Erythropoietin Protects against Local Anesthetic Myotoxicity during Continuous Regional Analgesia

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Background: Local anesthetics offer the benefits of extended analgesia with greater patient satisfaction and faster rehabilitation compared with intravenous morphine. These benefits, however, can be offset by adverse iatrogenic muscle pain caused by bupivacaine. Here, the authors describe the mechanisms of local anesthetic-induced myotoxicity and a partial protective effect of recombinant human erythropoietin (rhEPO).

Methods: The authors developed a rat analgesia model with femoral nerve catheter and a cell culture model of human skeletal muscle myoblasts to study local anesthetic effects. Rats were randomly assigned to four different groups: daily intraperitoneal injection with 5,000 U/kg rhEPO or saline coupled to a perineural catheter injection with 1 ml/kg bupivacaine, 0.25%, or saline. In psoas rat muscle, oxygen consumption rates were measured using a Clark-type electrode in saponin-skinned fibers. Mitochondrial adenine triphosphate synthesis rates were determined by bioluminescence. Enzymatic activity of mitochondrial respiratory chain complexes was measured on tissue homogenates using spectrophotometric procedures, and mitochondrial morphology was analyzed by transmission electron microscopy. In addition, the interaction between bupivacaine and rhEPO was investigated on human skeletal muscle myoblasts by fluorescence microscopy using mitotracker green and using the lipophilic cation JC-1.

Results: Bupivacaine caused impairment of mitochondrial structure and bioenergetics in rats. Human myoblasts treated with bupivacaine showed a dose-dependent decrease in mitochondrial membrane potential associated with unusual morphologies. Impairment of mitochondrial bioenergetics was prevented partially by the use of rhEPO coadministered with bupivacaine.

Conclusions: The authors demonstrated a dose- and time-dependent protective effect of rhEPO against bupivacaine-induced myotoxicity in regional analgesia.

LOCAL anesthetics (LAs) offer the benefits of extended analgesia with greater patient satisfaction compared with intravenous morphine after orthopedic surgery.1,2 Challenges remain, however, for the use of LAs with regard to improving the comfort and postoperative pain relief of patients receiving continuous regional blocks for surgical procedures. These patients can have problems with postoperative iatrogenic muscle pain or dysfunction caused by the toxicity of certain LAs, including bupivacaine.1,3,4 The frequency of these symptoms is largely unknown because they remain underreported.5 Therefore, a fundamental understanding of the mechanisms of LA-induced myotoxicity is needed to develop efficient clinical strategies to protect against adverse outcomes due to LAs. Based on previous work, bupivacaine myotoxicity likely involves the inhibition of mitochondrial energy metabolism.5,7 The aim of our work was to investigate the protective effects of recombinant human erythropoietin (rhEPO) on bupivacaine-induced myotoxicity in mitochondria. This hypothesis was based on recent observations suggesting that rhEPO has a cytoprotective effect on cardiomyocytes and neurons injured by ischemia–reperfusion, which typically involves mitochondrial function.8 However, no studies have investigated the effects of rhEPO on mitochondrial energetic function or its implications for muscle cell viability.

Numerous in vitro studies have shown deleterious effects of lidocaine and bupivacaine on respiratory chain activity and on the coupling of oxidative phosphorylation (OXPHOS) in isolated mitochondria.6,7,9 To investigate this hypothesis in physiologic conditions, we developed a rat model and a human cell culture model in which mitochondria retain the functional network organization described in the cellular context.10 We used bupivacaine concentrations similar to those used in perioperative analgesia protocols, and we used rhEPO concentrations described to have tissue-protective properties but higher than those usually used clinically to treat anemia.8,11

In this study, we evaluated the effects of bupivacaine on respiratory chain activity, adenosine triphosphate (ATP) synthesis, generation of reactive oxygen species, alterations in mitochondrial structure, and cell viability. Our findings confirm that bupivacaine-induced myotoxic-
Erythropoietin and Bupivacaine-Induced Myotoxicity

Erythropoietin and bupivacaine-induced myotoxicity is associated with alterations in mitochondrial structure and bioenergetic function both in vivo and in vitro. We also established that rhEPO can protect against these iatrogenic effects, suggesting the existence of preventive mechanisms in mitochondria.

Materials and Methods

This study, including care of the animals involved, was conducted according to the official edict presented by the French Ministry of Agriculture (Paris, France) and the recommendations of the Declaration of Helsinki. Therefore, these experiments were conducted in an authorized laboratory and under the supervision of an authorized researcher (K.N.-G.).

Chemicals

Bupivacaine hydrochloride, 0.5% (15 mM), was purchased from AstraZeneca (Rueil-Malmaison, France) for rat administration and from Sigma-Aldrich (St. Louis, MO; B5274) for cell experiments. rhEPO was obtained from Janssen-Cilag (Issy-Les-Moulineaux, France). All other reagents were purchased from Sigma, with the exception of the ATP monitoring kit (ATP Bioluminescence Assay Kit HS II; Roche Diagnostics GmbH, Mannheim, Germany) and the primary antibodies (OXPHOS Kit, MS601 MitoProfile®; Mitosciences, Eugene, OR).

Rat Model

Experiments were conducted on adult male Wistar rats, 10–12 weeks old, weighing 200–240 g. Rats were housed in a regulated facility with a 12-h light/12-h dark cycle, fed with chow, and allowed free access to tap water. After anesthesia with intraperitoneal pentobarbital sodium (40 mg/kg) and subcutaneous injection of lidocaine (10 mg), a plexus catheter (20 gauge, 0.9 mm OD; Pajunk, Geisingen, Germany) was inserted under the inguinal ligament near the left femoral nerve sheath (fig. 1). It was fixed with stitches on the quadriceps muscle, passed under the skin, and exited at the neck. Incisions were subsequently closed by suturing.

Animals were randomly assigned to four different groups according to the type of combined injections: daily intraperitoneal injection with 5,000 U/kg rhEPO (E) or saline (S) coupled to a perineural catheter injection of 1 ml/kg bupivacaine, 0.25% (EB and SB groups), or saline (ES and SS groups). Rats received one daily intraperitoneal injection for 3 days and seven perineural injections 8 h apart. This induced a decrease in pinprick sensation in the cutaneous distribution of the femoral nerve but not complete motor blockade in the first hour after each bupivacaine injection. Rats were killed by cervical dislocation 8 h after the last perineural injection, when the bupivacaine concentration in muscle was below the threshold of detection (< 0.3 μg/g tissue). Psoas muscle was quickly dissected adjacent to the femoral nerve, with the former tip region of the catheter located in the middle of the tissue block.

Cell Culture Model

Human skeletal muscle myoblasts (HSMMs) were obtained from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD) and grown according to the manufacturer’s recommendations in skeletal muscle basal medium 2 supplemented with skeletal muscle growth medium 2 Single-Quots (human epidermal growth factor, dexamethasone, L-glutamine, stain buffer, and gentamicin–amphotericin B). For bioenergetic and microscopic analysis, cells were analyzed during the exponential growth phase at 70% confluence. Bupivacaine treatment was performed by incubating cells for 1, 8, 24, or 48 h with 13 different concentrations of bupivacaine ranging from 0.1 to 5,000 μM. When indicated, pretreatment with 1 U/ml rhEPO was performed 8 or 24 h before bupivacaine treatment.

Dimethyl Thiazol Diphenyl Tetrazolium Bromide Reduction and Neutral Red Assay

Cytotoxicity of bupivacaine was analyzed using both a dimethyl thiazol diphenyl tetrazolium bromide (MTT) and neutral red assay. HSMM cells were seeded at a density of 2.10⁴/ml in 96-well plates and cultured until subconfluence in medium with or without bupivacaine and/or rhEPO. Staining with MTT or neutral red was performed as described by Mosmann and Borenfreund and Puerner, respectively. The MTT assay measures the reduction of the tetrazolium compound, primarily by...
respiratory chain activity of mitochondria using tetrazolium as an artificial electron acceptor. Absorbance was measured in a multiwell scanning spectrophotometer (Dynex MRXII; Chantilly, VA) at a wavelength of 570 nm for MTT and 540 nm for neutral red, with a reference set at 630 nm. Tests were performed in quadruplicate and repeated six times. The results were expressed as a percentage of control absorbance.

Bioenergetic Analyses: Polarography, Adenosine Triphosphate Synthesis, Enzyme Activity, Enzyme Content, and Mitochondrial Membrane Potential

To assess mitochondrial respiration in rats, we used a permeabilized muscle fiber technique with the respiratory substrates 10 mM malate plus 10 mM glutamate or 25 mM succinate plus rotenone (1 mg/ml dimethyl sulfoxide and ethanol 1:1) as indicated. Respiration was expressed as ng atom O/min/mg wet weight of the muscle fiber. Concomitant ATP synthesis measurements were performed by luminometry and expressed as nmol ATP/min/mg wet weight of the muscle fiber. This allowed for calculation of the efficiency of ATP production (ATP/O ratio).

For the HSMMs, endogenous respiratory rate was assayed in intact cells using a high-precision polarographic technique. Respiration was measured at 37°C on an Oroboros® oxygraph (Oroboros Instruments, Innsbruck, Austria) with 1 × 10^6 cells/ml in skeletal muscle growth medium 2. The experiment began with routine respiration, without additional substrates or effectors. After observing steady state respiratory flux, bupivacaine (0.1 mM, 1 mM, and 5 mM) was added. In addition, some HSMMs were pretreated with erythropoietin (1 U/ml) before conducting the experiment. Respiratory rates were expressed as ng atom O/min/1 × 10^6 cells.

Mitochondrial membrane potential (ΔΨ) was measured using the lipophilic cation JC-1 according to the manufacturer’s protocol in skeletal muscle growth medium 2. ΔΨ was expressed as the ratio of red (ex 490 nm/em 590 nm) to green (ex 490 nm/em 527 nm) fluorescence, measured on a Xenius spectrofluorometer (SAFAS, Monaco, France). The mitochondrial uncoupler carbonyl cyanide-p-trifluoromethoxyphenyl-hydrazone (15 mM) was used as positive control for mitochondrial depolarization. HSMMs were grown with or without bupivacaine (1 μM, 1 mM, and 5 mM) for 24 h and/or rhEPO (1 U/ml, 8 h before bupivacaine treatment).

To determine the effects of bupivacaine and rhEPO treatment on the mitochondrial respiratory chain, we measured the individual enzymatic activity of complexes I–IV and citrate synthase in tissue homogenates prepared from rat psoas muscle using spectrophotometric procedures as previously described. Briefly, approximately 100 mg psoas muscle was minced and homogenized with a glass potter homogenizer (Kimble/Kontes, Vine land, NJ) in ice-cold medium (10% wt/vol) containing 225 mM mannitol, 75 mM sucrose, 10 mM Tris-HCl, and 0.10 mM EDTA at pH 7.2. The homogenate was then centrifuged for 20 min at 650g. The supernatant was collected, and the protein concentration was determined. Citrate synthase activity was measured, as described by Srere, in the presence of 4% Triton (vol/vol) by monitoring the formation of thionitrobenzoate dianion from the reaction of coenzyme A and 5,5'-dithiobis (2-nitrobenzoic acid) at 412 nm and 30°C. Complex I activity, reduced nicotinamide adenine dinucleotide ubiquinone reductase, was measured as described by Birch-Machin et al. The oxidation of reduced nicotinamide adenine dinucleotide by complex I was recorded using the ubiquinone analog decylibiquinone as an electron acceptor. The decrease in absorption resulting from reduced nicotinamide adenine dinucleotide 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. 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 methosulfate. The oxidation of ubiquinol (UQH2) by complex III (decylibiquinol cytochrome c reductase) was determined using cytochrome c (III) as an electron acceptor. The reduction of cytochrome c (III) was recorded at 550 nm at 30°C. Complex IV (cytochrome c oxidase) activity was measured as described by Wharton and Tzagoloff using cytochrome c (II) as a substrate. The oxidation of cytochrome c was monitored at 550 nm at 30°C. Enzyme activities were expressed as nmol substrate/min/mg protein.

The content of respiratory chain complexes was measured by Western blot as described previously. Tissue samples from psoas muscle (SS and ES groups) were diluted in sodium dodecyl sulfate polyacrylamide gel tricine sample buffer (Bio-Rad Laboratories, Hercules, CA) containing 2% β-mercaptoethanol by incubation for 30 min at 37°C and separated on a 10–22% sodium dodecyl sulfate polyacrylamide gradient mini-gel (Bio-Rad Laboratories) at 150 V. Proteins were transferred electrophoretically to a 0.45-μm polyvinylidene difluoride membrane for 2 h at 100 mA in N-cyclhexyl-3-amino propane sulfonic acid buffer (3.3 g N-cyclohexyl-3-amino propane sulfonic acid; 1.51 methanol, 10%, pH 11) on ice. Membranes were blocked overnight in 5% milk-phosphate-buffered saline +0.02% azide, and incubated for 3 h with the primary antibodies. After three washes with phosphate-buffered saline −0.05% Tween 20, membranes were incubated for 2 h with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Bio-Rad Laboratories) diluted in 5% milk- phosphate-buffered saline. The secondary antibody was detected using chemiluminescent ECL Plus reagent (Amersham Biosciences, GE Healthcare, Uppsala, Sweden). The sig-
nal was quantified by densitometric analysis using Image J software (National Institutes of Health, Bethesda, MD).

**Oxidative Stress**

Changes in cytosolic levels of reactive oxygen species were monitored using the CM-H$_2$DCFDA fluorescent probe (Invitrogen) in HSMMs grown with or without bupivacaine (1 μM, 1 mM, and 5 mM for 24 h) and/or rhEPO (1 U/ml, 8 h before bupivacaine treatment). Fluorescence was measured at steady state in skeletal muscle basal medium 2 with a spectrofluorometer (SAFAS, Monaco, France) using excitation and emission wavelengths set at 495 and 520 nm, respectively. Adding H$_2$O$_2$ (100 μM) to the cuvette was used as a positive control. Results are expressed as a percentage of the control fluorescence.

**Mitochondrial and Muscle Morphology**

Profiles of mitochondrial sections were analyzed by transmission electron microscopy on a Hitachi H-7650 microscope (Tokyo, Japan) as described previously and catheter insertion sites in rat psoas muscle were analyzed by scanning electron microscopy. A series of at least five different samples was taken from four different muscles from each group of rats (SS, ES, SB, EB).

In HSMMs, the morphology of the mitochondrial network was studied by fluorescence microscopy using mitotracker green (150 nM, 20 min at 37°C) on a Nikon E 200 microscope (Kawasaki, Japan) with a 60×, 1.4 numerical aperture water immersion objective. A series of images was acquired using a Q-Imaging Retiga Exi fast camera driven by Fluoup (Explora Nova, La Rochelle, France). Image analysis was performed with Morpho pro version 2.8 (Explora Nova). HSMMs were grown with or without bupivacaine (1 μM, 1 mM, and 5 mM for 24 h) and/or rhEPO (1 U/ml, 8 h before bupivacaine treatment). Fifteen images were taken from three different cell culture dishes. We defined three main morphologic configurations: filamentous, outgrowth, and fragmented. One hundred twenty cells taken from three independent experiments were analyzed randomly for each condition. The results are expressed as a percentage of the counted cells for each configuration.

**Magnetic Resonance Imaging**

Magnetic resonance imaging experiments were conducted on a 4.7-T Biospec horizontal system (Bruker, Ettlingen, Germany), equipped with a 12-cm gradient system, capable of 200 mT/m maximum strength and 180 μs rise time. Measurements were performed with a rat-dedicated probe birdcage resonator (80 mm in diameter and 120 mm long) tuned to 200.3 MHz. Wistar rats were anesthetized with isoflurane (1.5% in air). A solution of 5 mM of magnetic resonance imaging contrast agent (Gd-DOTA, 5 mM, DOTAREM; Guerbet, Aulnay-sous-bois, France) was then injected by the peripheral nerve catheter, and diffusion was observed in the psoas muscle using a dedicated fast low angle shot sequence.

**Statistical Analysis**

For mitochondrial respiration and enzyme activities in the rat muscle model, quantitative data are reported as a median [25th and 75th percentiles], because of a non-normal distribution. Data from the groups (SS, SB, ES, EB) were then compared using a Kruskal–Wallis test; Mann–Whitney tests were performed by comparing the SS group with the other three groups (the P value required for statistical significance was determined by dividing 0.05 by the number of comparisons; therefore, $P = 0.05/3 = 0.0166$).

For the cell culture model, data are expressed as mean ± SD, and one-way or two-way analysis of variance ($α = 0.05$) was performed as appropriate, in addition to a Student–Newman–Keuls post hoc test (with $P < 0.05$ considered significant).

Tests were performed using SigmaStat 3.1 (Systat Software Inc., San Jose, CA).

**Results**

**Rat Analgesia Protocol**

Seventy rats were anesthetized according to the protocol summarized in figure 1A. No self-mutilation after catheter placement was observed. The localization of catheters and their tips inside living animals was verified by light microscopy (fig. 1B) and confirmed by magnetic resonance imaging analyses (fig. 1C), as well as by scanning electron microscopy observations (fig. 1D). The catheters were inserted into perimusium connective tissue and between muscle fibers without destruction to reach the vicinity of the femoral nerve, where bupivacaine was released. Five rats with catheter displacement were excluded from the analysis.

**Bupivacaine-induced Impairment of Energy Metabolism in Psoas Muscle**

Measurements of coupled oxygen consumption rate and ATP synthesis were performed in permeabilized fibers using glutamate plus malate or succinate as substrates. Bupivacaine induced a significant decrease in adenosine diphosphate–stimulated oxygen consumption along with a significant inhibitory effect on ATP synthesis. The efficiency of oxidative phosphorylation (ATP/O ratio) was also reduced. Erythropoietin cotreatment (5,000 U/kg/24 h, EB group) prevented the inhibitory effect of bupivacaine on mitochondrial bioenergetics (table 1).

**Alterations of Mitochondrial Respiratory Chain Enzyme Activities and Organelle Structure by Bupivacaine**

The activities of the respiratory chain complexes (I-IV) and citrate synthase were measured in the different
When Kruskal–Wallis analysis was significant, $P < 0.0166$ was considered significant. The global inhibition of respiratory chain activity by bupivacaine; E = recombinant human erythropoietin; IQR = interquartile range; O = oxygen; S = saline.

Electron micrographs of longitudinal psoas muscle tissue sections showed that mitochondrial morphology was also altered by bupivacaine (fig. 3). Subsarcolemmal aggregates of swollen mitochondria (fig. 3E) were observed with partial loss of interfibrillar mitochondria (fig. 3K). We also observed membranes thought to be autophagosomes containing either intact or degraded mitochondria (figs. 3K and J, respectively). The internal organization of mitochondria was also severely affected, forming an onion-like structure (figs. 3G and H) that included rearrangements of matrical space and a loss of cristae. These unusual mitochondrial morphologies were observed in all samples from bupivacaine-treated muscle. Cotreatment with rhEPO prevented this mitochondrial structural disorganization (figs. 3M–P). This also suggests an inhibition of mitochondrial degradation processes by rhEPO.

### Table 1. Effects of Bupivacaine and/or Recombinant Human Erythropoietin on Mitochondrial Oxidative Phosphorylations in Rat Psoas Muscle

<table>
<thead>
<tr>
<th>Glutamate</th>
<th>Complex I</th>
<th>Complex II</th>
<th>Complex III</th>
<th>Complex IV</th>
<th>Citrate Synthase</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP-stimulated Oxygen Consumption Rate</td>
<td>Median [IQR]</td>
<td>P Value</td>
<td>Median [IQR]</td>
<td>P Value</td>
<td>Median [IQR]</td>
</tr>
<tr>
<td>Kruskal–Wallis test</td>
<td>0.0018</td>
<td></td>
<td>0.0001</td>
<td></td>
<td>0.0012</td>
</tr>
<tr>
<td>SS</td>
<td>15.6 [13.1–22.1]</td>
<td>&lt; 0.001</td>
<td>34.5 [32.7–41.6]</td>
<td>&lt; 0.001</td>
<td>2.3 [1.8–2.6]</td>
</tr>
<tr>
<td>SB</td>
<td>8.2 [6.4–11.1]</td>
<td>0.005</td>
<td>14.1 [11.6–17.5]</td>
<td>&lt; 0.001</td>
<td>1.6 [1.3–1.9]</td>
</tr>
<tr>
<td>ES</td>
<td>14.6 [13.6–17.0]</td>
<td>0.534</td>
<td>31.1 [29.1–46.2]</td>
<td>0.207</td>
<td>2.3 [2.0–2.8]</td>
</tr>
<tr>
<td>EB</td>
<td>13.6 [10.2–17.3]</td>
<td>0.213</td>
<td>36.2 [19.3–44.8]</td>
<td>0.729</td>
<td>1.9 [1.4–2.9]</td>
</tr>
<tr>
<td>Succinate</td>
<td>Kruskal–Wallis test</td>
<td>0.035</td>
<td></td>
<td>0.129</td>
<td></td>
</tr>
<tr>
<td>SS</td>
<td>21.2 [16.8–23.9]</td>
<td>0.016</td>
<td>30.6 [24.8–33.0]</td>
<td>1.3 [1.1–2.0]</td>
<td></td>
</tr>
<tr>
<td>SB</td>
<td>15.8 [11.1–17.2]</td>
<td>0.021</td>
<td>16.9 [10.8–21.9]</td>
<td>0.11 [0.7–1.4]</td>
<td>0.203</td>
</tr>
<tr>
<td>ES</td>
<td>19.3 [13.5–21.3]</td>
<td>0.423</td>
<td>28.2 [21.4–37.8]</td>
<td>0.884</td>
<td>1.5 [1.4–1.8]</td>
</tr>
<tr>
<td>EB</td>
<td>20.3 [19.3–26.1]</td>
<td>0.630</td>
<td>34.8 [27.6–35.9]</td>
<td>0.315</td>
<td>1.4 [1.1–1.5]</td>
</tr>
</tbody>
</table>

$n = 9$ or 10 rats/group. Experimental conditions are described in the Material and Methods. Enzymatic activity was expressed in nmol substrate/min/mg protein.

### Table 2. Effects of Bupivacaine and/or Recombinant Human Erythropoietin on Enzymatic Activities of the Respiratory Chain in Rat Psoas Muscle

<table>
<thead>
<tr>
<th>Complex I</th>
<th>Complex II</th>
<th>Complex III</th>
<th>Complex IV</th>
<th>Citrate Synthase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median [IQR]</td>
<td>P Value</td>
<td>Median [IQR]</td>
<td>P Value</td>
<td>Median [IQR]</td>
</tr>
<tr>
<td>Kruskal–Wallis test</td>
<td>0.0002</td>
<td>0.0004</td>
<td>0.0017</td>
<td>0.0005</td>
</tr>
<tr>
<td>SS</td>
<td>165 [127–182]</td>
<td>&lt; 0.001</td>
<td>125 [109–147]</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>SB</td>
<td>77 [89–102]</td>
<td>0.001</td>
<td>72 [63–85]</td>
<td>0.001</td>
</tr>
<tr>
<td>ES</td>
<td>164 [158–165]</td>
<td>0.823</td>
<td>124 [119–138]</td>
<td>0.001</td>
</tr>
<tr>
<td>EB</td>
<td>133 [127–150]</td>
<td>0.252</td>
<td>108 [97–125]</td>
<td>0.001</td>
</tr>
</tbody>
</table>

$n = 9$ or 10 rats/group. Experimental conditions are described in the Materials and Methods. Enzymatic activity was expressed in nmol substrate/min/mg protein. When Kruskal–Wallis analysis was significant, $P < 0.0166$ was considered significant. B = bupivacaine; E = recombinant human erythropoietin; IQR = interquartile range; S = saline.
ERYTHROPOIETIN AND BUPIVACAINE-INDUCED MYOTOXICITY

Fig. 2. Effects of recombinant human erythropoietin (rhEPO) on Western blot analysis. Samples (n = 3) from the SS and ES groups were analyzed (experimental conditions are described in the Materials and Methods). (A) Electrophoretic separation of CI, CII, CIII, and F,F adenosine triphosphate synthase of psoas muscle. (B) Bars represent mean ± SD for three parallel experiments. The results obtained from rat muscles treated with rhEPO showed that rhEPO induced no increase in the expression levels of respiratory chain complexes (CI, CII, CIII, and F,F adenosine triphosphate synthase).

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Discussion

Local anesthetics (e.g., bupivacaine) are widely used for postoperative regional analgesia, even though some studies have demonstrated a potential muscle toxicity risk. Our study showed that this risk can be primarily explained by the impairment of mitochondrial structure and function and can be partially blocked by treatment with rhEPO.

Our results indicate that bupivacaine myotoxicity combines two deleterious synergistic effects, leading to a reduction in the activity of respiratory chain complexes I-V and diminished ATP synthesis. This had already been suggested from previous studies with isolated mitochondria. Our work validates those findings in a physiologic and clinically relevant rat model of reiterative exposure to bupivacaine. We also reproduced our findings using human myoblasts in culture. In contrast with previous studies using tumor-derived cell lines to investigate the toxicity of lidocaine and bupivacaine, our cell model was obtained from primary human myoblasts. The use of cancer cells might have been misleading because of their already abnormal bioenergetic properties and altered mitochondrial features. The general energy crisis caused by bupivacaine as observed in rats and HSMMs could be caused by the high liposolubility of bupivacaine and its accumulation in mitochondrial mem-

5% with and without rhEPO, respectively; P < 0.05 by Student-Newman-Keuls post hoc test) as compared with 5 mM bupivacaine alone. MTT reduction was also not decreased by increasing bupivacaine concentration (fig. 5C). Interestingly, the duration of pretreatment with 1 U/ml rhEPO determined the extent of MTT reduction (fig. 5C).
The toxic mechanisms of bupivacaine could also involve slipping of either the respiratory chain and/or the F$_1$F$_0$ ATP synthase, because we observed a reduction of ATP/O. At the molecular level, the tertiary amine of bupivacaine could potentially interact with respiratory chain complexes and/or the F$_1$F$_0$ ATP synthase to inhibit activity. Hence, mitochondrial structural changes induced by bupivacaine could be caused by a direct interaction with F$_1$F$_0$, given the close link between this enzyme complex and cristae formation. Indeed, the onion-like structure of mitochondria observed in our study is typically associated with abnormal F$_1$F$_0$ oligomerization. All rats that received bupivacaine without rhEPO showed significant inhibition of ATP production in the muscular region surrounding the catheter and exhibited changes in mitochondrial structure. These unusual morphologies could be consistent with autophagy, as indicated by the systematic observation of membrane structures presumed to be autophagosomes containing abnormal mitochondria. Abnormal mitochondrial autophagy was also recently described in various pathologic conditions, but it remains poorly understood.

Current hypotheses propose that a decrease in $\Delta\Psi$ can activate mitophagy. Our cell culture model confirmed that bupivacaine exposure led to a decrease in $\Delta\Psi$, an effect that is well known with lidocaine. This observation might explain the fragmentation of the mitochondrial network, because fusion and fission mechanisms depend on $\Delta\Psi$. Moreover, a marked decrease in mitochondrial membrane potential with 0.001 and 1 mM bupivacaine at 24 h was shown, whereas no effect of these concentrations on MTT reduction was observed at 24 h. This might be due to the existence of nonmitochondrial sites of MTT reduction, or endocytosis and autophagy.

**Fig. 3.** Erythropoietin protects mitochondrial morphology. Mitochondria were divided into two different populations: subsarcolemmal mitochondria (SSM, the two first columns) and interfibrillar mitochondria (IFM, the two others columns). Mitochondrial morphology was observed using transmission electron microscopy. SSM and IFM of the SS group (A–B and C–D, respectively) were normal. Abnormal mitochondrial morphology induced by bupivacaine was seen in SSM (E–F and G–H), in addition to IFM with onion-like structures (G–H) and membrane structures presumed to be autophagosomes (K–L). Recombinant human erythropoietin pretreatment partially prevented these anomalies in SSM (M–N) and in IFM (O–P). ap = autophagosome; ols = onion-like structure. Scale bar, 500 nm.

**Fig. 4.** Respiration of human skeletal muscle myoblasts. Endogenous cellular respiration (ng atom O/min/1 × 10$^6$ cells) was measured for human skeletal muscle myoblast cells cultured in galactose medium. The rate of respiration during nonphosphorylating conditions was obtained from cells with increased concentrations of bupivacaine in the cuvette. Bars represent mean ± SD for five parallel experiments al analysis of variance and *P < 0.05 versus control and 0.1 μM, with Student–Newman–Keuls post hoc test.
extrusion of MTT,\textsuperscript{36} or the fact that MTT reduction is not directly dependent on $\Delta\Psi$, because there is no evidence that $\Delta\Psi$ controls respiratory chain complex II activity. The results of the MTT assay used in our study to evaluate the impact of bupivacaine on cell metabolism should be considered with caution. This assay does not give a rigorous measure of cell proliferation rate or cell viability, because it is based on the intracellular reduction of the added tetrazolium salt MTT to a colored water-insoluble formazan salt by the mitochondrial enzyme succinate dehydrogenase. Therefore, it cannot distinguish between cytotoxic molecules and mitotoxic compounds. Therefore, the inhibition of MTT reduction induced by bupivacaine could be interpreted as an alteration of mitochondrial metabolism and/or an inhibition of cell viability. Moreover, inhibition of mitochondrial respiratory chain complexes (genetically or pharmacologically) is usually compensated by different mechanisms so that MTT reduction can be maintained despite a large OXPHOS deficiency.\textsuperscript{37} An important threshold of respiratory chain inhibition must be passed to observe changes in cellular homeostasis. This phenomenon was recently highlighted in cells treated with rotenone, a complex I inhibitor.\textsuperscript{35}

The cell culture model allowed measurement of the toxic dose of bupivacaine, giving values between 1 and 3 mM. This toxic dose, however, cannot be extrapolated to in vivo experiments or clinical practice. Indeed, these concentrations are likely much higher than the bupivacaine concentration described in psoas muscle at 1 h after the last injection.\textsuperscript{10} This shows that there is a margin of safety above which the inhibition of cellular energy metabolism by bupivacaine triggers cell death, as reported in different experimental and pathologic situations with mitochondrial pathologic impairment.\textsuperscript{37}

The molecular mechanisms of the protective effects of rhEPO against bupivacaine-induced myotoxicity include mitochondrial $\Delta\Psi$ maintenance, as observed in our cell culture model. rhEPO might delay both fragmentation of the mitochondrial network and the initiation of either apoptosis and/or mitoptosis.\textsuperscript{38,39} In addition to this supposed antimitopotic effect, rhEPO restored the efficiency of OXPHOS in rats treated with bupivacaine and prevented the reduction of OXPHOS activity and mitochondrial content, suggesting that preservation of mitochondrial membrane organization stops the apoptotic pathway. This would be a novel physiologic role for rhEPO at the level of mitochondrial energetics and struc-

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**Fig. 5.** Dimethyl thiazol diphenyl tetrazolium bromide (MTT) reduction is decreased by bupivacaine and was partially recovered by recombinant human erythropoietin (rhEPO) pretreatment and cotreatment. (A) Time course of bupivacaine-induced MTT reduction. MTT tests were performed, and different bupivacaine concentrations were evaluated (control [•], 0.001 mM bupivacaine [◆], 1 mM [▲], 3 mM [●], and 5 mM [■]). Bupivacaine induced time-dependent and concentration-dependent MTT reduction; $P = 0.021$ with one-way analysis of variance and *$P < 0.05$ versus control and 0.001 at the same time, †$P < 0.05$ versus 1 mM at the same time with Student–Newman–Keuls post hoc test. (B) Determination of rhEPO pretreatment concentration with 3 mM bupivacaine treatment for a 48-h period. MTT reduction was measured using an MTT test. The rhEPO protective concentration was around 1 U/ml, which was used for the subsequent experiments. (C) Both bupivacaine concentration–dependent (3–5 mM) and rhEPO pretreatment duration–dependent (no rhEPO for white bars, 8 h of 1 U/ml rhEPO cotreatment for gray bars, and 24 h of 1 U/ml rhEPO cotreatment for dark gray bars) effects were evaluated for human skeletal muscle myoblast MTT reduction for a 48-h bupivacaine treatment. $P < 0.001$ with two-way analysis of variance ($\alpha = 0.05$) and *$P < 0.05$ versus control, †$P < 0.5$ versus bupivacaine at the same concentration; ‡versus 8 h of rhEPO treatment with bupivacaine at the same concentration, with Student–Newman–Keuls post hoc test. Tests were performed in quadruplicate and repeated six times. Results are reported as mean ± SD.
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Fig. 6. Fission of mitochondrial reticulum coupled with changes in mitochondrial membrane potential in human skeletal muscle myoblasts. Human skeletal muscle myoblasts were grown with or without bupivacaine (24 h) and with or without recombinant human erythropoietin (1 U/ml, 8 h before bupivacaine treatment). Normal mitochondrial reticulum visualized by fluorescence microscopy and mitochondrial membrane potential obtained by spectrofluorometry are shown in A and E. Control (A), concentrations of 1 μM (B), 1 mM (C), and 5 mM bupivacaine (D) led to alterations in the mitochondrial network morphology, coupled with the loss of mitochondrial membrane potential (ΔΨ) (F–H, respectively). The images in E–H and M–P are pseudocolor, merged images using the two emission wavelengths monitored for JC-1 quantitation. Recombinant human erythropoietin alone induced no changes in either mitochondria network morphology (I) or ΔΨ (M). Recombinant human erythropoietin pretreatment prevented fission of the mitochondrial reticulum for low concentrations of bupivacaine (1 μM–1 mM, J–K) but not for the 5 mM concentration (L). Parallel effects were observed with regard to ΔΨ (N–P, respectively). Scale bar, 80 μm.
treatment to assess the risk of hypertension, venous thromboembolism, and mortality, which are routinely described in the elderly and in patients with cancer. The risk of rhEPO side effects combined to the extremely high cost of rhEPO has to be considered when minor clinical implications such as bupivacaine-induced myotoxicity are being discussed.

In conclusion, our findings demonstrate that bupivacaine-induced myotoxicity is associated with changes in mitochondrial structure and function in vivo. We show that rhEPO could protect against bupivacaine-induced myotoxicity, suggesting the existence of a protective mechanism in mitochondria. rhEPO not only reduced unusual morphologies consistent with autophagy but also partially rescued the bioenergetic suppression caused by bupivacaine. We examined both the dose dependence and time dependence of bupivacaine and rhEPO interactions with mitochondrial function. The clinical impact of our results remains to be evaluated in practice.

Fig. 7. Bupivacaine induced a loss of mitochondrial membrane potential, which was partially preserved by recombinant human erythropoietin (rhEPO). Human skeletal muscle myoblasts were grown in the presence or absence of the indicated concentration of bupivacaine (24 h, in absence of rhEPO, black bars) and/or rhEPO (1 U/ml, 8 h before bupivacaine treatment, gray bars). The mitochondrial membrane potential (ΔΨ) was expressed as the ratio red/green fluorescence. Two-way analysis of variance with Student–Newman–Keuls post hoc test showed a significant difference between levels of bupivacaine, and between experiments with or without rhEPO. The effects of different concentrations of bupivacaine depended on the presence of rhEPO (P < 0.001). Therefore, the fraction of human skeletal muscle myoblasts with reduced ΔΨ was dose dependent and increased with increasing bupivacaine concentration. rhEPO treatment led to partial preservation of ΔΨ. Bars represent mean ± SD for five parallel experiments. * P < 0.05 versus control, † P < 0.05 versus without rhEPO for the same bupivacaine concentration, with Student–Newman–Keuls post hoc test.

Fig. 8. Morphometric analysis of the mitochondrial network. Mitochondrial network morphology was analyzed by fluorescence microscopy of living cells during the exponential phase of growth. One hundred twenty cells taken from three independent experiments were analyzed randomly for each of the following conditions: 1 μM bupivacaine, 1 mM bupivacaine, 5 mM bupivacaine, recombinant human erythropoietin (rhEPO) alone, rhEPO pretreatment and 1 μM bupivacaine, rhEPO pretreatment and 1 mM bupivacaine, rhEPO pretreatment and 5 mM bupivacaine. Manual counting was performed according to the morphology of the mitochondrial network. Three main classes were defined: filamentous, outgrowth, and fragmented. The results are expressed as a percentage of the counted cells with each configuration.
Fig. 9. Potential mechanism of recombinant human erythropoietin (rhEPO) cytoprotection in skeletal muscle. rhEPO protects against bupivacaine-induced myotoxicity and dimethyl thiazol diphenyl tetrazolium bromide reduction through a series of pathways that originate with changes in mitochondria. Bupivacaine induces metabolic and structural changes at the level of pathways that originate with changes in mitochondria. rhEPO maintains the integrity of the mitochondrial membrane potential, allowing recovery. Lancet 2003; 362:1921–8

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