogenized tissue was added 100 μl pentacaine (internal standard, at 5 μg/ml in distilled water) and 200 μl NaOH, 1N. The mixture was extracted with 6 ml ethylacetate by rotative shaking for 20 min. After centrifugation, the organic phase was acidified by 200 μl HCl, 0.01N, and shaken for 20 min. After centrifugation, 20 μl of the acid aqueous phase was injected into the chromatograph. The high-performance liquid chromatography system consisted of a constant flow pump M 510, a 717 Plus Autoinjector (Waters Corp., Milford, MA), a UV 1000 ultraviolet model detector, and a Chromjet integrator (Thermoquest, San Jose, CA). The chromatographic separation was performed at room temperature on an XTerra RP 18 analytical column (Waters, Saint Quentin en Yvelines, France; 150 × 4.6 mm, 5-μm particle size). The mobile phase consisted of a binary mixture (17/83, vol/vol) of acetonitrile and potassium dihydrogen phosphate buffer (0.01 M, adjusted pH at 2.1 with concentrated orthophosphoric acid) with a 2-ml/min flow rate. Compounds were chromatographed at 210 nm within 12 min.

This method was specially developed for the assay of long-acting LA concentration in muscle tissue. A good linearity was obtained for the two molecules tested [r = 0.996, with a slope variation coefficient (n = 4) below 4% for ropivacaine, y = (1.711 ± 0.069)x − (0.01295 ± 0.003); r = 0.989, with a slope variation coefficient (n = 3) below 7% for bupivacaine, y = (1.229 ± 0.0775)x − (0.018 ± 0.003)]. In these chromatographic conditions, ropivacaine and bupivacaine were sufficiently resolved from endogenous tissue compounds. The method was accurate for the two concentration ranges and the two compounds. For bupivacaine determination, intraday precision ranged from 3.27% to 5.05% and interday precision ranged from 8.1% to 12.8% with less than 11% bias. The lower limit of quantification was 100 ng/ml (300 ng/g tissue). For ropivacaine determination, intraday precision ranged from 1.8% to 4.1% and interday precision ranged from 4.6% to 6.4% with less than 5% bias. The lower limit of quantification was 50 ng/ml (150 ng/g tissue).

Oxidative Phosphorylation

Psoas muscle was quickly removed and placed into a normoxic (*i.e.*, equilibrated with air) cooled (4°C) relaxing solution (solution 1:10 mM EGTA, 3 mM Mg^{2+}, 20 mM taurine, 0.5 mM dithiothreitol, 5 mM ATP, 15 mM phosphocreatine, 20 mM imidazole, and 0.1 M K^+2-(N-morpholino]ethane sulfonic acid, pH 7.2); chemicals were from Sigma Chemical Company (St. Louis, MO). Bundles of fibers between 2 and 5 mg were excised from the surface of psoas and then permeabilized in solution 1 added with saponin 50 μg/ml. The bundle was then washed twice for 10 min each time in solution 2 (10 mM EGTA, 3 mM Mg^{2+}, 20 mM taurine, 0.5 mM dithiothreitol, 3 mM phosphate, 1 mg/ml fatty acid–free bovine serum albumin, 20 mM imidazole, and 0.1 M K^+2-[N-morpholino]ethane sulfonic acid, pH 7.2) to remove saponin. All procedures were conducted at 4°C with extensive stirring. The success of the permeabilization procedure was estimated by determining the activity of the cytosolic lactate dehydrogenase and the mitochondrial citrate synthase in the medium. After 15–20 min of permeabilization, more than 60% of the cytosolic lactate dehydrogenase was found in the external medium, and the mitochondrial citrate synthase activity in the medium remained below 5%.[18,19] The oxygen consumption rate was measured polarographically at 30°C using a Clark-type electrode (Strathkelvin Instruments, Glasgow, United Kingdom) connected to a personal computer that displayed on-line the respiration rate value (949 oxygen System, Strathkelvin Instruments). Solubility of oxygen in the medium was considered to be equal to 450 nmol/ml. Respiratory rates were determined in a 2-ml oxygraph cuvette containing one bundle of fibers in solution 2 with 10 mM malate plus 10 mM glutamate or succinate plus rotenone (1 mg/ml DMSO and ethanol 1:1) as substrates; 50 μM di(adenosine 5′)-pentaphosphate, 20 μM EDTA and 1 mM iodoacetate were also added to the cuvette to inhibit extramitochondrial adenosine triphosphate (ATP) synthesis (*via* the glycolysis or the adenylate kinase) and ATP hydrolysis.[20] Adenosine diphosphate (ADP)–stimulated respiration, associated with ATP synthesis, was determined in the presence of 1 mM ADP. Basal respiration without ATP synthesis was measured after addition of 70 μM atractyloside and 1 μM oligomycin. After measurements, fibers were removed, dried on a precision wipe, and weighed. Results were expressed in nanomoles of atom oxygen consumed per minute and per milligram wet weight of fiber. Under identical conditions, the mitochondrial ATP synthesis rate in skinned fibers was determined by bioluminescence measurement (luciferin–luciferase system) of the ATP produced after addition of 1 mM ADP.[13] The ATP Bioluminescence Assay Kit HS II from Roche Diagnostics GmbH (Mannheim, Germany) was used. At various time intervals after addition of ADP, 10-μl aliquots were withdrawn from the oxygraph chamber, quenched in 100 μl DMSO, and diluted in 5 ml ice-cold distilled water. Standardization was performed with known quantities of ATP measured under the same conditions. ATP synthesis rate was expressed in nmol ATP produced per minute and per milligram wet weight of fiber. The efficiency of oxidative phosphorylation was taken as the ratio of ATP synthesis rate to oxygen consumption rate (ATP/O).[20]

Enzyme Activity

For enzymatic procedure, approximately 100 mg psoas muscle was minced and homogenized with a glass Potter homogenizer in a ice-cold medium (10% wt/vol) containing 225 mM mannitol, 75 mM sucrose, 10 mM Tris-HCl, and 0.10 mM EDTA, pH 7.2. The homogenate was then centrifuged for 20 min at 650*g*. The supernatant was collected, and the protein concentration was determined.[21] Enzymatic activity was assessed using previously described spectrophotometric procedures (Uvikon 940; BIO-TEK Instruments, Winooski, VT) and expressed in nanomoles of substrate transformed per minute and per milligram of protein. The citrate synthase activity was measured as described by Srere in the presence of 4% Triton (vol/vol) by monitoring at 412 nm wavelength at 30°C the formation of thionitrobenzoate dianion from the reaction of coenzyme A and 5,5′-dithiobis(2-nitrobenzoic acid).[22] The complex I activity, reduced nicotinamide adenine dinucleotide (NADH) ubiquinone reductase, was measured as described by Birch-Machin *et al.*[23] The oxidation of NADH by complex I was recorded using the ubiquinone analog decylubiquinone as the electron acceptor. The decrease in absorption resulting from NADH oxidation was measured at 340 nm at 30°C. Complex I activity was calculated from the difference in the rate before and after the addition of rotenone (2 μM), a specific inhibitor of complex I. The complex II (succinate dehydrogenase)–specific activity was measured by monitoring the reduction of 2,6-dichlorophenol indophenol at 600 nm at 30°C, in the presence of phenazine methosulphate.[24] The oxidation of ubiquinol (UQ_1H_2) by complex III (ubiquinol cytochrome *c* reductase) was determined using cytochrome *c*(III) as the electron acceptor.[23] The reduction of cytochrome *c*(III) was recorded at 550 nm at 30°C. Complex IV (cytochrome *c* oxidase) was measured by the method described by Wharton and Tzagoloff using cytochrome *c*(II) as substrate.[25] The oxidation of cytochrome *c* was monitored at 550 nm at 30°C.

Measurement of Cytoplasmic Ca^{2+} Variations

The muscle samples were immediately placed in an internal-like medium (140 mM K-glutamate, 10 mM HEPES, 20 mM phosphocreatine, 5 mM Na_2ATP, 4.53 mM $MgCl_2$, 1 mM EGTA, 0.29 mM $CaCl_2$, and 2 mM malate, pH 7.0). Single fibers were manually dissected under a dissecting microscope (Stemi 200; Zeiss, Le Peck, France), mounted in an experimental chamber containing an internal medium, and permeabilized by adding 0.01% saponin, 30 s at room temperature, for imaging experi-

ments. Fibers were slightly stretched to a sarcomere length approximately 2.8–3.2 μm to reduce movement artifacts. Fibers were bathed in an internal medium containing the fluorescent Ca^{2+} indicator Fluo-3 (pentapotassium salt; Tef-labs, Austin, TX; 50 μM). Global changes in intracellular Ca^{2+} were measured by time series (1 image/5 s) of x–y confocal fluorescent images (Zeiss LSM 510 Meta, 63× objective, numerical aperture = 1.2, H_2O immersion). Fluo-3 was excited with an argon/krypton laser at 488 nm, and fluorescence was recorded at 525 nm. Mean fluorescence was calculated in regions of interest and reported as a function of time.

Ca^{2+} Sparks Measurement

To characterize the *in vivo* effects of the LA treatment on the Ca^{2+} release process, we measured spontaneous Ca^{2+} sparks; their properties reflect the behavior of the SR-Ca^{2+} release channel (*i.e.*, ryanodine receptor [RyR]) *in situ*. Local Ca^{2+} release events, Ca^{2+} sparks, were measured as previously reported for rat skeletal muscle.[26] Fluorescence images were acquired with the confocal system operated in line-scan mode (x *vs.* t, 1.5 ms/line). Potential spark areas were empirically identified using an auto detection algorithm. The mean *F* value for the image was calculated by summing and averaging the temporal *F* at each spatial location while ignoring potential spark areas. This *F* value was then used to create a $\Delta F/F$ image pixel by pixel. Selection and analysis of Ca^{2+} sparks were performed essentially as previously described.[26] Determinations of spatiotemporal properties of individual Ca^{2+} sparks were made on spatial (x) and temporal (t) profiles of sparks centered at the peak amplitude. The $\Delta F/F$ amplitude as well as temporal parameters, rise time, and time constant decay were derived from the temporal profile. The width of the Ca^{2+} spark (full width at half maximum peak amplitude) was determined from the spatial profile.

Evaluation of Sarcoplasmic Reticulum Ca^{2+} Content

Bundles of fibers were prepared as described previously. Calcium release was elicited by rapidly exchanging the bathing solution to one containing 50 μM 4-chloro-*m*-cresol. Ca^{2+} transient was imaged using fast time series of x–y images (25× oil immersion, numerical aperture = 0.8; Zeiss). Fields of one or two fibers were imaged at the acquisition rate of one image every 400 ms.

Statistics

Results are given as mean ± SD or median [min, max] for long-acting LA muscle concentration. Data were plotted and analyzed using SigmaPlot 9.0 and Systat 10.0 (SPSS, Chicago, IL). Differences between long-acting LA groups were tested using the Kruskal–Wallis test. Differences between long-acting LA and the control group were tested using the Mann–Whitney test. A *P* value of less than 0.05 was required to reject the null hypothesis.

Table 1. Effects of Local Anesthetics on Mitochondrial Oxidative Phosphorylation with Glutamate as Substrate

	Oxygen Consumption			
	−ADP	+ADP	ATP Synthesis	ATP/O
Control	6.7 ± 2.4	18.9 ± 8.5	39.0 ± 10.8	2.2 ± 0.4
Bupivacaine	3.3 ± 1.1*	9.8 ± 5.2*	15.3 ± 4.2*	1.7 ± 0.3*
Levobupivacaine	4.2 ± 1.3*	11.8 ± 3.7*	15.4 ± 10.3*	1.2 ± 0.4*
Ropivacaine	5.2 ± 1.2	11.7 ± 2.8*	13.9 ± 3.4*	1.2 ± 0.2*

Data are mean ± SD (n = 6 rats/group). Experimental conditions are described in Materials and Methods. Basal, without adenosine diphosphate (ADP), and ADP-stimulated oxygen consumption rates supported by glutamate, in the presence of malate, are expressed in nmol atom oxygen · min^{-1} · mg^{-1} wet weight. Adenosine triphosphate (ATP) synthesis rate is expressed in nmol ATP · min^{-1} · mg^{-1} wet weight. ATP-to-oxygen ratio (ATP/O) is calculated as the ratio of the rate of ATP synthesis to the rate of the concomitant respiration in the presence of ADP.

* $P < 0.05$ *vs.* control animals.

Results

Muscle Long-acting LA Concentrations

Bupivacaine (n = 10) or ropivacaine (n = 10) muscle concentrations were 10.1 [0.6, 47.1] and 13.2 [1.9, 73.1] μg/g tissue, respectively, 1 h after the last injection. Eight hours after the last injection of long-acting LA (n = 6 rats/group), muscle concentrations were below the detection threshold, (below 300 ng/g or 150 ng/g tissue, respectively, for bupivacaine and ropivacaine).

Effects of Long-acting LA on Mitochondrial Oxidative Phosphorylation

Saponin-skinned fibers of psoas from rats that had been treated with long-acting LA (n = 6/group) oxidized substrates at lower rates than those from control rats (n = 6 rats). In the three long-acting LA groups, oxygen consumption rates (with and without ADP) were decreased with glutamate or pyruvate as substrates (tables 1 and 2). Likewise, the phosphorylation of ADP by ATP synthase after seven injections of these LA was strongly inhibited.

Table 2. Effects of Local Anesthetics on Mitochondrial Oxidative Phosphorylation with Pyruvate as Substrate

	Oxygen Consumption			
	−ADP	+ADP	ATP Synthesis	ATP/O
Control	5.4 ± 0.9	16.5 ± 5.5	39.4 ± 11.8	2.4 ± 0.4
Bupivacaine	3.9 ± 1.5	12.9 ± 6.6	15.8 ± 5.4*	1.4 ± 0.6*
Levobupivacaine	4.4 ± 1.1	13.4 ± 4.1	15.6 ± 5.6*	1.2 ± 0.3*
Ropivacaine	5.9 ± 1.9	14.1 ± 3.0	12.4 ± 2.8*	0.9 ± 0.1*

Data are mean ± SD (n = 6 rats/group). Experimental conditions are described in Materials and Methods. Basal, without adenosine diphosphate (ADP), and ADP-stimulated oxygen consumption rates supported by pyruvate, in the presence of malate, are expressed in nmol atom oxygen · min^{-1} · mg^{-1} wet weight. Adenosine triphosphate (ATP) synthesis rate is expressed in nmol ATP · min^{-1} · mg^{-1} wet weight. ATP-to-oxygen ratio (ATP/O) is calculated as the ratio of the rate of ATP synthesis to the rate of the concomitant respiration in the presence of ADP.

* $P < 0.05$ *vs.* control animals.

Table 3. Effects of Local Anesthetics on Mitochondrial Oxidative Phosphorylation with Succinate as Substrate

	Oxygen Consumption			
	−ADP	+ADP	ATP Synthesis	ATP/O
Control	9.5 ± 2.9	21.7 ± 7.1	29.3 ± 3.9	1.4 ± 0.4
Bupivacaine	5.9 ± 3.0	14.2 ± 3.5	18.7 ± 7.4*	1.3 ± 0.3
Levobupivacaine	8.4 ± 3.5	18.5 ± 6.2	19.5 ± 8.3*	1.1 ± 0.2
Ropivacaine	9.1 ± 4.9	21.1 ± 7.4	17.2 ± 8.7*	0.8 ± 0.2*

Data are mean ± SD (n = 6 rats/group). Experimental conditions are described in Materials and Methods. Basal, without adenosine diphosphate (ADP), and ADP-stimulated oxygen consumption rates supported by succinate, in the presence of rotenone, are expressed in nmol atom oxygen · min^{-1} · mg^{-1} wet weight. Adenosine triphosphate (ATP) synthesis rate is expressed in nmol ATP · min^{-1} · mg^{-1} wet weight. ATP-to-oxygen ratio (ATP/O) is calculated as the ratio of the rate of ATP synthesis to the rate of the concomitant respiration in the presence of ADP.

* $P < 0.05$ *vs.* control animals.

This inhibition was approximately 50% with glutamate and pyruvate. Therefore, long-acting LA reduced ATP/O in the same way. However, bupivacaine, levobupivacaine, and ropivacaine had the same effects on mitochondrial respiration supported by both substrates. The ATP synthesis was decreased equally, with no difference noticed between groups. The oxidative capacities with succinate as substrate might be altered less by the injections of LA (table 3).

Effects of Long-acting LA on Enzymatic Complexes of the Respiratory Chain

The enzyme activities of the complexes of the respiratory chain showed comparable changes according to the three LA groups (n = 6 rats/group) (table 4). The four enzyme complexes of the respiratory chain decreased after seven injections of long-acting LA. Citrate synthase, a matrix mitochondrial enzyme, was assayed to establish a possible relation between mitochondrial content and psoas muscle toxicity. This mitochondrial enzyme is closely correlated with mitochondrial volume fraction. Citrate synthase decreased significantly after injections of long-acting LA. There is no difference between groups of long-acting LA. Moreover, it is noteworthy that the ratio of the activity of each respiratory chain complex to citrate synthase activity was not significantly different, suggesting a global decrease in mitochondrial enzymes (fig. 1).

Effects of Intermittent Injections of Long-acting LA on Ca^{2+} Homeostasis

The levobupivacaine treatment induced a significant increase in the frequency of Ca^{2+} sparks (0.01054 ± 0.00254 and 0.00289 ± 0.00139 events · μm^{-1} · s^{-1}, respectively, in levobupivacaine and control groups; $P < 0.05$), whereas no changes were seen with bupivacaine and ropivacaine (fig. 2). As far as spatiotemporal properties of Ca^{2+} sparks were concerned, levobupivacaine induced an increase in the amplitude of Ca^{2+} sparks (0.74 ± 0.02 *vs.* 0.58 ± 0.02 ΔF/F; $P < 0.05$). Spatial spread (full width at half maximum) of Ca^{2+} sparks was increased in bupivacaine and levobupivacaine groups (1.43 ± 0.05 and 1.46 ± 0.02 *vs.* 1.24 ± 0.05 μm, respectively; $P < 0.05$). For the other properties of Ca^{2+} sparks, no significant differences between groups have been reported regarding decay and rise time parameters. Finally, in the levobupivacaine group, a significant decrease in SR Ca^{2+} content was detected (5.31 ± 1.22 and 12.52 ± 3.2 F/F_0, respectively, in levobupivacaine and control groups; $P < 0.05$). No significant changes were observed in either LA group (fig. 3).

Discussion

Clinically relevant concentrations of bupivacaine, levobupivacaine and ropivacaine *in vivo* produced equal changes in mitochondrial energy metabolism with a significant decrease in ATP synthesis and a global reduction in enzyme activities of the respiratory chain. On the other hand, only levobupivacaine had deleterious effects on calcium homeostasis in rat psoas muscle of rats. Although skeletal muscle damage induced by long-acting LA has been described for a few decades, the pathogenesis of bupivacaine myotoxicity *in vivo* is still unclear.[7,8,27–29]

Intermittent Long-acting LA Injections

To imitate a clinically relevant setting to compare the effects of bupivacaine, levobupivacaine, and ropivacaine on mitochondrial and calcium metabolism, we chose a rat model of intermittent injections with the femoral nerve block catheter in place during 56 h as described in clinical practice.[1,2,6] Although continuous or basal-bolus infusion is recommended in clinical practice, intermit-

Table 4. Effects of Local Anesthetics on the Enzymatic Activities of the Respiratory Chain

	Complex I	Complex II	Complex III	Complex IV	Citrate Synthase
Control	131 ± 35	116 ± 38	625 ± 138	1,085 ± 286	409 ± 104
Bupivacaine	70 ± 8*	68 ± 11*	389 ± 82*	718 ± 66*	289 ± 34*
Levobupivacaine	65 ± 27*	47 ± 20*	344 ± 148*	600 ± 200*	226 ± 76*
Ropivacaine	65 ± 32*	63 ± 32*	363 ± 145*	707 ± 264*	281 ± 103*

Data are mean ± SD (n = 6 rats/group). Experimental conditions are described in Materials and Methods. Enzymatic activity was expressed in nmol substrate · min^{-1}· mg^{-1} protein.

* $P < 0.05$ *vs.* control animals.

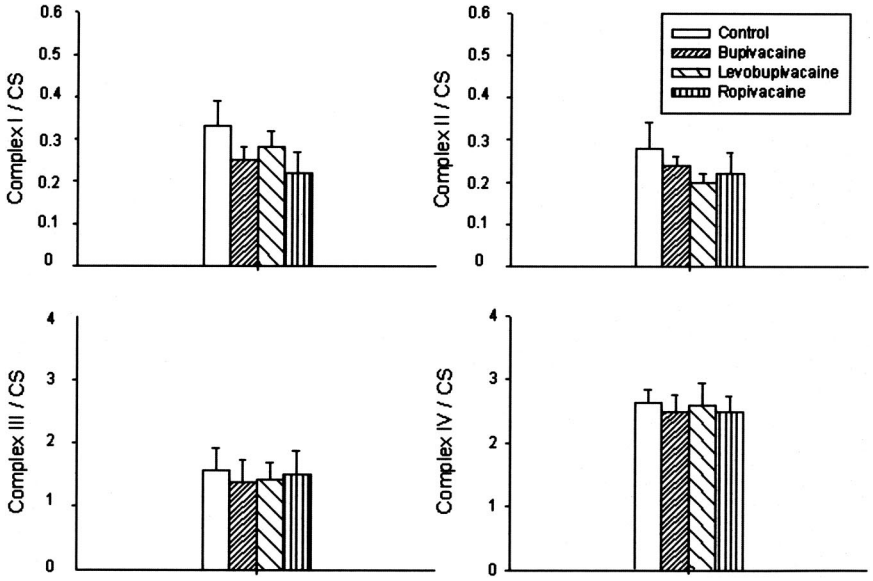

Fig. 1. Ratio of the enzyme activity of the respiratory chain complexes to the citrate synthase (CS) activity. Experimental conditions are described in Materials and Methods. Enzyme activities are reported in table 4. Values are mean ± SD (n = 6/group).

tent boluses every 8 h are better adapted to our experiment in rats.[30] Equipotent doses of bupivacaine and ropivacaine were administered.[15,31] On the other hand, identical volumes of bupivacaine and levobupivacaine were injected. The difference between solution concentrations was marginal considering the injected volume (1 ml/kg). Moreover, this method of prolonged nerve blockade using bupivacaine is well known to attenuate the local inflammatory edema in an experimental inflammatory pain model.[32] This effect does not depend on a mechanism shared by tetrodotoxin nor on the local activation of cytokines, but a mitochondrial dysfunction in this model remains to be investigated.[33] Considering all of the data, both local mitochondrial toxicity and systemic antiinflammatory effect appear as unexpected properties of bupivacaine. A common approach may permit a rational evaluation of benefit-to-risk ratio for bupivacaine use in each case.

Effects of Long-acting LA on Mitochondrial Energy Metabolism

Surprisingly, ATP synthesis and most enzyme activities of the respiratory chain decreased in psoas muscle. *In vitro*, it is now established that in mitochondria, long-acting LA uncouple oxidative phosphorylation and inhibit complex I activity. The uncoupling effect of LA corresponds to the dissipation of the transmembrane proton gradient that reduces the efficiency of ATP synthesis, with a large decrease in ATP/O ratio (*i.e.*, the efficiency of ATP synthesis). The mechanism of bupivacaine uncoupling has been extensively investigated.[9,34–37] In our rat model, this effect may occur after each injection of LA. But the decrease in observed ATP/O ratio is probably due both to the effects of LA on complexes of respiratory chain in the inner membrane and to an inhibition of ATP synthase. The decrease in ATP synthase activity may be explained by a direct inhi-

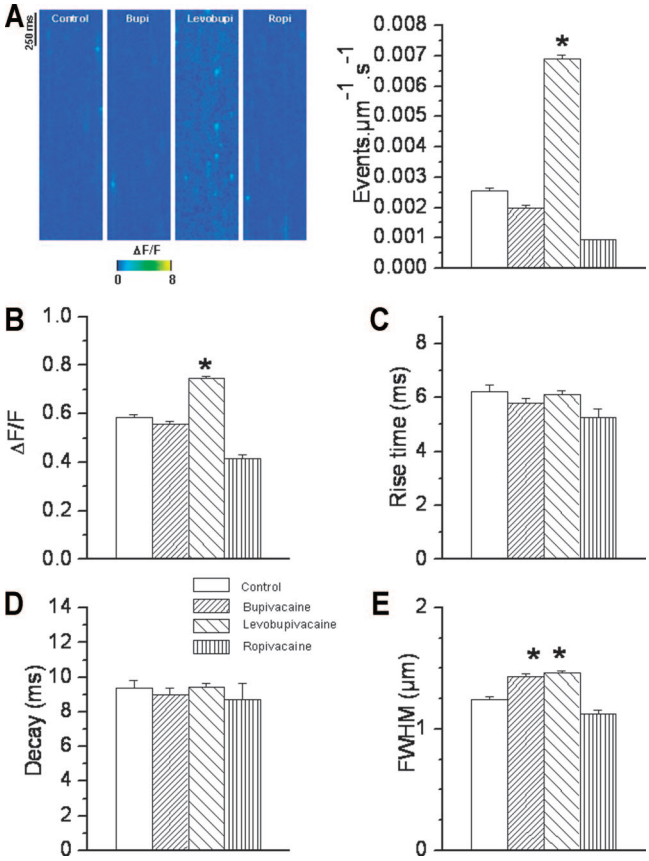

Fig. 2. Effects of intermittent injections of local anesthetic on Ca^{2+} sparks frequency and on spatio-temporal properties of Ca^{2+} sparks in psoas muscle fibers. (*A*) Representative line-scan ΔF/F images acquired on psoas fibers from control and local anesthetic–treated animals with Ca^{2+} sparks frequency in psoas muscle of control and local anesthetic–treated animals. Spatio-temporal analysis of brief (< 100 ms) and localized increases in fluorescence, corresponding to Ca^{2+} sparks, was performed using a biexponential fitting procedure.[12,26] Spark amplitude (*B*), rise time (*C*), decay time (*D*), and full width at half maximum (FWHM; *E*) were analyzed and compared between control and local anesthetic–treated animals. Values are mean ± SD (n = 6/group). * $P < 0.05$ *versus* control group.

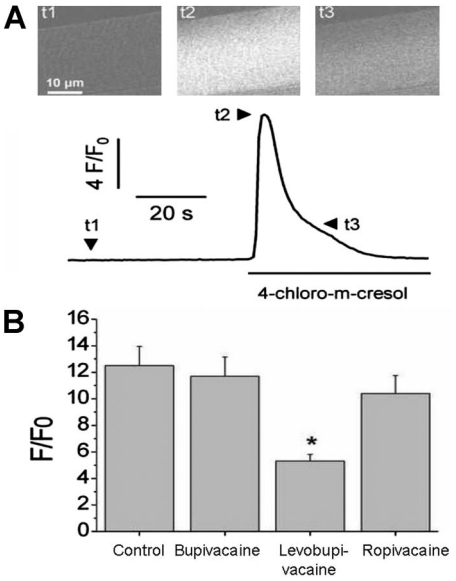

Fig. 3. Effects of intermittent injections of local anesthetic on psoas sarcoplasmic reticulum Ca^{2+} content. (*A*) Examples of x–y fluorescence images (*top*) recorded in a control bundles of skinned psoas muscle fibers loaded with Fluo-3 (50 μM) at different times (t1, t2, t3) before and after acute application of 50 μM 4-chloro-*m*-cresol. The time course of fluorescence is obtained from the averaged fluorescence measured in region of interest in the fibers (*bottom*) and exhibits a large transient calcium release after application of 4-chloro-*m*-cresol reflecting sarcoplasmic reticulum Ca^{2+} store. (*B*) This experiment has been performed after intermittent injections of local anesthetic vehicle, bupivacaine, levobupivacaine, and ropivacaine and indicates a lower sarcoplasmic reticulum Ca^{2+} store after levobupivacaine treatment. Values are mean ± SD (n = 6/group). * $P < 0.05$ *versus* control group.

bition of mitochondrial F0F1-ATPase or by a global decline in cellular protein synthesis with a decrease in mitochondrial proteins as a consequence.[38–40]

Likewise, the mechanism of the decrease in oxidative phosphorylation can be related to the reduction in the enzyme activities of the different respiratory chain complexes of the mitochondrial inner membrane.[41,42] In the current study, normalization of enzyme activities of the respiratory chain complexes by citrate synthase significantly yields no changes between LA injections, suggesting that a reduction in functional mitochondria has been induced. Therefore, the decrease in oxidation observed in skinned fibers could be explained by the reduction in mitochondrial number rather than a decrease in the specific activity of the complexes. In a previous study, the decrease in citrate synthase activity and in state 3 rate of respiration observed after high concentration of bupivacaine in rat muscle was closely correlated with disjointed fibers, interstitial edema, and infiltrating cells.[43] Moreover, after a single injection of ropivacaine, mitochondria appear with an edematous and disintegrated aspect.[17] So, we hypothesized that after several injections, the decrease in oxidative capacities is coupled with such histopathologic mitochondria alterations. Histologic studies and reverse-transcription polymerase chain reaction comparing expression of genes involved in mitochondria biogenesis could confirm these results.

Therefore, lack of decrease in a specific activity of complex I is unexpected.[10,11] Moreover, the complex I is involved in the control of free radical production and interacts with the opening of the permeability transition pore through membrane depolarization.[9] Both of the latter involvements might explain in part the global alterations of enzyme activities we observed. An additional mechanism that may be involved in mitochondrial LA myotoxicity is the inhibition of carnitine transport, which has been demonstrated for heart mitochondria.[12,14,44]

Lack of Difference Between Long-acting LA Effects on Energy Metabolism

Bupivacaine, levobupivacaine, and ropivacaine alter the mitochondrial energy metabolism in the same way, despite the stereoselectivity or the liposolubility of the molecule. Lack of stereoselectivity in the uncoupling effect has been reported in isolated mitochondria of the heart.[11] Stereoselectivity corresponds to a difference of affinity or potency between the isomers for a specific target or receptor in general. On Na^+ channel, isomers of LA exhibit stereospecific effects and *R*(+)-bupivacaine blocks nerve conduction with a higher potency.[45] Likewise in the rat heart, atrioventricular conduction time shows evident stereoselectivity for bupivacaine isomers.[46] In contrast to effects on voltage-gated Na^+ channel, both anesthetics have negative inotropic effects without evidence of stereoselectivity in the same conditions. These findings for bupivacaine isomers were confirmed in the current study. Our results partly contradict the results of a few experimental studies on the comparison of ropivacaine and bupivacaine toxicity. As well as in the heart or in the striated muscle, equipotent concentration of ropivacaine is clearly associated with significantly reduced toxic effects in comparison with bupivacaine.[15,46] In case of systemic toxicity in rats, seizures, bradycardia, and severe hypotension occur at significantly larger doses in the ropivacaine group.[47] After local administration of both anesthetics in the same animal, ropivacaine is characterized by no qualitative differences and histopathologic changes in comparison with bupivacaine, but the extent of tissue damage is significantly smaller.[15] Both drugs disturb the skeletal muscle fiber organization, but most mitochondria, in light microscopic evaluation, remain unaffected in this study. These findings are confirmed when long-term myotoxic effects of long-acting LA are evaluated.[48] This lack of specific effect of ropivacaine is confirmed in our results. Whereas LA muscle concentration was below threshold 8 h after the seventh injection, muscle concentrations of bupivacaine and ropivacaine 1 h after the last injection were around 30 nmol/mg tissue or around 30 μM if we consider the molecular weight of bupiva-

caine (288) or ropivacaine (274). This concentration is clearly below that used in *in vitro* experiments (1 mM) and above the measured plasmatic concentration after a perineural blockade or a systemic toxicity.[9,49] On the other hand, *in vivo* tissue concentration has been only determined with lidocaine in nerve. This concentration was in the same range as in our study (less than 10 nmol/mg tissue), with a small percentage of the molecules of LA administered found in the muscle 1 h after the last injection.[50]

Effects of Intracellular Calcium Homeostasis

Intermittent injections of LA in psoas muscle of rats induced Ca^{2+} signaling alterations mainly with levobupivacaine treatment, whereas few changes were observed either with bupivacaine or ropivacaine. In the levobupivacaine group, the important decrease in SR Ca^{2+} content is contrary to the increase of Ca^{2+} spark amplitude, which is strongly dependent on the Ca^{2+} load. As a matter of fact, ryanodine receptor channel activation and spark frequency have been correlated to the level of SR calcium content.[51,52] Then, the first effect of levobupivacaine should be focused on the ryanodine receptor channel, increasing its Ca^{2+} release potential and then increasing the Ca^{2+} leak, which induces a decrease in SR Ca^{2+} content. A reduction of SR Ca^{2+} ATPase activity after intermittent injections of levobupivacaine might also contribute to the lower SR Ca^{2+} content. This effect might be explained by a more potent effect of *S*(−)-isomer, although this last point remains controversial.[14] However, interactions between calcium release and ATP synthesis remain unclear. The hypothesis that calcium signals match the demand for ATP in the cell to its production in mitochondria and, thus, to the control of respiration is insufficient to explain our results.[53] For excitable cells with high and rapidly fluctuating energy fluxes such as skeletal muscle, mitochondrial depolarization might be the cause rather than the consequence of dysregulation of Ca^{2+} homeostasis.[9,54] As such, LA alter mitochondrial energy metabolism is probably the priming effects in our model. The time course of this mitochondrial dysfunction is not linked to an increase in calcium release except in the case of levobupivacaine. The fact that only levobupivacaine induces alterations in ryanodine receptor channel properties as well as on the SR content might be the consequence of the LA-triggered weak calcium release at low concentration.[14] Moreover, both the observed liposolubility and stereoselectivity of levobupivacaine suggest effects on specific reticulum proteins, the ryanodine RyR1 receptor, and the Ca^{2+}-ATPase pump, rather than nonspecific increase in Ca^{2+} permeability.[55]

In conclusion, bupivacaine, levobupivacaine, and ropivacaine induce a decrease in the mitochondrial energy metabolism when they are injected in rats *via* femoral nerve block catheters. On the other hand, only levobupivacaine has a marked effect on Ca^{2+} homeostasis. These alterations probably result in both lipophilic and stereospecific properties of the long-acting LA. Therefore, no myotoxic-free LA could be identified when using these three drugs in these three concentrations. The clinical impact of these results must be explored and confirmed in the future. Reversibility of all these lesions remains to be confirmed. Further studies are necessary to evaluate other mechanisms that are involved, such as oxidative stress and the consequences on functional properties of fibers. Moreover, if mitochondria play a key role in cell death, mitochondria-targeting drugs, such as antioxidants, might prevent long-acting LA myotoxicity with potential clinical relevance.

The authors thank Evelyne Déridet (Research Engineer, Laboratoire de Pharmacologie, Unité INSERM U 657, Université Victor Ségalen Bordeaux 2, Bordeaux, France) for technical support in high-performance liquid chromatography method and Françoise Masson, M.D. (Service d'Anesthésie et Réanimation I, Centre Hospitalier Universitaire Pellegrin, Bordeaux, France), for statistical analysis.

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