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Erythropoietin Protects against Local Anesthetic Myotoxicity during Continuous Regional Analgesia

Karine Nouette-Gaulain, M.D., Ph.D., Nadège Bellance, Ph.D.,† Baptiste Prévost, Ph.D.,† Emilie Passerieux, Ph.D.,‡ Claire Pertuiset, Ph.D.,† Olivier Galbes, Ph.D.,§ Katarina Smolkova, Ph.D.,† Françoise Masson, M.D.,‖ Sylvain Miraux, Ph.D.,# Jean-Paul Delage, Ph.D.,** Thierry Letellier, Ph.D.,†† Rodrigue Rossignol, Ph.D.,** Xavier Capdevila, M.D., Ph.D.,‡‡ François Sztark, M.D., Ph.D.§§*

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 adenosine 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.[6,7] 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 myotox-

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icity 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).[10] 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.

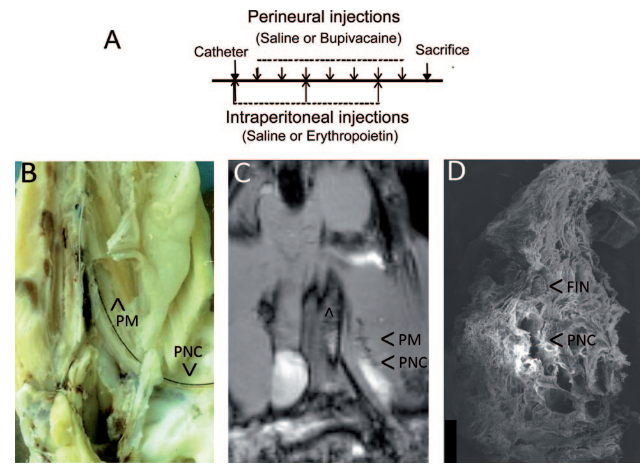

Fig. 1. Animal model. The study protocol for rats is described in the timeline (*A*), with daily intraperitoneal injections (saline or recombinant human erythropoietin, the first occurring 8 h before the first perineural injection) and seven iterative injections 8 h apart *via* peripheral nerve catheter (saline or bupivacaine). Rats were killed 8 h after the last perineural injection. The localization of catheters inside the living animals was verified by optic microscopy (*B*), characterized by magnetic resonance imaging analyses (*C*) and scanning electron microscopy observations (*D*). FlN = femoral nerve; PM = psoas muscle; PNC = peripheral nerve catheter.

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 2.10^4/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[12] and Borenfreund and Puerner,[13] 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[14] 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.[15] 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.[16] The experiment began with routine respiration, without additional substrates or effectors. After observing steady state respiratory flux, bupivacaine (0.1 μM–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 ($\Delta\Psi$) was measured using the lipophilic cation JC-1 according to the manufacturer's protocol in skeletal muscle growth medium 2.[17] $\Delta\Psi$ 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.[10] Briefly, approximately 100 mg psoas muscle was minced and homogenized with a glass potter homogenizer (Kimble/Kontes, Vineland, 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 650*g*. The supernatant was collected, and the protein concentration was determined.[18] Citrate synthase activity was measured, as described by Srere,[19] 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.*[20] The oxidation of reduced nicotinamide adenine dinucleotide by complex I was recorded using the ubiquinone analog decylubiquinone 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 methosulphate.[21] The oxidation of ubiquinol (UQ_1H_2) by complex III (decylubiquinol cytochrome *c* reductase) was determined using cytochrome *c*(III) as an electron acceptor.[20] 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[22] 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.[17] 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 polyvinylidine difluoride membrane for 2 h at 100 mA in *N*-cyclohexyl-3-aminopropanesulfonic acid buffer (3.3 g *N*-cyclohexyl-3-aminopropanesulfonic acid; 1.5 l 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_2O_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,[23,24] 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 1394 digital 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 ($\alpha = 0.05$) 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 perimysium 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

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

	ADP-stimulated Oxygen Consumption Rate		ATP Synthesis		ATP/O Ratio	
	Median [IQR]	*P* Value	Median [IQR]	*P* Value	Median [IQR]	*P* Value
Glutamate						
Kruskal–Wallis		0.018		0.001		0.050
SS	15.6 [13.1–22.1]		34.5 [32.7–41.6]		2.3 [1.8–2.6]	
SB	8.2 [6.4–11.1]	0.005	14.1 [11.6–17.5]	< 0.001	1.6 [1.3–1.9]	0.016
ES	14.6 [13.6–17.0]	0.534	31.1 [29.1–46.2]	0.207	2.3 [2.0–2.8]	0.674
EB	13.6 [10.2–17.3]	0.213	36.2 [19.3–44.8]	0.729	1.9 [1.4–2.9]	0.728
Succinate						
Kruskal–Wallis		0.035		0.016		0.129
SS	21.2 [16.8–23.9]		30.6 [24.8–33.0]		1.3 [1.1–2.0]	
SB	15.8 [11.1–17.2]	0.021	16.9 [10.8–21.9]	0.004	1.1 [0.7–1.4]	0.203
ES	19.3 [13.5–21.3]	0.423	28.2 [21.4–37.8]	0.884	1.5 [1.4–1.8]	0.329
EB	20.3 [19.3–26.1]	0.630	34.8 [27.6–35.9]	0.315	1.4 [1.1–1.5]	0.958

n = 9 or 10 rats/group. Experimental conditions are described in the Material and Methods. Adenosine diphosphate (ADP)–stimulated oxygen consumption rates are expressed in ng atom O/min/mg wet weight. Adenosine triphosphate (ATP) synthesis rate is expressed in nmol ATP/min/mg wet weight. 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* values in front of medians are those of Mann–Whitney tests for *post hoc* paired comparisons between SS group and other groups (according to Bonferroni correction, $P < 0.0166$ was considered significant).

B = bupivacaine; E = recombinant human erythropoietin; IQR = interquartile range; O = oxygen; S = saline.

groups (table 2). Results indicated a significant and global inhibition of respiratory chain activity by bupivacaine, with a prominent effect on complex I. This was associated with a reduction in mitochondrial content, as suggested by the decrease in citrate synthase activity. These toxic effects were fully blocked, however, by cotreatment with rhEPO (table 2). To explore the underlying mechanisms involved in the recovery of citrate synthase activity after rhEPO treatment, we looked at the possible activation of mitochondrial biogenesis by performing Western blot experiments (fig. 2). The results obtained from rat muscles treated with rhEPO showed that rhEPO induced no increase in the expression level of respiratory chain complexes (CI, CII, CIII, and F_1F_0 ATP synthase).

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).[25] The internal organization of mitochondria was also severely affected, forming an onion-like structure (figs. 3G and H) that included rearrangements of matricial 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.

rhEPO Maintains Energy Status in Human Skeletal Muscle Myoblast Cells Treated with Bupivacaine

Bupivacaine treatment (0.1 μM–5 mM) of intact HSMM cells resulted in dose-dependent alteration of endogenous respiration with uncoupling, followed by signifi-

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

	Complex I		Complex II		Complex III		Complex IV		Citrate Synthase	
	Median [IQR]	*P* Value	Median [IQR]	*P* Value	Median [IQR]	*P* Value	Median [IQR]	*P* Value	Median [IQR]	*P* Value
Kruskal–Wallis		0.0002		0.0004		0.0017		0.0005		0.012
SS	165 [127–182]		125 [109–147]		611 [531–741]		915 [841–1,072]		367 [337–446]	
SB	77 [69–102]	< 0.001	72 [63–85]	< 0.001	389 [346–445]	< 0.001	543 [428–599]	< 0.001	289 [270–323]	0.0013
ES	164 [158–165]	0.823	124 [119–138]	0.787	571 [535–626]	0.684	1015 [901–1,119]	0.478	352 [327–408]	0.386
EB	133 [127–150]	0.252	108 [97–125]	0.144	571 [543–678]	0.772	790 [729–863]	0.041	338 [287–404]	0.159

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* values in front of medians are those of Mann–Whitney tests for *post hoc* paired comparisons between SS group and other groups (according to Bonferroni correction, $P < 0.0166$ was considered significant).

B = bupivacaine; E = recombinant human erythropoietin; IQR = interquartile range; S = saline.

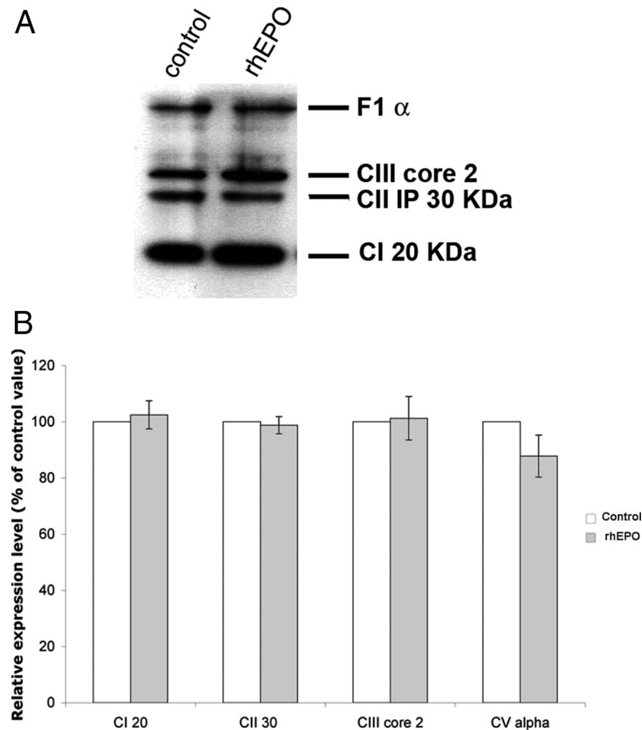

Fig. 2. Effects of recombinant human erythropoietin (rhEPO) on Western blot analysis. Samples (n = 3) from the SS and ES groups were analyzed (experimental conditions are described in the Materials and Methods). (*A*) Electrophoretic separation of CI, CII, CIII, and F_1F_0 adenosine triphosphate synthase 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_1F_0 adenosine triphosphate synthase).

cant inhibition (fig. 4). These changes were not prevented by the direct addition of rhEPO to the cuvette of the oxygraph (data not shown).

The impact of this inhibition of respiratory rate on the MTT reduction assay was investigated (fig. 5A) and exhibited a threshold profile. Similar results were obtained with the neutral red assay (data not shown). Above 1 mM, bupivacaine exerted a dose- and time-dependent effect that reached 17 ± 5% and 18 ± 6% cell viability with 5 mM bupivacaine after 48 h, as measured by the MTT and neutral red assays, respectively. MTT reduction was decreased by bupivacaine and was partially recovered by rhEPO pretreatment and cotreatment (fig. 5), while treatment with 0.1–100 U/ml rhEPO alone for 24 and 48 h had no effect on MTT reduction (data not shown). No significant difference in this protective effect was observed for rhEPO concentrations ranging from 0.25 to 100 U/ml for 3 mM bupivacaine and 48-h incubation (fig. 5B), and we chose rhEPO at 1 U/ml for subsequent experiments. MTT reduction was significantly less diminished when rhEPO (1 U/ml) was added concomitantly with 5 mM bupivacaine for 24 h (57 ± 4 *vs.* 31 ± 2% with and without rhEPO, respectively; $P < 0.05$ by Student–Newman–Keuls *post hoc* test) or 48 h (44 ± 10 *vs.* 17 ± 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).

rhEPO Prevents Bupivacaine-induced Reduction of Mitochondrial Membrane Potential and Fragmentation of Mitochondria in HSMMs

Treatment of HSMMs for 24 h (fig. 6) with variable doses of bupivacaine (1 μM–5 mM) resulted in a progressive decrease in $\Delta\Psi$ (figs. 6E–H and 7) with complete depolarization using 1 mM bupivacaine. Interestingly, 1 U/ml rhEPO for an 8-h pretreatment and cotreatment protected against this phenomenon (figs. 6M–P). In contrast, we observed no change in cytosolic reactive oxygen species levels in cells treated with bupivacaine or cotreated with rhEPO (data not shown).

Bupivacaine treatment of HSMMs also led to progressive fragmentation of the mitochondrial network (figs. 6B–D and 8), which was initiated with 1 μM bupivacaine (24 h) and led to complete fragmentation at a dose of 5 mM (fig. 6D). Such changes in the mitochondrial network were delayed by pretreatment and cotreatment with 1 U/ml rhEPO for 8 h (figs. 6I–L, 8, and 9).

Discussion

Local anesthetics (*e.g.*, bupivacaine) are widely used for postoperative regional analgesia, even though some studies have demonstrated a potential muscle toxicity risk.[4,5,26] Our study showed that this risk can be primarily explained by the impairment of mitochondrial structure and function[6,7] 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.[6,7] 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,[9] 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-

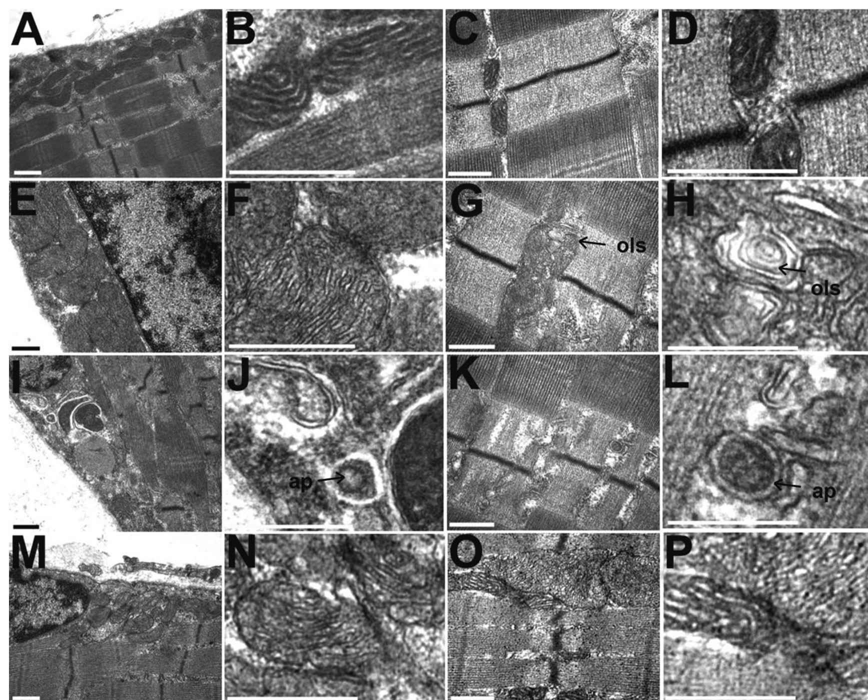

Fig. 3. Erythropoietin protects mitochondrial morphology. Mitochondria were divided into two different populations: subsarcolemmal mitochondria (SSM, the two first columns) and interfibrillar mitochondria (IFM, the two others columns). Mitochondrial morphology was observed using transmission electron 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 *I–J*), 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.

branes. The toxic mechanisms of bupivacaine could also involve slipping of either the respiratory chain and/or the F_1F_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_1F_0 ATP synthase to inhibit activity.[27] Hence, mitochondrial structural changes induced by bupivacaine could be caused by a direct interaction with F_1F_0, given the close link between this enzyme complex and cristae formation.[28,29] Indeed, the onion-like structure of mitochondria observed in our study is typically associated with abnormal F_1F_0 oligomerization.[30] 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.[31]

Current hypotheses propose that a decrease in $\Delta\Psi$ can activate mitophagy.[32] Our cell culture model confirmed that bupivacaine exposure led to a decrease in $\Delta\Psi$,[6] an effect that is well known with lidocaine.[33,34] This observation might explain the fragmentation of the mitochondrial network, because fusion and fission mechanisms depend on $\Delta\Psi$.[35] 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

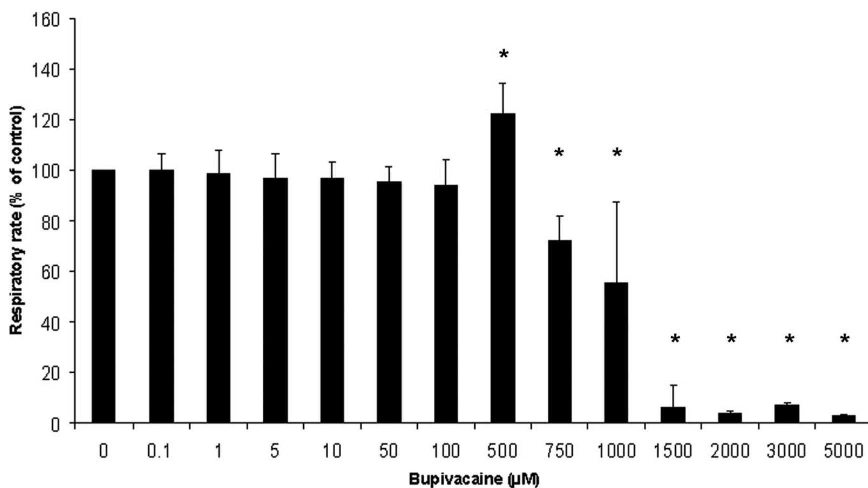

Fig. 4. Respiration of human skeletal muscle myoblasts. Endogenous cellular respiration (ng atom O/min/1 × 10^6 cells) was measured for human skeletal muscle myoblast cells cultured in galactose medium. The rate of respiration during nonphosphorylating conditions was obtained from cells with increased concentrations of bupivacaine in the cuvette. *Bars* represent mean ± SD for five parallel experiments; $P < 0.001$ with one-way analysis of variance and $^*P < 0.05$ *versus* control and 0.1 µM, with Student–Newman–Keuls *post hoc* test.

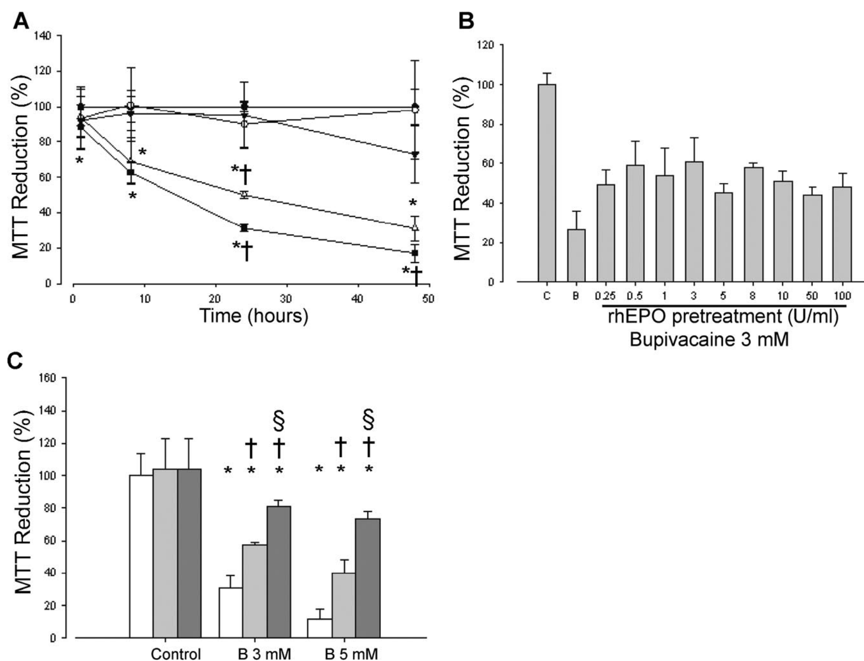

Fig. 5. Dimethyl thiazol diphenyl tetrazolium bromide (MTT) reduction is decreased by bupivacaine and was partially recovered by recombinant human erythropoietin (rhEPO) pretreatment and cotreatment. (*A*) Time course of bupivacaine-induced MTT reduction. MTT tests were performed, and different bupivacaine concentrations were evaluated (control [●], 0.001 mM bupivacaine [○], 1 mM [▼], 3 mM [Δ], 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.

extrusion of MTT,[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.[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.[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.[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.[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.[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-

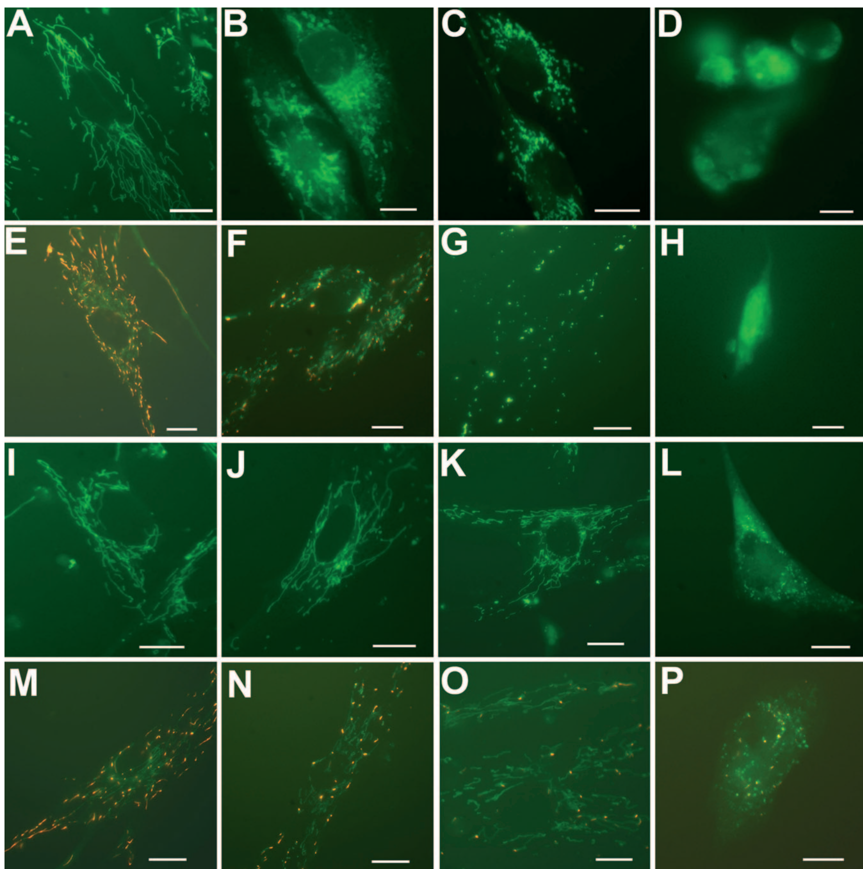

Fig. 6. Fission of mitochondrial reticulum coupled with changes in mitochondrial membrane potential in human skeletal muscle myoblasts. Human skeletal muscle myoblasts were grown with or without bupivacaine (24 h) and with or without recombinant human erythropoietin (1 U/ml, 8 h before bupivacaine treatment). 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.

ture (see model fig. 9). With regard to the mechanism, citrate synthase measurements indicated that rhEPO prevented the decrease in mitochondrial content, implying a role in mitochondrial biogenesis or degradation. rhEPO alone did not improve citrate synthase activity, indicating no activation of mitochondrial biogenesis. Western blot analysis of respiratory chain complexes (fig. 2) validated this finding. Therefore, rhEPO could prevent the degradation of mitochondria triggered by bupivacaine, perhaps explaining the recovery of both the ATP/O ratio and citrate synthase. This indicates that rhEPO could interact with mitochondrial degradation processes to prevent the decrease in citrate synthase activity. However, no effect of rhEPO was reported on regulation of the mitochondrial energy system and biogenesis, which involve mitogen-activated protein kinase signaling, the peroxisome proliferator–activated receptor γ coactivator-1α pathway, and various posttranslational modifications (*e.g.*, phosphorylation, nitrosylation) of complexes I and IV. Most studies of rhEPO and mitochondria have described activation of phosphoinositide-3 kinase/Akt and extracellular signal–regulated kinase 1/2 in the regulation of mitochondrial apoptotic processes.[40,41] Alternatively, our results suggest that rhEPO might prevent bupivacaine-induced degradation of mitochondria (*i.e.*, recovery of citrate synthase activity and absence of autophagosomes) through a possible interaction with the regulatory pathway of mitochondrial autophagy. Indeed, among the different kinases activated by rhEPO,[42] extracellular signal–regulated kinase/mitogen-activated protein kinase plays a role in autophagy.[43]

Our study is the first to show that rhEPO can protect against LA-induced myotoxicity. Interestingly, rhEPO is often used in the perioperative period for its hematopoietic effects.[44] In our study, pretreatment with 5,000 U/kg rhEPO partially prevented myotoxicity in both rats and cultured human myoblasts. The rhEPO doses used in these experiments were an order of magnitude greater than those typically used clinically to treat anemia.[45] Interestingly, rhEPO had no effect in our cell model on preventing bupivacaine-induced inhibition of endogenous cell respiration, suggesting a time-dependent protective effect. Likewise, higher doses at 8,000 U/kg were shown to be beneficial for neuroprotection in patients with progressive multiple sclerosis,[46] with no deleterious secondary effects being reported in that preliminary study. A recent review highlights that the cardioprotective effects of rhEPO are species specific and have not been seen in pig and sheep models, and that initial human trials have been disappointing, with definitive study results still pending.[42] Moreover, our findings suggest that the mitochondrial protective effects of rhEPO should be carefully evaluated in the general context of mitochondrial pathologic dysfunction.[47,48] rhEPO utilization at high doses or for long periods of time, however, requires rigorous and continuous evaluation during

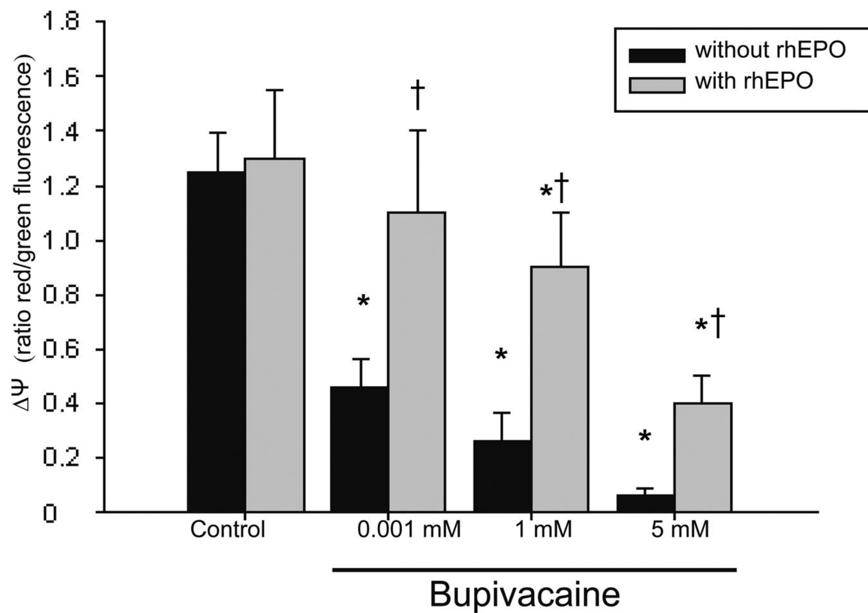

Fig. 7. Bupivacaine induced a loss of mitochondrial membrane potential, which was partially preserved by recombinant human erythropoietin (rhEPO). Human skeletal muscle myoblasts were grown in the presence or absence of the indicated concentration of bupivacaine (24 h, in absence of rhEPO, *black bars*) 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.

treatment to assess the risk of hypertension,[49] venous thromboembolism, and mortality, which are routinely described in the elderly and in patients with cancer.[50] 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.[45,51]

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.

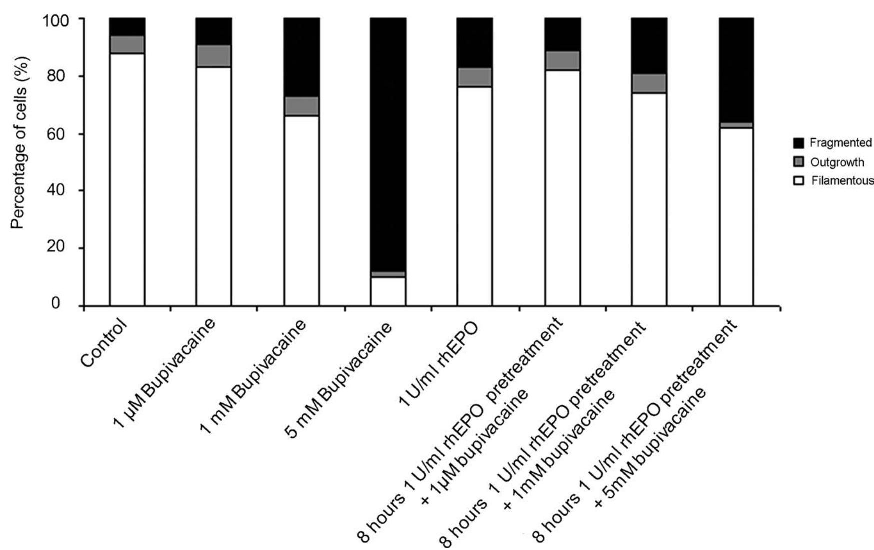

Fig. 8. Morphometric analysis of the mitochondrial network. Mitochondrial network morphology was analyzed by fluorescence microscopy of living cells during the exponential phase of growth. One hundred twenty cells taken from three independent experiments were analyzed randomly for each of the following 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.

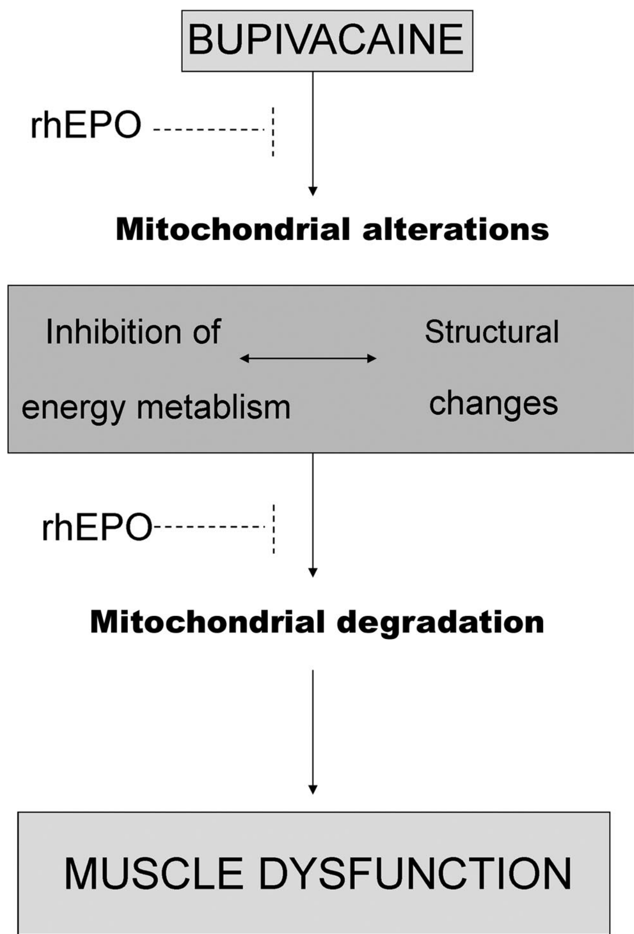

Fig. 9. Potential mechanism of recombinant human erythropoietin (rhEPO) cytoprotection in skeletal muscle. rhEPO protects against bupivacaine-induced myotoxicity and dimethyl thiazol diphenyl tetrazolium bromide reduction through a series of pathways that originate with changes in mitochondria. Bupivacaine induces metabolic and structural changes at the level of mitochondria. rhEPO maintains the integrity of the mitochondrial network and prevents autophagy and mitochondrial degradation through a number of pathways, including maintenance of the mitochondrial membrane potential, allowing oxidative phosphorylation. Bupivacaine-induced myotoxicity leads to muscle dysfunction, which could be prevented by rhEPO coadministration.

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