N-Acetylcysteine Protects against Bupivacaine-induced Myotoxicity Caused by Oxidative and Sarcoplasmic Reticulum Stress in Human Skeletal Myotubes

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ABSTRACT

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. Here, the authors investigate the mechanisms of local anesthetic-induced myotoxicity and assess the protective effect of N-acetylcysteine.

Methods: The authors used primary cell cultures of human skeletal muscle myoblasts to study local anesthetic adverse effects. Production of reactive oxygen species was investigated in human skeletal myotubes by fluorescence microscopy. Expression of sarcoplasmic/endoplasmic reticulum stress markers and induction of apoptosis were followed by immunofluorescence and Western blot analysis. Finally, the effect of N-acetylcysteine on bupivacaine-induced myotoxicity was investigated in vitro.

Results: Bupivacaine sequentially induced reactive oxygen species production, oxidative stress, sarcoplasmic/endoplasmic reticulum stress, and activation of caspases 9 and 7 in human differentiated myoblasts. These iatrogenic effects were prevented by N-acetylcysteine.

Conclusions: The authors demonstrated a protective effect of N-acetylcysteine against bupivacaine-induced sarcoplasmic/endoplasmic reticulum stress and apoptosis in primary human skeletal muscle cell.

Local anesthetic injections can produce myotoxicity although the clinical significance is not entirely clear.

Mechanisms for myotoxicity by local anesthetics are not understood.

In cultured human muscle cells, local anesthetics produced myotoxicity that was reduced by the antioxidant N-acetylcysteine.

In the future, perhaps N-acetylcysteine could be used to prevent local anesthetic induced myotoxicity when this is anticipated.

Background

Background: Local anesthetics offer the benefits of extended analgesia with greater patient satisfaction compared with intravenous morphine after orthopedic surgery.1–3 Challenges remain, however, for the use of LAs to improve the comfort and postoperative pain relief in patients who receive continuous regional blocks for surgical procedures. These benefits, however, can be offset by adverse iatrogenic muscle pain caused by bupivacaine.4,5 LA-induced myotoxicity seems to be a rather uncommon side effect of local and regional anesthesia. However, studies revealed that certain anesthesia techniques, especially retrobulbar and peribulbar blocks, are related to a relatively high postoperative rate of myopathies.
significant muscular dysfunction directly caused by these agents. Therefore, skeletal muscle damage has to be considered a potentially serious complication after LA application. The frequency of these symptoms is largely unknown because they remain underreported. Moreover, a better understanding of the mechanisms of LA-induced myotoxicity is needed to develop efficient clinical strategies to protect against LA adverse outcomes.

After LA administration, the affected muscle fibers undergo an intrinsic degenerative phase with increase in pycnotic myonuclei and pathologically condensed chromatin. However, the cellular mechanisms, particularly the apoptotic pathway(s) involved in LA myotoxicity, remain to be characterized (reviewed in1). Several studies indicate that in lidocaine-induced neurotoxicity the intrinsic mitochondrial death pathway, rather than the extrinsic death receptor pathway, might be implicated in the apoptotic response to LA administration.8,9 In addition, bupivacaine could interfere with the mitochondrial energy metabolism in skeletal muscle,10,11 leading to Ca2+ deregulation. It has been proposed that increased intracellular Ca2+ concentrations play a role in myocyte injury.12,13 Indeed, in skinned fiber preparations of skeletal muscle, LAs cause Ca2+ release from the sarcoplasmic reticulum (SR) and simultaneously inhibit Ca2+ reuptake,14–16 thus resulting in persistently increased Ca2+ levels. On the other hand, in psos muscle of rats anesthetized with bupivacaine and ropivacaine administered by catheter for femoral nerve block, a decrease in the mitochondrial energy metabolism was detected, but without changes in SR Ca2+ content.3 Interestingly, a microarray analysis revealed that, in HL-60 promyelocytic leukemia cells, bupivacaine treatment up-regulated the expression of several apoptosis-related genes and also of X-box-binding protein 1 (XBP-1).17 XBP-1 is a transcription factor that operates as a downstream component of the unfolded protein response against sarcoplasmic or endoplasmic reticulum (SR/ER) stress caused by accumulation of unfolded/misfolded proteins.18 XBP-1 also controls expression of C/EBP homologous protein (CHOP),19 a key signaling component of ER stress-induced apoptosis, which, therefore, could mediate bupivacaine-induced myotoxicity.

To better characterize the mechanism(s) of LA-induced myotoxicity and, particularly, the involvement of SR/ER stress, we investigated bupivacaine effects in human primary myotubes. We have identified the caspases activated during the apoptotic response to bupivacaine and demonstrated that bupivacaine-induced apoptosis is mediated by SR stress caused by increased reactive oxygen species (ROS) production. Finally, we report that N-acetylcysteine protects against these iatrogenic effects.

Materials and Methods

Reagents

Anti-tubulin monoclonal antibody was from Sigma-Aldrich (St. Quentin Fallavier, France). Anti-CHOP, anti-XBP-1, and anti-ATF-6 (activating transcription factor 6) polyclonal antibodies were from Abcam (Paris, France). Anti-CHOP monoclonal antibody, anti-PARP (poly(ADP-ribose)polymerase) and anti-cleaved-PARP, anti-caspases 9, 7, 6, and 3, and anti-cleaved caspases 9, 7, 6, and 3 polyclonal antibodies were from Cell Signaling Technology (Ozyme; Montigny, France). The Alexa Fluor secondary antibodies were purchased from Invitrogen (Cergy-Pontoise, Paris, France). Bupivacaine (1-butyl-N-(2,6-dimethylphenyl)-2-piperidinercarbamide), staurosporine, thapsigargin, and N-acetylcysteine were purchased from Sigma-Aldrich, and CM-H2DCFDA derivatives (5-(and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester) were from Invitrogen.

Primary Cell Cultures

Human primary myoblasts were isolated as described previously20 from quadriceps muscle biopsies obtained from the “AFM-BTR Banque de Tissus pour la Recherche ” (Hôpital de la Pitié-Salpêtrière, 75013 Paris). 30,000 myoblasts/ml were grown in growth medium (Dulbecco’s modified Eagle’s medium; Cambrex, Lonza, Verviers, Belgium) supplemented with 10% fetal calf serum (Perbio Science, Brebieres, France) and 1% Ultraisor G (PALL Life Sciences, Cergy St. Christophe, France) for 5 days. At day 5, myoblasts started to fuse, and cultures were switched to differentiation medium composed of Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and penicillin/streptomycin (Cambrex). Cultures of myotubes grown in differentiation medium were used for all experiments.

Immunoblotting and Immunofluorescence Analysis

Cell extracts prepared as described previously21 were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted onto nitrocellulose membranes, and probed with the indicated antibodies. Bound antibodies were detected with a chemiluminescence detection kit (PerkinElmer, Courtaboeuf, France). Analysis and quantification were performed with Kodak 1D3.6 software (Kodak, Carestream Health France, Bagnolet, France).

For immunofluorescence, cells were fixed in 4% paraformaldehyde in phosphate-buffered saline and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline. Cells were incubated with primary antibodies for 2 h, followed by Alexa Fluor secondary antibodies and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) staining. Cell imaging was performed at the “Centre Regional d’Imagerie Cellulaire de Montpellier.” Fluorescent cells were viewed with a Leica Microscope (Leica DM6000, Wetzlar, Germany) using a

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40×/NA = 1.30 EC Plan-Neofluar grade oil objective. Images were captured as 16 TIFF files with a MicroMax 1300 CCD cameras (RS-Princeton Instruments, Inc., Roper Scientific SAS, Evry, France) driven by the MetaMorph (version 7; Universal Imaging Corp., Roper Scientific SAS). Images were processed using ImageJ software (National Institutes of Health, Bethesda, MD; ImageJ is in the public domain).

**ROS Production and Oxidative Stress Analysis**

We used CM-H₂DCFDA as a cell-permeant indicator of ROS. Myotubes were cultured in 6-well plates, treated with 0.5, 1.0, and 1.5 mM bupivacaine for 8 h, washed, and incubated with 5 μM CM-H₂DCFDA and DAPI in phosphate-buffered saline at 37°C in the dark for 60 min. Cells were then washed with phosphate-buffered saline three times, fixed in 4% paraformaldehyde for 5 min, and stored at 4°C for up to 16 h before image acquisition. Cells were visualized with Zeiss (Carl Zeiss SAS; Le Pecq, France) fluorescent microscope (AxioCam MRm equipped with a charged-coupled device camera), using excitation sources and filters appropriate for fluorescein isothiocyanate as described by the manufacturer. Fluorescence quantification was performed with ImageJ using for each experimental condition. Ten images from at least three independent experiments (30 images, more than 300 myotubes analyzed in total/experimental condition) were then combined to allow quantitative analysis of changes in ROS levels. In all experiments, fluorescence levels were normalized by the number of nuclei.

The OxyBlot Protein Oxidation Detection kit was purchased from Millipore (Division Bioscience Millipore SAS, St. Quentin en Yvelines, France), and analysis of the oxidation status of protein samples (dinitrophenylhydrazine derivatization) was performed following the manufacturer’s instructions. One-dimensional electrophoresis was carried out using 12.5% sodium dodecyl sulfate-polyacrylamide gels after dinitrophenylhydrazine derivatization. Proteins were transferred onto nitrocellulose membranes and then stained with Red Ponceau to control for sample loading. After labeling with the rabbit anti-dinitrophenyl antibody included in the kit, blots were developed using a chemiluminescence detection system.

**Fig. 1.** Bupivacaine-induced apoptosis in human primary myotubes. (A) Human myotubes were treated with vehicle alone (Ctl) or with the indicated concentrations of bupivacaine or 1 μM staurosporine (S) as a positive control. After 18 h, myotubes were fixed and incubated with anticleaved poly(ADP-ribose)polymerase (PARP) antibodies, followed by fluorescently labeled secondary antibodies. DNA was stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) and F-actin with rhodamine-conjugated phalloidin. (B) After 18 h, 24 h, and 31 h, the percentage of nuclei positive for cleaved PARP was determined. Values are the mean ± SEM (n = 4); P < 0.05 with one-way analysis of variance and *P < 0.05 with post hoc unpaired t test versus control (Ctl) with Holm’s procedure. Scale bars = 10 μm.
Statistical Analysis

Distribution of the quantitative variables was fitted. Transformation to ensure normal distribution was performed when necessary. Means were compared between each experimental conditions by applying a one-way analysis of variance or a unpaired $t$ test when a priori comparisons were performed versus control. When analysis of variance was significant, post hoc unpaired $t$ test were applied, and the $P$ value was adjusted to control the false discovery rate according to the Holm’s procedure. All $P$ values were two-tailed and were considered significant at 0.05 or less. All data are presented as mean ± SEM, and the statistical analysis was performed using Stata Statistical Software version 6.0 (Stata Corporation, College Station, TX).

Results

Bupivacaine Induces Apoptosis of Human Skeletal Muscle Cells and the Cleavage of Caspases 9 and 7

The myotoxicity of the LA bupivacaine was investigated in primary cell cultures of human myoblasts that fused in vitro to form mature differentiated myotubes. Because the cleavage of PARP is an indicator of apoptosis, the presence of its cleaved form was determined by immunofluorescence in

![Fig. 2. Bupivacaine (Bupi)-induced caspase activation in human primary myotubes. (A) Human myotubes were treated with vehicle alone (Ctl) or with the indicated concentrations of bupivacaine or 1 $\mu$M staurosporine (S) as a positive control. After 18 h, myotubes were lysed and immunoblotted with the indicated antibodies. Analysis of tubulin expression served as loading control. (B) Human myotubes were treated with 1.5 $\mu$M bupivacaine, and caspase activation was analyzed at the indicated time points. (C) The histograms show the quantification of the expression level of the proteins obtained in experiments (A) and (B). Data are the mean ± SEM ($n = 4$); $P < 0.05$ with one-way analysis of variance and *$P < 0.05$ with post hoc unpaired $t$ test versus without bupivacaine for the same time with Holm’s procedure. PARP = poly(ADP-ribose)polymerase.](image)

![Fig. 3. Bupivacaine-induced C/EBP homologous protein (CHOP) expression in human myotubes. (A) Human myotubes were treated with vehicle alone (Ctl) or with the indicated concentrations of bupivacaine and 1 $\mu$M staurosporine (S) or 100 $nM$ thapsigargin (T), a known inducer of CHOP expression and sarco/endoplasmic reticulum stress. After 18 h, myotubes were fixed and incubated with anti-CHOP antibody as indicated, followed by fluorescently labeled secondary antibodies. DNA was stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). (B) The percentage of CHOP-positive nuclei was determined in fluorescent experiments. Values are the mean ± SEM ($n = 4$); $P < 0.05$ with one-way analysis of variance and *$P < 0.05$ with post hoc unpaired $t$ test versus control (Ctl) with Holm’s procedure; §$P < 0.05$ with unpaired $t$ test versus control (Ctl). Scale bars = 10 $\mu$m.](image)
myotubes incubated with different concentrations (from 0.75 to 1.5 mM) of bupivacaine and with 1 μM staurosporine, a known initiator of apoptosis in vitro, for 18 h. Cleaved PARP was clearly detected in myotubes treated with staurosporine and in cells incubated with 1.25 and 1.5 mM bupivacaine (fig. 1A). In addition, the number of PARP-positive nuclei in myotubes treated with 1.25 mM bupivacaine increased proportionally to the duration of the incubation (fig. 1B). Thus, induction of apoptosis by bupivacaine is dose- and time-dependent in primary human myotubes. We then assessed whether and which caspases were implicated in bupivacaine-induced apoptosis by analyzing the expression of their cleaved forms. Immunoblot analysis showed that cleaved PARP and cleaved caspase 9 and 7 were detected in human myotubes treated with 1.5–2 mM bupivacaine but not in cells treated with 1 mM bupivacaine (fig. 2A), consistent with the results described in figure 1. Similarly, expression of cleaved caspase 9 and 7 increased with longer incubation times (figs. 2B and C). Cleavage of caspase 3 and 6 was not induced by bupivacaine differently from staurosporine, which induced strong expression of all the cleaved caspases tested (fig. 2A). These results indicate that bupivacaine induces apoptosis in human myotubes by a mechanism probably different from that of staurosporine.

**Bupivacaine Induces Activation of the Unfolded Protein Response and CHOP Expression**

Because the activation of caspase 7 is involved in ER stress-mediated apoptosis, we next tested whether bupivacaine myotoxicity could be mediated via induction of SR/ER stress in human myotubes. To this aim, myotubes were incubated with different concentration (from 0.75 to 1.5 mM) of bupivacaine for 18 h and with 100 nM thapsigargin, an irreversible inhibitor of the ER Ca^{2+}-ATPase, for 4 h. Expression of CHOP, a key downstream transcription factor in SR stress-induced apoptosis, was stimulated by thapsigargin (fig. 3A). Bupivacaine (1–1.5 mM) also strongly up-regulated CHOP expression (fig. 3A), and this effect was dose-dependent (fig. 3B). Moreover, 1 mM bupivacaine induced CHOP expression (figs. 3A, 4A, and 4B) but not activation of

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**Fig. 4.** Bupivacaine-induced C/EBP homologous protein (CHOP) expression precedes caspase activation. (A) Human myotubes were treated with vehicle alone (Ctl) or with the indicated concentrations of bupivacaine, 1 μM staurosporine (S), or 100 nM thapsigargin (T). After 18 h, myotubes were lysed and immunoblotted using the indicated antibodies. (B) Histograms show the quantification of the experiments described in (A). Data are the mean ± SEM (n = 4); P < 0.05 with one-way analysis of variance and *P < 0.05 with post hoc unpaired t test versus without bupivacaine with Holm’s procedure. (C) Myotubes treated with 1 mM bupivacaine were lysed at different time points as indicated. Cell extracts were immunoblotted using the indicated antibodies. Analysis of tubulin expression served as loading control. The active and inactive forms of activating transcription factor 6 (ATF-6) are indicated, and * marks nonspecific bands. (D) Histograms show the quantification of the experiments described in (C). Data are the mean ± SEM (n = 4); P < 0.05 with one-way analysis of variance and *P < 0.05 with post hoc unpaired t test versus without bupivacaine for the same time with Holm’s procedure. cl-CSP9 = cleaved caspase 9; cl-CSP7 = cleaved caspase 7; PARP = poly(ADP-ribose)polymerase; XBP = X-box-binding protein 1.
caspase 9 and 7 (figs. 4A and B) as observed also with thapsigargin. Thus, induction of SR stress seems to precede activation of caspases. To test this hypothesis, the kinetics of the expression of XBP-1 and ATF-6 (two transcription factors that control expression of the unfolded protein response genes like CHOP), CHOP, and cleaved caspase 9 and 7 were determined in myotubes treated with 1 mM bupivacaine. ATF-6, XBP-1, and CHOP were detected after 8 h of bupivacaine treatment, and their expression was maintained, except for XBP-1, which was transient (figs. 4C and D). Cleaved caspases were detected only at 24 h (figs. 4C and D). Together, these results show that after exposure to bupivacaine, SR stress and expression of the unfolded protein response genes are induced before apoptosis, suggesting that the apoptotic events depend on SR stress induction.

Production of ROS and Oxidative Stress Are Increased in Human Myotubes Treated with Bupivacaine

ROS alter cellular redox reactions, interfere with protein disulfide bonding, and result in protein misfolding leading to SR stress and the unfolded protein response activation (reviewed in26). Therefore, primary myotubes were incubated with different concentrations (from 0.5 to 1.5 mM) of bupivacaine for 8 h, and ROS production was evaluated indirectly by measuring the oxidation status of CM-H$_2$DCFDA derivatives. Dichlorofluorescein oxidation was significantly increased in myotubes treated with 1 mM and 1.5 mM bupivacaine compared with control, untreated cells (fig. 5A). Moreover, ROS production in bupivacaine-treated myotubes was inhibited by the antioxidant drugs N-acetylcysteine, trolox (a water-soluble derivative of vitamin E), and resveratrol (antioxidant polyphenol) in a dose-dependent manner (fig. 5B and data not shown). To confirm that bupivacaine-induced ROS caused oxidative stress in myotubes, the content in carbonylated proteins of samples treated with 1.5 mM bupivacaine for 8 h was assessed. As shown in figure 5C, carbonylated proteins increased in response to bupivacaine treatment, and this effect was prevented by antioxidant treatment.

N-Acetylcysteine Protects against SR Stress and Apoptotic Events Induced by Bupivacaine

To test whether inhibition of ROS production could protect against bupivacaine-induced SR stress and apoptosis, primary myotubes were incubated with 1 or 1.5 mM bupivacaine and increasing concentrations of the antioxidant N-acetylcysteine for 18 h. Addition of N-acetylcysteine decreased CHOP expression and inhibited expression of activated caspases in myotubes (figs. 6A and B). CHOP expression was also monitored by immunofluorescence, and the percentage of nuclei expressing CHOP decreased from 48% in bupivacaine-treated myotubes to 26%, 16%, and 9% in cells coinubcated with 2, 5, and 10 mM N-acetylcysteine, respectively (fig. 6C).

These results show that, in primary human myotubes, blocking ROS production with the antioxidant N-acetylcysteine has a protective effect against bupivacaine-induced myotoxicity. These findings could have an important clinical impact, which will have to be evaluated in practice.

Discussion

A variety of experimental findings indicate that LA myotoxicity has a complex pathogenesis. The experiments described indicate that treatment of human primary myotubes with bupivacaine induces ROS production and SR stress leading to apoptosis characterized by expression of cleaved caspase 9 and 7. Coincubation of myotubes with bupivacaine and antioxidant drugs, especially N-acetylcysteine, inhibits ROS production and SR stress and expression of the unfolded protein response genes are induced before apoptosis, suggesting that the apoptotic events depend on SR stress induction.

Fig. 5. Antioxidants protect against bupivacaine-induced oxidative stress. (A) Reactive oxygen species (ROS) production was assessed by oxidation of CM-H$_2$DCFDA (5- and 6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester) derivatives in myotubes treated, or not (Ctl), with the indicated concentrations of bupivacaine for 8 h. Values are the mean ± SEM (n = 4); *P < 0.01 with one-way analysis of variance and *P < 0.05 with post hoc unpaired t test versus control (Ctl) with Holm’s procedure. (B) ROS production was measured in myotubes incubated with the indicated concentrations of bupivacaine, N-acetylcysteine (NAC) for 8 h. Values are the mean ± SEM (n = 4). NAC effect is analyzed for the indicated concentrations of bupivacaine, P < 0.01 with one-way analysis of variance; *P < 0.05 with post hoc unpaired t test versus control (Ctl) with Holm’s procedure; ##P < 0.05 with post hoc unpaired t test versus without NAC (baseline value) for the same bupivacaine concentration, with Holm’s procedure. (C) Oxyblot analysis of carbonylated proteins from myotubes treated with vehicle (Ctl, lane 1) or with 1.5 mM of bupivacaine (lanes 2, 3, and 4) and the indicated concentrations of NAC (lanes 3 and 4) for 8 h. Histograms show the quantification of carbonylated proteins. Data are the mean ± SEM (n = 4); *P < 0.05 with unpaired t test versus control (Ctl, lane 1); #P < 0.01 with one-way analysis of variance and ##P < 0.05 with post hoc unpaired t test versus without NAC (baseline value) for the same bupivacaine concentration, with Holm’s procedure.

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Fig. 6. *N*-Acetylcysteine (NAC) protects against the iatrogenic effects of bupivacaine in human myotubes. (A) Human myotubes were treated with vehicle alone (Ctl) or with the indicated concentrations of bupivacaine, 1 μM staurosporine (S), 100 nM thapsigargin (T), and NAC. After 18 h, myotubes were lysed and immunoblotted using the indicated antibodies. (B) Histograms show the quantification of the experiments described in (A). Data are the mean ± SEM (n = 4), for C/EBP homologous protein (CHOP) expression analysis; for 1 and 1.5 mM bupivacaine, *P < 0.01 with unpaired t test versus control (without bupivacaine and NAC); P < 0.01 with one-way analysis of variance of NAC effect with 1 and 1.5 mM bupivacaine and #P < 0.05 with post hoc unpaired t test versus without NAC (baseline value) for the same bupivacaine concentration, with Holm’s procedure. For cleaved caspase 9 (cl-CSP9) and cleaved caspase 7 (cl-CSP7) expression analysis, for 1.5 mM bupivacaine, *P < 0.01 with unpaired t test versus control (without bupivacaine and NAC); P < 0.01 with one-way analysis of variance of NAC effect with 1.5 mM bupivacaine and #P < 0.05 with post hoc unpaired t test versus without NAC (baseline value) for the same bupivacaine concentration, with Holm’s procedure. (C) Human myotubes were incubated with 1 mM bupivacaine and without or with the indicated concentrations of NAC. After 18 h, myotubes were fixed and incubated with anti-CHOP antibody, followed by fluorescently labeled secondary antibodies. DNA was stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) and F-actin with rhodamine-conjugated phalloidin, respectively. The percentage of CHOP-positive nuclei was determined in four independent experiments. Values are the mean ± SEM (n = 4); *P < 0.05 with unpaired t test versus control (Ctl); P < 0.05 with one-way analysis of variance of NAC effect and #P < 0.05 with post hoc unpaired t test versus without NAC (baseline value) for the same bupivacaine concentration, with Holm’s procedure.
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Bupivacaine increases the production of reactive oxygen species (ROS) which induce sarcoplasmic reticulum (SR) stress and the unfolded protein response and may cause SR calcium depletion. Bupivacaine sequentially induces ROS production and SR stress leading to apoptosis mediated by the mitochondrial death pathway. Blocking ROS production, the antioxidant NAC protects against bupivacaine-induced myotoxicity. ATF-6 = activating transcription factor 6; CHOP = C/EBP homologous protein; RyR = channel ryanodine receptors; SERCA = sarcoplasmic reticulum Ca^{2+}-ATPase; XBP-1 = X-box-binding protein 1.

Fig. 7. Potential mechanism of bupivacaine-induced myotoxicity and N-acetylcysteine (NAC) cytoprotection in skeletal muscle. Bupivacaine increases the production of reactive oxygen species (ROS) which induce sarcoplasmic reticulum (SR) stress and the unfolded protein response and may cause SR calcium depletion. Bupivacaine sequentially induces ROS production and SR stress leading to apoptosis mediated by the mitochondrial death pathway. Blocking ROS production, the antioxidant NAC protects against bupivacaine-induced myotoxicity. ATF-6 = activating transcription factor 6; CHOP = C/EBP homologous protein; RyR = channel ryanodine receptors; SERCA = sarcoplasmic reticulum Ca^{2+}-ATPase; XBP-1 = X-box-binding protein 1.

production and SR stress and consequently caspase activation (fig. 7).

Skeletal muscle fiber degeneration induced by bupivacaine has been confirmed in multiple studies, but the pathway of cell death remained to be clarified.\(^7\) In neuroblastoma cells, the LA lidocaine induces a cell death that is prevented by the lack of caspase 9,\(^9\) indicating an apoptosis mediated by the intrinsic mitochondrial death pathway. Consistent with these observations, we detected the cleaved form of PARP, which is an indicator of apoptosis, and expression of activated caspase 9 and 7 in human myotubes treated with bupivacaine, suggesting the involvement of the intrinsic pathway. LA also can decrease the mitochondrial membrane potential,\(^10,11\) leading to progressive fragmentation of the mitochondrial network in human myoblasts.\(^27\) However, mitochondria are unlikely to be the only targets of LAs. The increase of the intracellular Ca^{2+} concentrations by LAs could play a role in myocyte injury by inducing pathways of cell death.\(^12,13\) In isolated myofibers, bupivacaine induces Ca^{2+} release from the SR by acting on the Ca^{2+} release channel ryanodine receptors at SR membranes and inhibits Ca^{2+} reuptake into the SR, which is mainly regulated by SR Ca^{2+}-ATPase activity.\(^16,28\) Bupivacaine could inhibit these receptors by direct binding, but this has never been demonstrated. Therefore, and based on the results of this work, we propose an alternative mechanism linked to induction of oxidative stress. In human myotubes, bupivacaine treatment increases production of ROS, which may then modify oxidizable residues (cysteine, tyrosine) of SR-associated calcium channels, such as ryanodine receptors (S-nitrosylation) and SR/ER Ca^{2+}-ATPases (by tyrosine nitration), causing their dysfunction and SR calcium depletion.\(^29–31\) However, disturbance of intracellular Ca^{2+} homeostasis should not be considered the only mechanism by which bupivacaine induces apoptosis in muscle. Indeed, \textit{in vivo}, bupivacaine injected \textit{via} femoral nerve block catheters has deleterious effects on mitochondrial energy without affecting calcium homeostasis,\(^5\) and in our experiments, bupivacaine induced apoptosis at concentrations that do not influence Ca^{2+} release from SR. Another mechanism, associated with SR/ER stress, should therefore be considered. Here, we show that, in myotubes, bupivacaine induces activation of caspase 7, which is involved in ER stress-mediated apoptosis\(^22\) as well as expression of the ER stress markers XBP-1, ATF-6, and CHOP.

Oxidative stress can cause SR/ER stress. Production of ROS, which alter cellular redox reactions, and production of nitric oxide, a mediator of protein nitrosylation, interfere with protein disulfide bonding and result in protein misfolding.\(^26\) The excessive presence of misfolded proteins disrupts SR/ER function, resulting in accumulation of unfolded or misfolded proteins in the reticulum lumen, which leads to SR/ER stress and the unfolded protein response (reviewed in\(^19\)). In bupivacaine-treated myotubes, addition of the antioxidant N-acetylcysteine protects against ROS production, as expected, but also inhibits its expression of CHOP and of activated caspases. These results strongly suggest that bupivacaine causes muscle injury by increasing ROS production, which leads to SR/ER stress and finally to apoptosis. The implication of ROS production in bupivacaine-mediated toxicity was also shown in a Schwann cell line,\(^32\) but how bupivacaine induces ROS production remains a major question for future studies.

Furthermore, the dose of 1–2 mM bupivacaine used for the experiments described in this paper was chosen to mimic the effects of direct exposure of myotubes to this agent at clinically relevant concentrations (7.7 mM = 0.25% bupivacaine solution used in peripheral nerve blocks). These concentrations are in the same range as those that altered mitochondrial energy metabolism in human myoblasts (1–5 mM bupivacaine)\(^27\) and induced apoptosis in a Schwann cell line (0.5 mM bupivacaine).\(^32\) \textit{In vivo}, lipophilic LAs accumulate in tissues, but the real concentrations in the cells remain unknown. Bupivacaine concentration in the muscle 1 h after the last injection of a clinically relevant protocol performed in rats was approximately 30 nmol/mg of tissue or 30 μM.\(^5\) Nevertheless, this concentration is underestimated because the au-
thors could not measure precisely the local concentration of bupivacaine in the strict diffusion space within the muscle because of the limited sensitivity of the high-performance liquid chromatography system that would have required a larger biopsy. Thus, 1 mM bupivacaine is probably not so far away from real concentrations in the vicinity of the nerve catheter. Anyhow, in clinical practice, the relevance of concentration depends mainly on the dose effect, which play a key role in bupivacaine toxicity. This suggests that the current clinical protocols for LA administration should be reevaluated and adapted to use dose close to the minimal LA concentration needed.

The observation that an antioxidant protects against LA-induced myotoxicity constitutes an important result for clinical practice. Recently, we showed that recombinant human erythropoietin (hrEPO), often used for its hematopoietic effects, can partially protect against LA-induced myotoxicity in rats and cultured human myoblasts.27 Nevertheless, the recombinant human erythropoietin doses used in these experiments were much higher than those typically used clinically to treat anemia.33 Therefore, the risk of side effects combined with its extremely high cost does not make of recombinant human erythropoietin an ideal treatment against LA-induced myotoxicity. Conversely, N-acetylcysteine might be an interesting alternative, although the clinical impact of our results remains to be evaluated in practice.

References


ANESTHESIOLOGY REFLECTIONS

A “French” Charrière Ether Inhaler

When surgeons request 36-French (12 mm-diameter) chest tubes, the term “French” honors the originator of that sizing system, Joseph-Frédéric-Benoît Charrière, a Parisian manufacturer of surgical instruments. By 1847, Charrière’s firm had generated brass-and-glass inhalers for etherizing and later chloroforming patients. Sharing the glass-and-brass design flaws of the original 1846 Morton ether inhaler, Charrière inhalers’ volatile contents chilled progressively, leading to inefficient vaporizing and patients’ early emergence from anesthesia. Curatorial donations of travel costs and of the inhaler itself (see above) expedited hand-carrying this “French” Charrière Ether Inhaler aboard jets from Montpelier to Chicago via London. (Copyright © the American Society of Anesthesiologists, Inc. This image appears in color in the Anesthesiology Reflections online collection available at www.anesthesiology.org.)

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