Impaired Autophagosome Clearance Contributes to Local Anesthetic Bupivacaine-induced Myotoxicity in Mouse Myoblasts

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ABSTRACT

Background: The current study examined the role(s) of autophagy in myotoxicity induced by bupivacaine in mouse myoblast C2c12 cells.

Methods: C2c12 cells were treated with bupivacaine. Myotoxicity was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (n = 3 to 30), live/dead assay (n = 3 to 4), and morphological alterations (n = 3). Autophagosome formation was reflected by microtubule-associated protein light chain 3 conversion (n = 4 to 12) and light chain 3 punctation (n = 4 to 5). Autophagosome clearance was evaluated by p62 protein level (n = 4) and autolysosomes generation (n = 3).

Results: Bupivacaine induced significant cell damage. Notably, there was a significant increase in autophagosome generation as evidenced by light chain 3 puncta formation (72.7 ± 6.9 *vs.* 2.1 ± 1.2) and light chain 3 conversion (2.16 ± 0.15 *vs.* 0.33 ± 0.04) in bupivacaine-treated cells. Bupivacaine inactivated the protein kinase B/mammalian target of rapamycin/p70 ribosomal protein S6 kinase signaling. However, cellular levels of p62 protein were significantly increased upon bupivacaine treatment (1.29 ± 0.15 *vs.* 1.00 ± 0.15), suggesting that the drug impaired autophagosome clearance. Further examination revealed that bupivacaine interrupted autophagosome–lysosome fusion (10.87% ± 1.48% *vs.* 32.94% ± 4.22%). Administration of rapamycin increased autophagosome clearance and, most importantly, improved the survival in bupivacaine-treated cells. However, knockdown of autophagy-related protein 5 (atg5) exacerbated bupivacaine-induced impairment of autophagosome clearance and myotoxicity.

Conclusions: The data suggest that autophagosome formation was induced as a stress response mechanism after bupivacaine challenge; however, autophagosome clearance was impaired due to inadequate autophagosome–lysosome fusion. Therefore, impairment of autophagosome clearance appears to be a novel mechanism underlying bupivacaine-induced myotoxicity.
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LOCAL anesthetics (LAs) are widely used during regional blocks for improving postoperative analgesia and postoperative rehabilitation in children and adult patients.[1,2] However, LA solution during continuous blocks comes into contact with muscles and can provoke muscle injury.[2] Some of the patients who receive LA develop postoperative iatrogenic muscle pain, dysfunction, and degeneration due to myotoxic effects.[1–5] Although the potential myotoxicity of LA has been investigated for more than 50 yr,[3–6] the mechanisms by which they induce muscle cell injury are not fully understood. Therefore, it is necessary to identify these mechanisms if we are to develop effective clinical strategies for preventing adverse outcomes after the administration of LA.

Autophagy is an evolutionarily conserved process by which cytoplasmic materials, including damaged proteins and organelles, are sequestered for lysosome-dependent

What We Already Know about This Topic

- Some patients who receive local anesthetic solution during continuous blocks develop muscle pain, dysfunction, and degeneration due to myotoxic effects
- Autophagy maintains cellular homeostasis by sequestering damaged proteins and organelles in autophagosomes for lysosome-dependent degradation after autophagosome–lysosome fusion
- Autophagy may be involved in local anesthetic–induced myotoxicity

What This Article Tells Us That Is New

- Bupivacaine stimulates autophagosome formation as a stress response and impairs autophagosome clearance by interfering with autophagosome–lysosome fusion, resulting in muscle cell injury
- Rapamycin attenuates bupivacaine myotoxicity by increasing both autophagosome formation and autophagosome–lysosome fusion

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degradation.[7,8] Thus, the autophagy machinery is responsible for the maintenance of cellular homeostasis. Three types of autophagy occur in mammalian cells: macroautophagy, microautophagy, and chaperone-mediated autophagy.[7] Macroautophagy (referred to as autophagy hereafter) is a dynamic process comprising two consecutive stages. The first step is autophagosome formation, which begins with the formation of isolation membranes that engulf substrates. The second step is autophagosome clearance, which involves autolysosome formation *via* autophagosome–lysosome fusion, followed by degradation of the inner membrane together with its luminal contents.[7,9] Evidence suggests that inhibition of autophagy flux may either result in cell death directly or sensitize cells to stimuli-induced damage.[7,10–12]

Intriguingly, recent evidence suggests a possible involvement of autophagy in LA-induced cytotoxicity. Morissette *et al.*[13] reported that smooth muscle cell death induced by two LA, bupivacaine and lidocaine, was accompanied by increases in microtubule-associated protein light chain 3 (LC3)-II levels and LC3 puncta. In another study, Peropadre *et al.*[14] showed that autophagosomes accumulated in vero cells treated with dibucaine. These findings suggest that LA can increase the number of autophagosomes within cells. However, the following questions arise and have not been answered yet. First, is the LA-induced increase in the number of autophagosomes due to increased autophagosome formation (activation of early phase of autophagy) or due to impaired autophagosome clearance (suppression of late phase of autophagy)? Second, how does LA affect autophagy? Finally and most importantly, what role does altered autophagy play in LA-induced cytotoxicity?

To address these questions, we treated mouse muscle myoblast C2c12 cells with bupivacaine and examined its effect on autophagy. Our results suggest that impairment of autophagosome clearance is a novel mechanism underlying bupivacaine-induced myotoxicity.

Materials and Methods

Reagents

Bupivacaine, procaine, chloroquine (CQ), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, and primary antibody for α-tubulin were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's Modified Eagle Medium, Hoechst 33342, the Live/Dead Viability/Cytotoxicity Kit, LysoSensor Yellow/Blue DND-160, LysoTracker Red DND-99, and Lipofectamine 2000 reagent were from Invitrogen (Carlsbad, CA). The Magic Red Cathepsin Assay Kit was a product of ImmunoChemistry (Bloomington, MN). Protease inhibitor cocktail and X-tremeGENE HP DNA Transfection Reagent were from Roche (Mannheim, Germany). Bicinchoninic acid protein assay kit and the Supersignal West Pico Chemiluminescent Substrate were from Thermo Scientific (Rockford, IL). The primary antibodies for LC3-I/II, p62 protein, protein kinase B (Akt), phosphor-Akt, p70 ribosomal protein S6 kinase (p70S6K), phosphor-p70S6K, mammalian target of rapamycin (mTOR), phosphor-mTOR, and lysosomal-associated membrane protein 1 were from Cell Signaling Technology (Beverly, MA). Rapamycin was from LC Laboratories (Woburn, MA), and bafilomycin A1 was from Calbiochem (San Diego, CA). The plasmid expressed rat LC3 fused to enhanced green fluorescent protein (pEGFP-LC3) was provided by Addgene (Cambridge, MA).[15]

Cell Culture and Treatment

Mouse muscle myoblast C2c12 cells were obtained from the American Type Culture Collection and maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum. To induce myotoxicity, C2c12 cells were challenged with bupivacaine at the indicated concentrations for the indicated times. For the experiments involving rapamycin, cells were pretreated with rapamycin (500 nM) 30 min before bupivacaine exposure. All the experiments were grouped randomly.

Examination of Cell Injury

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide Assay. After stimulation with bupivacaine at the indicated concentrations for the indicated times, cell viability was determined in a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described previously.[16]

Morphology. After stimulation with bupivacaine (600 μM) for 24 h, cell morphology was examined under a phase-contrast light microscope (Zeiss Ltd., Oberkochen, Germany).

Live/Dead Assay. Thirty-six hours after bupivacaine (600 μM) stimulation, cell death was analyzed using a Live/Dead Viability/Cytotoxicity Kit according to the manufacturer's instructions, as previously described.[17] The Live/Dead Viability/Cytotoxicity Assay provides two-color fluorescence based on the number of living (green) and dead (red) cells. Intracellular esterase activity is detected by calcein AM, and plasma membrane integrity is detected by an ethidium homodimer. In brief, cells were stained for 30 min with 4 μM of ethidium homodimer and 2 μM of calcein AM. Staining was then examined under a fluorescence microscope (Zeiss Ltd.). More than three images per culture well were taken at random. Cell death was expressed as a percentage of dead cells to total cells.

Image taking and cell scoring were conducted by an investigator blinded to the treatment of experiments.

Examination of EGFP-LC3 Punctation

C2c12 cells were grown on cover slips and then transiently transfected with the pEGFP-LC3 using the X-tremeGENE HP DNA Transfection Reagent according to the manufacturer's instructions. Twenty-four hours later, the cells were stimulated with bupivacaine (600 μM) for 6 h and then fixed with 4% formaldehyde for 20 min. EGFP-LC3 puncta were observed under a confocal microscope (Zeiss Ltd.). The number of LC3 puncta per cell was counted in more than 50 randomly selected cells based on one batch of four to five independent experiments.[7]

Evaluation of Lysosomal pH Changes

Changes in lysosomal pH were evaluated using the LysoSensor Yellow/Blue DND-160 reagents. These reagents exhibit a pH-dependent increase in fluorescence intensity upon acidification.[7,18] In brief, C2c12 cells were grown on cover slips in 24-well plates. After stimulation with bupivacaine (600 μM) or CQ (10 μM) for 6 h, the cells were incubated with 5 μM of the LysoSensor reagents for 1 h. Fluorescence intensity was examined under a confocal microscope (Zeiss Ltd.).

Examination of Autophagosome–Lysosome Fusion

C2c12 cells grown on cover slips were transiently transfected with the pEGFP-LC3. Twenty-four hours later, cells were stimulated with bupivacaine (600 μM) for 6 h and then incubated with LysoTracker Red DND-99 (75 nM) for 30 min to visualize lysosomes as previously described.[19–22] The colocalization of EGFP-LC3 puncta and LysoTracker was examined under a confocal microscope (Zeiss Ltd.). The rate of autophagosome–lysosome fusion was expressed as the percentage of autolysosomes number over autophagosome number.

Cathepsin B Activity Assay

Cathepsin B activity was analyzed using a Magic Red Cathepsin B assay kit according to the manufacturer's instructions. In brief, C2c12 cells were exposed to bupivacaine for 6 h and then incubated with Magic red staining solution for 45 min in the dark. The fluorescence intensity of the cells was then measured in a fluorometer (BIO-TEK, Winooski, VT) at an excitation/emission wavelength of 590/645 nm. The images were observed and captured under a confocal microscope (Zeiss Ltd.).

Western Blot Analysis

Western blotting was performed as described previously.[16] In brief, C2c12 cells were collected at 6 h after bupivacaine treatment. Cytosolic fractions were prepared, and equal amounts of protein extract were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto Immobilon-P membranes (Millipore Corp., Bedford, MA). After blocking with 5% fat-free milk, the membrane was incubated with the appropriate primary antibody overnight at 4°C, followed by the appropriate secondary antibody. The same membranes were also probed with anti-α-tubulin as a loading control. Signals were detected with an enhanced chemiluminescence kit and quantified by scanning densitometry.

Knockdown of Autophagy-related Protein 5 (atg5) by Small-interfering RNA (siRNA)

C2c12 cells were transfected with atg5 siRNA with Lipofectamine 2000 reagent. Cells transfected with scrambled RNA served as negative controls. The atg5 knockdown

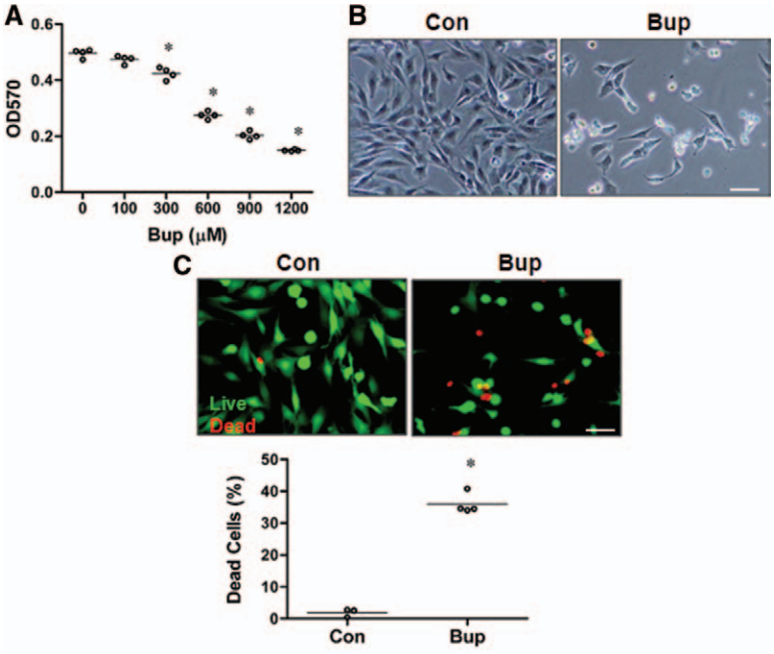

Fig. 1. Bupivacaine provoked myotoxicity. (*A*) Cell survival. C2c12 cells were treated with bupivacaine at the indicated concentrations for 24 h. Cell viability was examined in a 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide assay. **P* < 0.01 *versus* untreated controls (0 μM). n = 4 per group. (*B*) Morphological changes. C2c12 cells were treated with bupivacaine (600 μM) for 24 h. Cell morphology was observed under a phase-contrast microscope. Representative images from three independent experiments are shown. *Scale bar* = 100 μm. (*C*) Live/dead assay. C2c12 cells were treated with bupivacaine (600 μM) for 36 h. Cell survival and cell death were estimated using the live/dead assay, and the cells were observed under a fluorescence microscope. Living and dead cells are indicated by *green and red fluorescence*, respectively. *Scale bar* = 100 μm. **P* < 0.01 *versus* Con group. n = 3–4 per group. Bup = bupivacaine; Con = control.

efficiency was evaluated by real-time quantitative polymerase chain reaction and immunoblotting analysis, respectively, at 24 and 48 h after transfection. For bupivacaine or procaine stimulation, the LAs were administrated to the cells 48 h after siRNA transfection.

Statistical Analysis

There was not *a priori* power analysis conducted before initiating the study. The sample size used in the study was based on the previous similar works by ours and others.[16,23,24] There were no missing data in this study. Data are presented as mean ± SD. Groups were compared using Student two-tailed unpaired *t* test or one-way ANOVA followed by Tukey *post hoc* test, as appropriate with SPSS 13.0 software (SPSS Inc., Chicago, IL). Statistical significance was set at *P* value less than 0.05.

Results

Bupivacaine Provokes Myotoxicity in C2c12 Cells

We first examined the effects of treating cells for 24 h with different doses of bupivacaine with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The doses of bupivacaine for treatment were chosen according to previous studies.[1,6,25] As shown in figure 1A, 100 μM of bupivacaine had no significant effect on cell survival. However, doses of 300, 600, 900, and 1,200 μM led to significant reductions in cell viability by 16.0, 49.0, 65.0, and 76.8%, respectively, compared with untreated controls ($P < 0.01$). Based on these results, 600 μM of bupivacaine was used for all subsequent experiments unless stated otherwise.

Figure 1B shows representative images of morphological changes induced by bupivacaine. Compared with the untreated controls, cells treated with bupivacaine were round and shrunken, and most cells lost their cellular integrity.

Cell survival and cell death were further evaluated in a live/dead assay. As shown in figure 1C, the number of dead cells in bupivacaine-treated cultures was 18-fold higher than that in untreated cultures ($P < 0.0001$).

Autophagosomes Accumulate in Bupivacaine-treated Cells

Autophagy plays an important role in cell survival and cell death under different pathological conditions.[26,27] To determine whether autophagy was involved in bupivacaine-induced myotoxicity, we examined EGFP-LC3 punctation (a well-known marker of autophagosomes)[7] in C2c12 cells. As shown in figure 2A, the number of EGFP-LC3 puncta in bupivacaine-treated cells was significantly higher by 34-fold

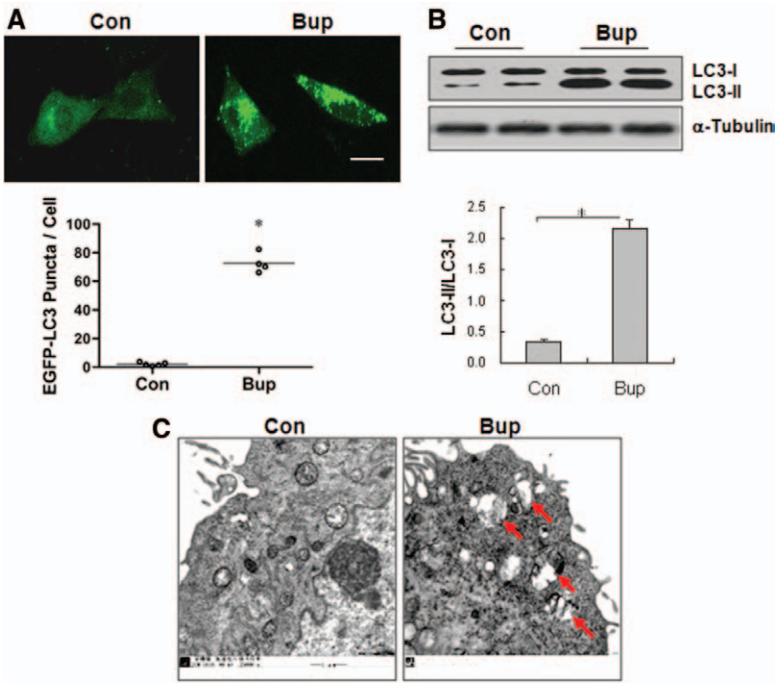

Fig. 2. Bupivacaine increased autophagic markers. (*A*) GFP-LC3 punctation. C2c12 cells transfected with the pEGFP-LC3 were exposed to bupivacaine ([Bup] 600 μM) for 6 h. Untreated cells served as controls (Con). LC3 punctuation was observed under a confocal microscope. Representative images from four to five independent experiments are shown. *Scale bar* = 20 μm. **P* < 0.01 *versus* Con group. n= 4–5 per group. (*B*) LC3 conversion in C2c12 cells. C2c12 cells were exposed to Bup (600 μM) for 6 h. Cells were collected and cellular extracts were prepared for Western blot with anti-LC3-I/II antibodies. The same membrane was blotted with an α-tubulin antibody as a loading control. **P* < 0.01. n = 12 per group. (*C*) Autophagosomes. C2c12 cells were exposed to Bup (600 μM) for 6 h. Ultrathin sections of cells were prepared for electron microscopy. The autophagic vacuoles are indicated by *arrows*. Images from three independent experiments are shown. *Scale bar* = 1 μm. GFP = green fluorescent protein; LC3 = microtubule-associated protein light chain 3.

than that in untreated controls ($P < 0.0001$). We next performed Western blot analysis to examine the changes in LC3 conversion, a marker for autophagosome formation according to the guidelines for monitoring autophagy and other studies.[27] The ratio of LC3-II/LC3-I was significantly higher by 5.5-fold in bupivacaine-treated cells than that in untreated controls ($P < 0.0001$) (fig. 2B).

The autophagosomes were then examined under an electron microscope. As shown in figure 2C, autophagosomes accumulated in bupivacaine-treated cells but not in untreated controls. Autophagosome frequency was significantly increased by 5.6-fold in bupivacaine group compared with control group ($P < 0.0001$).

Bupivacaine Suppresses Akt/mTOR/p70S6K Signaling and Increases p62 Protein Levels in C2c12 Cells

The bupivacaine-induced accumulation of autophagosomes could be due to either an increase in autophagosome generation (activation of early autophagy) or blockade of autolysosome clearance (inhibition of late autophagy). Thus, we next asked whether bupivacaine increases autophagosome generation by examining the activation of Akt/mTOR/p70S6K signaling because this signaling pathway plays an important role in negatively regulating autophagy initiation.[26,28] Figure 3A shows that bupivacaine treatment led to a significant reduction in the phosphorylation of Akt by 46.9%, p70S6K by 36.4%, and mTOR by 46.6%, respectively, compared with that in untreated controls ($P < 0.01$).

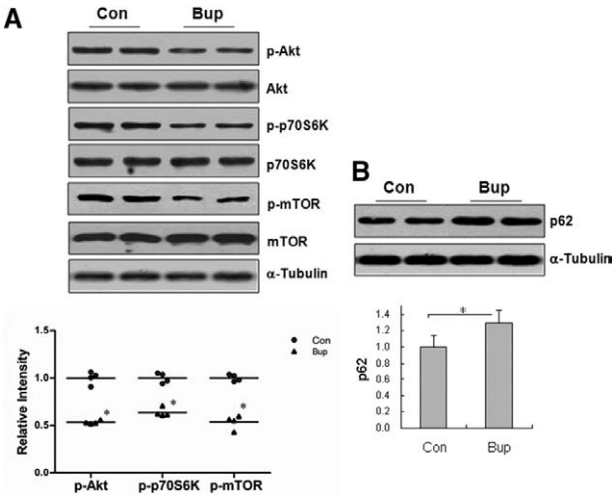

Fig. 3. Bupivacaine inhibited the phosphorylation of protein kinase B (Akt), mammalian target of rapamycin (mTOR), and p70 ribosomal protein S6 kinase (p70S6K) and increased the level of p62 protein in C2c12 cells. C2c12 cells were treated with bupivacaine ([Bup] 600 μM) for 6 h. Untreated cells served as controls (Con). Cellular extracts were prepared for Western blot with antibodies against phosphor-Akt (p-Akt) and Akt, phosphor-mTOR (p-mTOR) and mTOR, phosphor-p70S6K (p-p70S6K) and p70S6K (*A*, n = 4 per group), and p62 (*B*, n = 10 per group). The same membrane was blotted with an α-tubulin antibody as a loading control. **P* < 0.01 *versus* the respective Con group.

These data suggest that autophagosome formation is activated in bupivacaine-treated cells.

We next asked whether autophagosome clearance was impaired in bupivacaine-treated cells by examining p62 protein levels because p62 is selectively incorporated into autophagosomes through direct binding to LC3 and is efficiently degraded by autophagy.[7,12,26] Figure 3B shows that bupivacaine treatment significantly increased p62 protein levels in C2c12 cells by 29.1% compared with that in the untreated controls ($P = 0.0018$). These results suggest that bupivacaine impairs the clearance of autophagosomes.

Bupivacaine Increases Both Lysosomal Abundance and Cathepsin B Activity

We next asked how bupivacaine impairs autophagosome clearance. Because lysosomal pH is a critical factor that determines lysosomal activity, we first measured lysosomal pH using LysoSensor fluorescence.[7] Cells treated with CQ served as positive controls for the neutralization of lysosomal pH. As shown in figure 4A, the fluorescence was not changed by bupivacaine but was effectively quenched by CQ in comparison with the untreated controls. These results indicate that lysosomal pH was neutralized by CQ but not affected by 600 μm of bupivacaine.

Cathepsin B is an important proteinase contained within lysosomes. Unexpectedly, cathepsin B activity was 23.7% higher in bupivacaine-treated cells than that in untreated controls ($P = 0.0148$) (fig. 4B).

Lysosomal abundance is critical for autophagosome clearance. Therefore, we next evaluated lysosomal abundance by examining lysosomal-associated membrane protein 1 levels and LysoTracker fluorescence intensity according to previous studies.[19] Surprisingly, we found that lysosomal-associated membrane protein 1 levels were significantly higher by 36.4% after bupivacaine challenge (fig. 4C) ($P < 0.0001$). Consistent with this, LysoTracker fluorescence intensity was 15.2% greater in bupivacaine-treated cells than that in control cells ($P < 0.0001$) (fig. 4D).

Collectively, the results suggest that lysosomal abundance and activity are not impaired after bupivacaine treatment. Therefore, bupivacaine-induced impairment of autophagosome clearance is not likely contributed by dysregulation of lysosomal abundance and activity.

Bupivacaine Interferes with Autophagosome–Lysosome Fusion

To better understand how bupivacaine impairs autophagosome clearance, we examined autolysosome formation. Autophagosomes were visualized *via* EGFP-LC3 puncta (green fluorescence), and lysosomes were visualized using LysoTracker (red fluorescence, an acidic pH marker for lysosomes) according to previous studies.[20–22,29] If autolysosome formation proceeds normally, the green and red signals will overlap and appear yellow. Conversely, if the autophagosome does not fuse with the lysosome, the red and green signals will

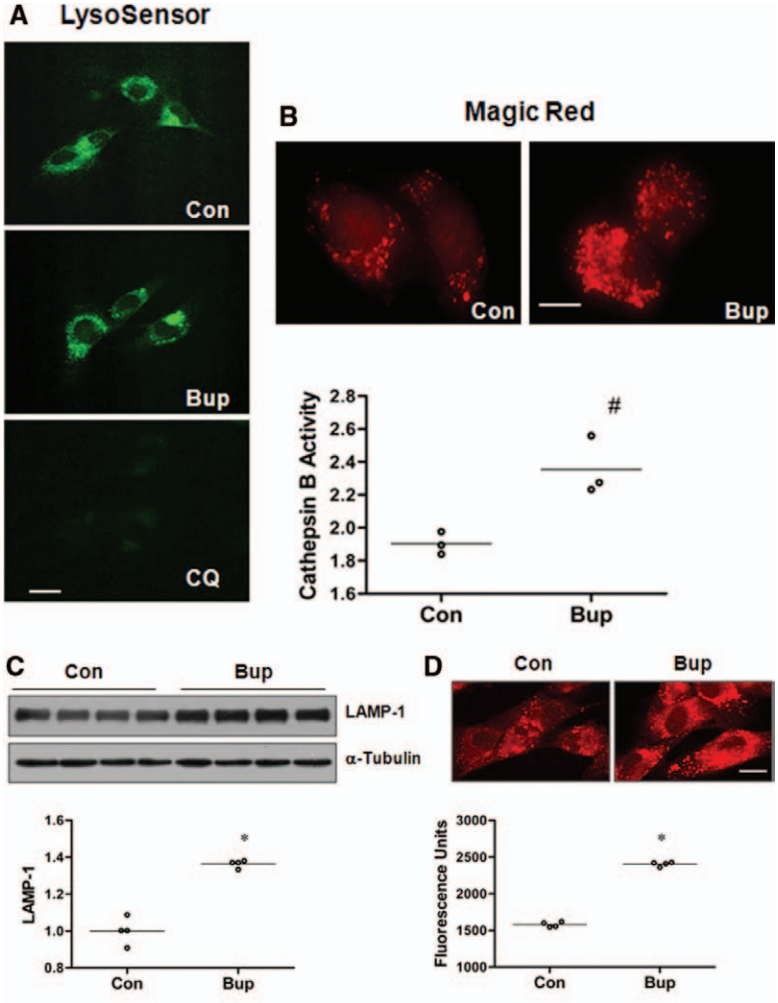

Fig. 4. Bupivacaine increased cathepsin B activity and lysosomal abundance. C2c12 cells were treated with bupivacaine ([Bup] 600 μM) for 6 h and the following experiments were performed. Untreated cells served as controls (Con). (*A*) Lysosomal pH. Cells were stained with LysoSensor, and fluorescence was observed under a confocal microscope (*scale bar* = 20 μm). Representative images from three independent experiments are shown. (*B*) Cathepsin B activity. The activity of cathepsin B was examined using the Magic Red Cathepsin B Assay Kit (ImmunoChemistry, Bloomington, MN). Fluorescence images were taken under a confocal microscope (*scale bar* = 20 μm). Fluorescence intensity was quantified using a fluorometer. #$P < 0.05$ *versus* Con group. n = 3 per group. (*C*) Lysosomal-associated membrane protein 1 (LAMP-1) levels. Cellular extracts were prepared for Western blot with an antibody against LAMP-1. The same membrane was probed with an α-tubulin antibody as a loading control. *$P < 0.01$ *versus* Con group. n = 4 per group. (*D*) LysoTracker staining. Cells were stained with LysoTracker. Fluorescence images were taken under a confocal fluorescence microscope (*scale bar* = 20 μm). Fluorescence intensity was quantified using a fluorometer. *$P <$ 0.01 *versus* Con group. n = 4 per group. CQ = chloroquine.

appear separated. As shown in figure 5, the red and green signals merged in controls cells; however, in bupivacaine-treated cells, the most green and red signals were not colocalized. The colocalization rate of EGFP-LC3 puncta with LysoTracker signals was significantly decreased in bupivacaine group by 67.0% compared with control group ($P = 0.001$). The results suggest that bupivacaine inhibits autolysosome formation by interfering with autophagosome–lysosome fusion.

Rapamycin Promotes Autophagosome Clearance in Bupivacaine-treated Cells

Administration of rapamycin increased LC3 conversion in bupivacaine-treated cells. By contrast, rapamycin prevented bupivacaine-induced increases in p62 protein levels. As shown in figure 6A, the ratio of LC3-II/LC3-I was significantly higher by 60.8% in bupivacaine-treated cells after exposure to rapamycin than that in cells treated with bupivacaine alone ($P < 0.0001$). The p62 protein level in bupivacaine-treated cells was reduced by 57.6% in the presence of rapamycin compared with that in the absence of rapamycin ($P < 0.0001$). Also, compared with untreated controls, there was a significant increase in the LC3-II/LC3-I ratio and a reduction in p62 protein levels in cells treated with rapamycin alone ($P < 0.01$). These data suggest that rapamycin promotes both autophagosome formation and autophagosome clearance in bupivacaine-treated cells.

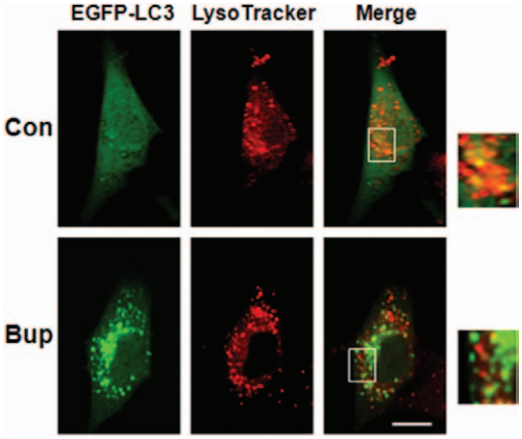

Fig. 5. Bupivacaine impaired autophagosome–lysosome fusion. C2c12 cells transfected with the plasmid expressed rat LC3 fused to enhanced green fluorescent protein (pEGFP-LC3) were stimulated with bupivacaine (Bup, 600 μM) for 6 h. Untreated cells served as controls (Con). Cells were stained with LysoTracker, and the fluorescence was observed under a confocal microscope (*scale bar* = 20 μm). Higher magnification images of the *boxed areas* are shown in the *right-hand panels*. Representative images from three independent experiments are shown. LC3 = microtubule-associated protein light chain 3.

Figure 6B shows confocal images of autophagosomes fusing with lysosomes. Compared with the cells solely treated with bupivacaine, rapamycin increased the colocalization (yellow) of EGFP-LC3 puncta (green) and LysoTracker (red) in bupivacaine-treated cells. The colocalization rate was significantly increased in bupivacaine group by rapamycin by 312.5% compared with that in the cells treated solely with bupivacaine ($P < 0.0001$). The results suggest that rapamycin promotes the fusion of autophagosomes with lysosomes.

Rapamycin Attenuates Bupivacaine-induced Myotoxicity

As shown in figure 6C, rapamycin attenuated morphological abnormalities in bupivacaine-treated cells. These observations were confirmed by the results of the live/dead assay (fig. 6D), which showed that rapamycin markedly attenuated bupivacaine-induced cell death by 48.6% compared with that in cells treated with bupivacaine alone ($P < 0.0001$). Compared with untreated controls, rapamycin alone did not induce significant cell death (fig. 6D).

Autophagy Inhibition by atg5 Knockdown Aggravated the Bupivacaine-induced Myotoxicity

Finally, we investigated the effects of autophagy inhibition by atg5 knockdown on bupivacaine myotoxicity. As shown in figure 7A, atg5 siRNA decreased atg5 messenger RNA levels by 83.8% compared with negative controls ($P < 0.0001$). Consistent with this, atg5 protein levels were decreased by 91.1% compared with negative controls ($P = 0.0003$) (fig. 7B). After atg5 knockdown by siRNA, the LC3-II/LC3-I ratio was decreased by 80.8%, whereas p62 level increased by 144.2%, respectively, compared with negative controls ($P < 0.01$) (fig. 7B). The results suggest an effective autophagy inhibition by atg5 knockdown.

Figure 7C shows the cell viability in bupivacaine-treated cells in the presence of atg5 knockdown. The bupivacaine-provoked cell injury was aggravated by 26.4% by atg5 knockdown ($P < 0.0001$).

Discussion

The most significant finding of this study is that impairment of autophagosome clearance contributed to LA bupivacaine-induced myotoxicity. Autophagy manipulation may be a potential therapeutic intervention to manage or prevent LA-induced myotoxicity.

There was a significant increase in the levels of autophagic markers (LC3 conversion and EGFP-LC3 punctuation) in bupivacaine-treated C2c12 cells. One possible reason for the bupivacaine-induced increase in autophagic markers is the activation of autophagosome generation. This is supported by the significant reduction in Akt, mTOR, and p70S6K phosphorylation detected after bupivacaine stimulation. Akt, mTOR, and p70S6K are three pivotal kinases belonging to the Akt/mTOR/p70S6K signaling pathway, a pathway that plays an important role as a negative regulator of autophagy initiation.[26,28] The results presented herein are generally consistent with those of earlier reports by ours and others showing that bupivacaine suppresses Akt activation in neuronal cells and renal cells.[30–32] Therefore, the observations made in the current study suggest that bupivacaine activates autophagosome formation as a stress response by inhibiting the Akt/mTOR/p70S6K signaling pathway.

However, increased expression of autophagic markers may also be caused by impaired autophagosome clearance. Therefore, we asked whether bupivacaine impaired autophagosome clearance. We selected p62 as a marker of autophagosome clearance because p62 binds to LC3 and is selectively degraded through autophagy.[7,26] If autophagosomes are not appropriately cleared, then p62 protein will accumulate within cells.[7] Indeed, we observed a significant increase in p62 levels after bupivacaine stimulation in both C2c12 cells and skeletal muscles (data not shown). Taken together, the results indicate that bupivacaine impairs autophagosome clearance.

Autophagosome clearance is dependent upon the maturation and degradation of autophagosomes, which involves the fusion of autophagosomes with lysosomes to form autolysosomes and the subsequent degradation of luminal substrates.[7] In this study, we present evidence that bupivacaine is a potent inhibitor of autophagosome–lysosome fusion. First, bupivacaine increased the levels of autophagic markers by inhibiting autophagy clearance; and second, bupivacaine suppressed the colocalization of EGFP-LC3 puncta (a marker for autophagosomes) with LysoTracker (a marker for lysosomes). These effects were similar to those of CQ and bafilomycin A1, both of which block the

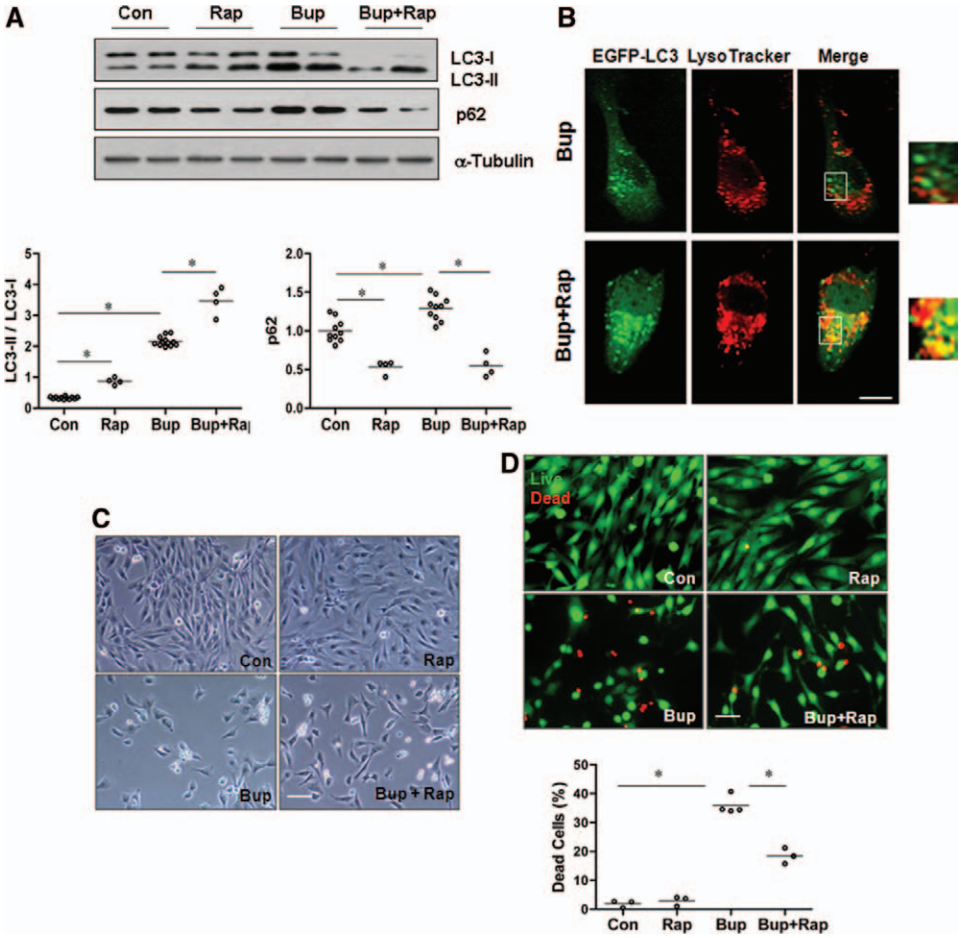

Fig. 6. Rapamycin promoted autophagosome formation and autophagosome clearance in bupivacaine-treated cells. C2c12 cells were pretreated with rapamycin (Rap, 500 nM) for 30 min, followed by challenge with bupivacaine ([Bup] 600 μM) for 6 h. Untreated cells served as controls (Con). The following experiments were then performed: (*A*) LC3 conversion and p62 protein levels. Cellular extracts were prepared for Western blot with antibodies against LC3-I/II and p62. The same membrane was then probed with an antibody against αsss-tubulin as a loading control. *$P < 0.01$, n = 4–12 per group. (*B*) Colocalization of the plasmid expressed rat LC3 fused to enhanced green fluorescent protein (pEGFP-LC3) puncta and LysoTracker staining. C2c12 cells were transfected with the pEGFP-LC3 and treated with rapamycin for 30 min before bupivacaine exposure. Cells were stained with LysoTracker 6 h after bupivacaine challenge. Fluorescence was observed under a confocal microscope (*scale bar* = 20 μm). Higher magnification images of the *boxed areas* are shown in the *right-hand panels*. Representative images from three independent experiments are shown. (*C*) Morphological changes. C2c12 cells were pretreated with rapamycin for 30 min, followed by challenge with bupivacaine for 24 h. Changes in cell morphology were observed under a phase-contrast microscope (*scale bar* = 100 μm). Representative images from three independent experiments are shown. (*D*) Live/dead assay. C2c12 cells were pretreated with rapamycin for 30 min, followed by challenge with bupivacaine for 36 h. A live/dead assay was then performed, and fluorescence was observed under a fluorescence microscope (*scale bar* = 100 μm). Representative images from three to four independent experiments are shown. *$P < 0.01$. n = 3–4 per group. LC3 = microtubule-associated protein light chain 3.

autophagosome–lysosome fusion process. The exact mechanisms underlying autophagosome–lysosome fusion are still unclear. Bupivacaine had no effect on lysosomal pH, whereas it increased cathepsin B activity and lysosomal abundance, which may be due to the reduced consumption of lysosomes as they fail to fuse with autophagosomes. Our results expanded the bioactivities of LAs in the autophagic process.

This study provides evidence that impaired autophagosome clearance contributes to bupivacaine-induced myotoxicity based on the following observations. First, increased autophagosome clearance by rapamycin attenuated bupivacaine-induced myotoxicity; second, autophagy inhibition with atg5 knockdown aggravated bupivacaine myotoxicity; and finally, the combined effects of autophagy inhibitors CQ and bafilomycin A1 exacerbated bupivacaine-induced myotoxicity (data not shown). Previous studies show that altering autophagosome clearance plays a critical role in several pathophysiological processes. For example, increased autophagosome clearance improves survival and attenuates pulmonary injury in cecal ligation and puncture-induced septic mice.[33] However, impairing autophagosome clearance results in cell death,

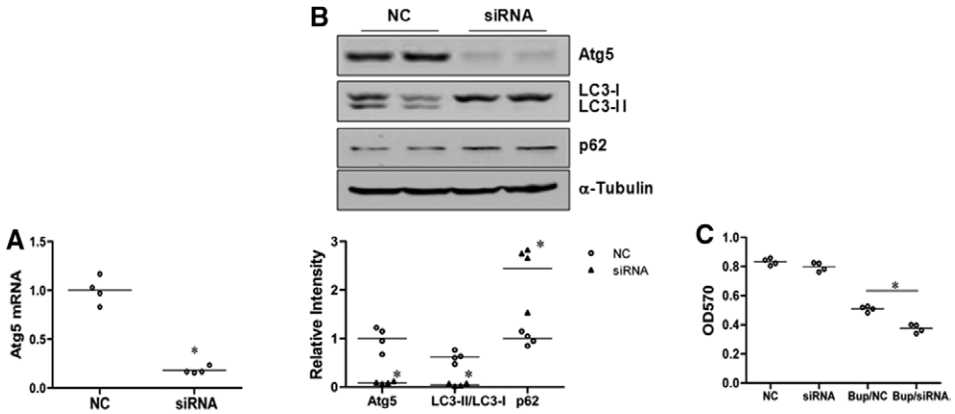

Fig. 7. Autophagy inhibition by autophagy-related protein 5 (atg5) knockdown aggravated bupivacaine myotoxicity in C2c12 cells. (*A*) atg5 messenger RNA (mRNA) levels after small interfering RNA (siRNA). C2c12 cells were transfected with atg5 siRNA. Cells transfected with scrambled RNA served as negative control (NC). atg5 mRNA levels were analyzed by real-time quantitative polymerase chain reaction 24 h after siRNA transfection. * $P < 0.01$ *versus* NC group, n = 4 per group. (*B*) atg5 protein levels, microtubule-associated protein light chain 3 (LC3) conversion and p62 protein levels after siRNA. C2c12 cells were collected 48 h after siRNA transfection. Cellular extracts were then prepared for Western blot with the indicated antibodies. The same membrane was then probed with an antibody against α-tubulin as a loading control. *$P < 0.01$ *versus* the respective NC group, n = 4 per group. (*C*) Cell survival. C2c12 cells were treated with bupivacaine (600 μM) at 48 h after atg5 siRNA. Cell viability was examined in a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay 24 h after bupivacaine exposure. * $P < 0.01$. n = 4 per group.

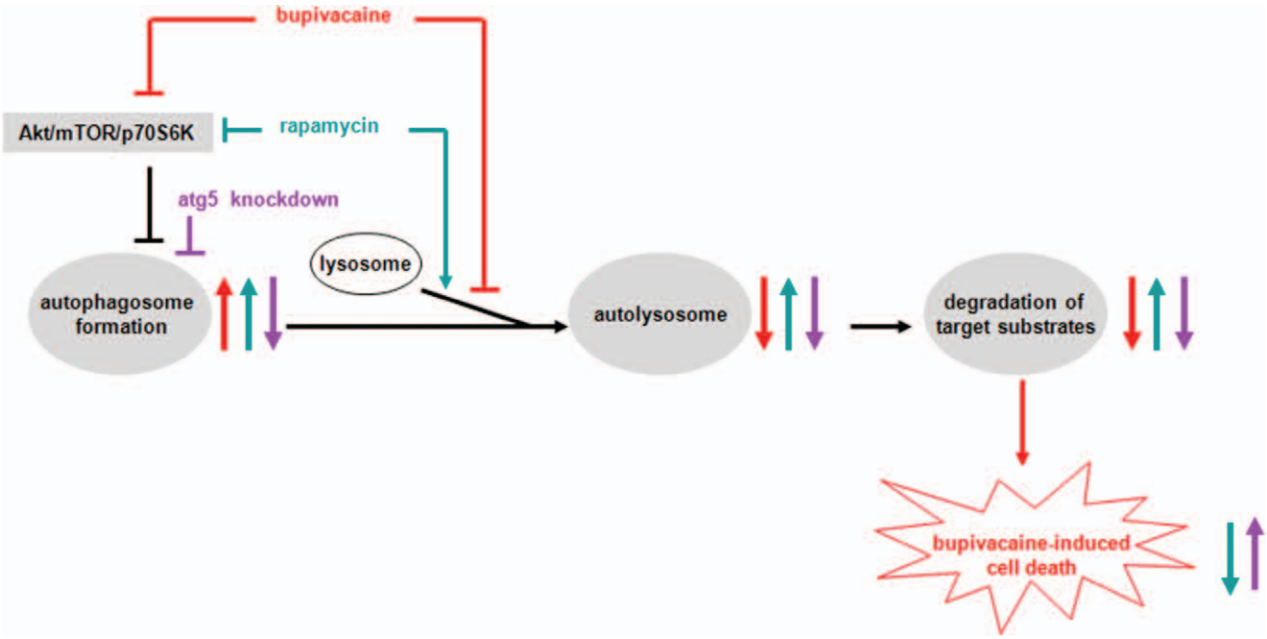

Fig. 8. Scheme summarizes the proposed mechanism for bupivacaine-induced impairment of autophagosome clearance and myotoxicity. Bupivacaine administration stimulates autophagosomes formation, which could be initiated by inactivation of protein kinase B/mammalian target of rapamycin/p70 ribosomal protein S6 kinase (Akt/mTOR/p70S6K) signaling. However, bupivacaine interferes with autophagosome–lysosome fusion, a critical step for autophagosome clearance. Impaired autophagosome clearance results in the injury in muscle cells. Rapamycin Increases both autophagosome formation and autophagosome–lysosome fusion, which in turn attenuates bupivacaine myotoxicity. However, bupivacaine myotoxicity is aggravated by inhibiting autophagosome formation by atg5 knockdown. *Red symbols* illustrate effects of bupivacaine; *green symbols* illustrate effects of rapamycin; *purple symbols* illustrate effects atg5 knockdown.

which contributes to the development of diverse disorders such as cardiac ischemia–reperfusion injury, Alzheimer disease, and myocilin glaucoma.[12,34,35] The results presented herein show that impairment of autophagosome clearance is a novel mechanism underlying bupivacaine-induced myotoxicity. Importantly, we observed increases

in LC3-II/LC3-I ratios and p62 protein levels in the cells exposed to procaine with myotoxic dosage, a less myotoxic LA (figs. 1 and 2, Supplemental Digital Content 1, http://links.lww.com/ALN/B129, which are figures showing the effects of procaine on cell viability and changes of autophagic markers). Moreover, autophagy inhibition with atg5 knockdown aggravated procaine myotoxicity (fig. 3, Supplemental Digital Content 1, http://links.lww.com/ALN/B129, which is a figure showing the effects of atg5 knockdown on procaine-induced myotoxicity). Taken together, impaired autophagosome clearance is likely serving as a common mechanism responsible for the myotoxicity of tertiary amine LAs.

Rapamycin activates autophagosome formation by inhibiting mTOR signaling. Interestingly, we observed that bupivacaine also showed an inhibition role on mTOR activation, suggesting that bupivacaine and rapamycin share similar role in the induction of autophagosome generation. However, the bupivacaine-induced impairment of autophagosome–lysosome fusion and increases in p62 protein levels were attenuated by pretreatment with rapamycin, suggest that bupivacaine and rapamycin oppositely regulate autophagosome clearance. The results of the current study suggest that rapamycin rescues the bupivacaine-induced impairment of autophagic flux by promoting autophagosome clearance. As rapamycin has been used in clinical treatment for many years,[36–38] short-term administration of rapamycin may be a potential approach to preventing LA-induced myotoxicity.

In conclusion, this study demonstrates for the first time that the impairment of autophagosome clearance is a novel mechanism underlying LA bupivacaine-induced myotoxicity (fig. 8). The data suggest that autophagy manipulation could be an alternative intervention for preventing and managing the potential myotoxicity induced by LA.

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Competing Interests

The authors declare no competing interests.

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