

Mitochondrial Injury and Caspase Activation by the Local Anesthetic Lidocaine

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Background: Lidocaine, a local anesthetic, can be neurotoxic. However, the cellular mechanisms of its neurotoxicity at concentrations encountered during spinal anesthesia remain unclear.

Methods: The authors examined the mechanisms of lidocaine neurotoxicity in the ND7 cell line derived from rat dorsal root ganglion. Individual neurons were assayed by flow cytometry or microscopy using fluorescent probes of plasma membrane integrity, mitochondrial membrane potential, caspase activity, phospholipid membrane asymmetry, and mitochondrial cytochrome c release.

Results: In the ND7 cell line, lidocaine at 185 mM \times 10 min to 2.3 mM \times 24 h caused necrosis or late apoptosis. Equimolar Tris buffer and equipotent tetrodotoxin controls were not toxic, indicating that neither osmotic nor Na⁺-blocking effects explain lidocaine neurotoxicity. The earliest manifestation of lidocaine neurotoxicity was complete loss of mitochondrial membrane potential within 5 min after exposure to lidocaine at a concentration of 19 mM or greater. Consistent with these data, 37 mM lidocaine (1%) induced release of mitochondrial cytochrome c into the cytoplasm, as well as plasma membrane blebbing, loss of phosphatidylserine membrane asymmetry, and caspase activation, with release of mitochondrial cytochrome c to the cytoplasm within 2 h. Treatment with z-VAD-fmk, a specific inhibitor of caspases, prevented caspase activation and delayed but did not prevent neuronal death, but did not inhibit the other indicators of apoptosis.

Conclusions: Collectively, these data indicate that lidocaine neurotoxicity involves mitochondrial dysfunction with activation of apoptotic pathways.

LIDOCAINE has been considered a benign and specific Na⁺ channel blocker and is widely used as a local anesthetic. However, recurrent reports of cauda equina syndrome and persistent lumbosacral neuropathy have suggested that lidocaine can be neurotoxic at concentrations injected into the subarachnoid space for spinal anesthesia when poor mixing with cerebrospinal fluid (CSF) and maldistribution lead to undiluted high local concentrations, with an incidence of approximately 1 in 1,000,¹ and this has been confirmed in an animal model.² A more frequent syndrome of pain or dysesthesia in the

buttocks or legs after recovery also occurs after lidocaine spinal anesthesia, with an incidence of approximately 1 in 3, without any apparent maldistribution. Although the pain typically resolves within 1 week without lasting sequelae, it can be severe in up to a third of patients with the syndrome.³

At concentrations below its ED₅₀ for Na⁺ block (approximately 50 μ M = 0.001%), lidocaine is neuroprotective.^{4,5} However, much higher concentrations of 37–185 mM (1–5% lidocaine HCl) must be injected into the subarachnoid space to achieve complete Na⁺ channel blockade over a large number of dermatomes, because of dilution with CSF and incomplete control of distribution after injection. Even with good mixing, the lidocaine CSF concentration is approximately 10 mM (0.27%) for the first 15 min after spinal injection.⁶

The mechanism of neurotoxicity at such concentrations, three orders of magnitude greater than the ED₅₀ of lidocaine for Na⁺ block, has not been elucidated. Disturbances in Ca²⁺ homeostasis were associated with necrotic neuronal death from 185 mM (5%) lidocaine within an hour of exposure, but lower concentrations of lidocaine caused much less change in cytoplasmic Ca²⁺ and minimal overt necrosis within an hour.^{7,8} Work in non-neuronal subcellular particles has suggested that local anesthetics may adversely affect mitochondrial energetics,^{9–11} and mitochondrial insults can induce either apoptosis or necrosis, with less severe injuries leading to apoptosis, a form of programmed cell death.^{12,13} Recent studies have reported that low concentrations of lidocaine can induce 15–30% apoptotic cell death in a dorsal root ganglion cell line (9 mM, 0.25% lidocaine \times 45 min),¹⁴ in a neuroblastoma line (3 mM, 0.08% lidocaine \times 3 h),¹⁵ and in the Jurkat human lymphoma T-cell line (22 mM, 0.6% lidocaine \times 4–6 h).¹⁶

The proximate or upstream activator of apoptosis in lidocaine neurotoxicity is unknown. Multiple apoptotic pathways exist.^{17–21} Apoptosis can be initiated by activation of a death receptor on the plasma membrane, by the effects of Bcl-2 family proteins on mitochondria, or by direct toxic effects on mitochondria leading to loss of mitochondrial membrane potential ($\Delta\psi$) and release of apoptosis-activating mitochondrial intermembrane proteins. Activation of different caspases (apoptosis-related proteases) can either initiate or amplify the apoptotic response, upstream or downstream of mitochondria, and caspase-independent pathways to apoptosis also exist. The mitochondrion plays a central role in most apoptotic pathways, as initiator, effector, or amplifier of apoptotic responses. Multiple cross-talk and feedback loops

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among different mitochondrial death pathways make it likely that most components of apoptotic pathways will eventually be activated after apoptotic stimuli,¹⁹ so that the time course of apoptotic events is important for determining the dominant apoptotic pathway for a given cellular injury.

Hence, using a spinal sensory neuron model, we tested the hypotheses that lidocaine causes (1) concentration-dependent apoptosis and necrosis, (2) activation of caspases as an essential part of its apoptotic pathway, (3) loss of mitochondrial membrane potential ($\Delta\psi$) as an early upstream event, and (4) subsequent release of the mitochondrial apoptosis-inducing factor cytochrome c.

Materials and Methods

Chemicals and Buffers

Chemicals were obtained from Sigma-Aldrich (St. Louis, MO), except where specifically noted, and were of the highest purity available. Lidocaine, bupivacaine, and tris(hydroxymethyl)-aminomethane (Tris buffer) were obtained as their hydrochloride salts, dissolved in buffer, and adjusted to a pH of 7.4 before use. Concentrations of lidocaine tested were selected as fractions of those approved and commercially available for subarachnoid injection, e.g., 185 mM lidocaine = 5.0% lidocaine HCl. Experimental buffer was HEPES-buffered KRH: 5 mM *D*-glucose, 25 mM HEPES, 115 mM NaCl, 5 mM KCl, 1.2 mM MgSO_4 , 1.0 mM KH_2PO_4 , and 2.0 mM CaCl_2 , + NaOH to a pH of 7.4.⁷ Compound 1799 was obtained from DuPont (Wilmington, DE).

The fluorescent probes propidium iodide (PI), annexin V-Alexa 488 conjugate, rhodamine 123 (R123), Mitofluor Green (MFG), and tetramethyl-rhodamine methyl ester (TMRM) were obtained from Molecular Probes (Eugene, OR). Sulforhodamine-labeled fluoromethyl ketone peptide inhibitor of caspase (SR-VAD-fmk) was obtained as part of the *in situ* FLICA sulforhodamine pan-caspase detection kit from Chemicon (Temecula, CA).

Tris buffer was used at equimolar concentrations as a control for lidocaine in all experiments. Because it is a weak amine base with an acid dissociation constant similar to that of lidocaine, it served as an equiosmotic and equicharged control. Tetrodotoxin was used as another control in some experiments at a concentration at least equipotent to lidocaine in terms of Na^+ channel blocking. As a Na^+ channel blocker structurally unrelated to lidocaine and other clinical local anesthetics, tetrodotoxin control allowed separation of lidocaine neurotoxicity from its Na^+ channel-blocking property. In all experiments, controls were prepared in the same buffer and added at the same time as lidocaine would have been.

Cell Culture

All neuronal studies were conducted using the ND7-104 subclone of the ND7 cell line, obtained from Dr. Patrick Hogan of the Department of Neurobiology, Harvard Medical School (Boston, Massachusetts). ND7 was derived from rat dorsal root ganglion, was immortalized by fusion with mouse neuroblastoma, and has been extensively characterized as a sensory neuron model.²²⁻²⁵ Neurons were cultured and differentiated as previously described.⁷

Experimental Design and Presentation of Data

All experiments were performed at 37°C. For time course graphs, time of local anesthetic or Tris buffer addition to the neurons is always presented graphically as 0 min, so that baseline values before local anesthetic addition are at negative times. Local anesthetic or Tris was present for the rest of the experiment after it was added, except where specifically noted.

Flow Cytometry to Determine Neuronal Death

At the end of lidocaine or control exposure, the medium was decanted and saved (so that all nonadherent cells were included). Trypsin, 0.05% (1 ml), was added to the culture plate and incubated at 37°C for 2.5 min. The resultant cell suspension was added to the previously decanted media, together with an additional 1 ml buffer washing of the culture plate, then stained with 500 nM PI for 1 min, and then analyzed immediately by flow cytometry (FACStar Plus; Becton-Dickinson, Franklin Lakes, NJ): excitation 488 nm, emission greater than 630 nm; more than 10^5 neurons/injection. Neurons staining with PI were dead either by necrosis or late apoptosis.²⁶

Microscopy (Nonconfocal; All Experiments Except Cytochrome c Release)

Phase contrast and fluorescence images were obtained with an Axiovert 135 TV inverted microscope (Carl Zeiss, Inc., Heidelberg, Germany) equipped with a Zeiss 40 \times Plan Neofluor 0.75 NA lens, with the stage heated to 37°C. Images for quantitative analysis were acquired and analyzed using software of the Attofluor RatioVision system (Atto Instruments, Rockville, MD). Photomicrographs were recorded using an AxioCam digital microscope camera (Carl Zeiss, Inc.) at 1,300 \times 1,030 pixel, 8-bit, 256-color resolution. In control experiments, lidocaine did not interfere with any fluorescent stain except annexin V-Alexa 488 when lidocaine was present at 92 mM or higher (data not shown).

Cell Death Assays by Microscopy

Necrosis and late apoptosis were detected by adding 500 nM PI after lidocaine exposure and acquiring images at 488 nm excitation, greater than 630 nm emission. Necrotic and late apoptotic cell nuclei stained red,

whereas live and early apoptotic cells were not stained with PI. Early apoptosis was detected by staining with annexin V-Alexa 488 and SR-VAD-FMK after lidocaine exposure, following the manufacturers' instructions. Annexin V-Alexa 488 binds to phosphatidylserine exteriorized on the plasma membrane early in apoptosis to give a green stain,^{27,28} whereas SR-VAD-FMK binds to caspases only after they have been activated, to give a red stain.²⁹

Mitochondrial Membrane Potential Assays by Microscopy

The cationic fluorophore R123 concentrates in energized mitochondria and yields a punctate intracellular fluorescence in proportion to $\Delta\psi$.³⁰ Neurons were loaded before local anesthetic exposure with R123 at 500 nM for 30 min at 37°C, and then the neurons were washed and covered with experimental buffer and observed at 488 nm excitation, 530 \pm 15 nm emission. Local anesthetic was added after a stable baseline fluorescence was attained. For R123, changes in $\Delta\psi$ were determined from the decrease in fluorescence in a region of interest within the neuron but excluding the nucleus, because the concentration of mitochondria is sparse in the small amount of cytoplasm overlaying the nucleus in the two-dimensional microscope image.³⁰ For quantitation of R123 fluorescence as a semiquantitative assay of $\Delta\psi$, fluorescent intensity of each neuron at 5 and 60 min after lidocaine addition was (1) corrected by subtraction of background fluorescence and (2) normalized to baseline prelidocaine values by dividing by the average net fluorescence of that neuron for the 5 min preceding lidocaine addition, to yield the fraction of original R123 fluorescence.

To test the reversibility of mitochondrial depolarization by lidocaine, additional experiments were performed with 37 mM lidocaine or Tris. Neurons were loaded with 500 nM R123 at 37°C for 30 min, and then single-cell fluorescence was determined as for the regular R123 assay described in the preceding paragraph. After a 5-min baseline, lidocaine or 37 mM Tris was added, and the neurons were observed for 30 min. The buffer over the neurons was then exchanged twice and replaced with lidocaine-Tris-free buffer. Neurons were then reloaded with 500 nM R123 at 37°C for 30 min, and the assay was repeated with lidocaine or 37 mM Tris.

An additional assay of $\Delta\psi$ was based on fluorescence resonance energy transfer (FRET) between mitochondrial (MFG) and TMRM, and performed similarly to a previous description.³¹ MFG localizes to mitochondria independent of $\Delta\psi$, whereas TMRM localizes to mitochondria in proportion to $\Delta\psi$. MFG fluorescence is quenched by FRET with TMRM so that when $\Delta\psi$ decreases and TMRM leaves the mitochondria, MFG fluorescence increases. ND7 neurons on coverslips were loaded with 200 nM MFG for 30 min and then with 20 μ M

TMRM for 15 min, all at 37°C, and were then washed twice with KRH and used immediately for experiments. Individual neuronal MFG fluorescence was quantified at 488 \pm 5 nm excitation, 530 \pm 15 nm emission. The MFG FRET assay of $\Delta\psi$ was quantitated as for the R123 assay of $\Delta\psi$, except that an increase in MFG fluorescence as a fraction of original fluorescence indicated a decrease in $\Delta\psi$ rather than an increase.

Oxygen Measurements

Neurons were trypsinized, washed, and resuspended at a concentration of 1.0×10^6 /ml in KRH buffer supplemented with 1 mM l-pyruvate and 1 mM l-glutamine. Oxygen consumption was measured polarographically at 25°C in a closed glass reaction chamber (15 \times 45 mm; Kimble Glass, Vineland, NJ) completely filled with neuronal suspension, with a mini-Clark oxygen electrode inserted through a gas-tight gasket and monitored with a Chemical Microsensor oxygen meter (Diamond General, Ann Arbor, MI).

Confocal Microscopy Assay of Release from Mitochondria to Cytochrome c-GFP Fusion Protein

ND7 neurons were transfected with the expression vector pE-GFP-cytochrome c as previously described.³² Transfection efficiency for neurons used in these experiments was greater than 15%. Two dimensional confocal images of transfected neurons were obtained sequentially before and during lidocaine exposure, using an LSM510 confocal microscope (Carl Zeiss, Inc.) with C-Apochromat 63 \times /1.2NA water immersion lens and stage heated at 37°C. Excitation was provided by an argon-krypton laser at 488 nm, with emission at greater than 505 nm. The pinhole was set for a 1.5- μ m focal slice. Images were acquired at 512 \times 512 pixels with an 8 \times line-averaging mode. In a few experiments, transfected neurons were also loaded with TMRM at 500 nM for 30 min at 37°C and then washed and covered with experimental buffer. Images were then acquired with both green (cytochrome c-green fluorescent protein) and red (TMRM) emission channels to illustrate colocalization of cytochrome c-GFP and TMRM to mitochondria and to qualitatively confirm the mitochondrial depolarization shown in nonconfocal microscopy assays of $\Delta\psi$.

After stable baseline images were obtained, lidocaine was added. Images were acquired every 2 min for 92 and 185 mM lidocaine and every 10 min for 37 mM lidocaine and 37 and 185 mM Tris controls, until all neurons had released mitochondrial cytochrome c-GFP, or for 150 min lidocaine or control exposure if all neurons did not release cytochrome c-GFP. The time of release of mitochondrial cytochrome c-GFP into the cytoplasm was determined as previously described.³³ The SD of the green fluorescence histogram for each neuron expressing cytochrome c-GFP at each time point was determined using ImageJ software (National Institute of Men-

tal Health, Bethesda, MD)[#] and the time of release from mitochondria to cytoplasm taken as the time of sudden decrease in SD.

Statistical Analysis

All error bars indicate SD. All statistics calculations were performed using the algorithms in Systat 7.0 (Systat Software, Richmond, CA). For flow cytometry to determine neuronal death, statistical analysis of percent PI⁺ neurons as a dependent variable and drug treatment as an independent variable was performed by analysis of variance separately at each lidocaine/Tris concentration and at each exposure time, with Tukey *post hoc* correction of individual comparisons among lidocaine, lidocaine with z-VAD-fmk, and equimolar Tris buffer.

For the R123 assay of mitochondrial membrane potential, the fraction of original R123 fluorescence was analyzed by analysis of variance with local anesthetic/buffer as a factor at each concentration. When the analysis of variance was significant ($P < 0.05$), *post hoc* analysis using the Bonferroni correction was performed, comparing each local anesthetic with its equimolar Tris control (e.g., 1.0% [37 mM] lidocaine compared with 37 mM Tris) and equipotent bupivacaine with equipotent lidocaine (e.g., 0.25% bupivacaine HCl compared with 1.0% lidocaine HCl). The effect of local anesthetic concentration was determined for each local anesthetic and each Tris control by linear regression analysis. For the MFG FRET assay of $\Delta\psi$, statistical analysis was performed as for the R123 assay of $\Delta\psi$, except that values at 30 rather than 60 min after lidocaine addition were analyzed.

For the R123 assay testing, the reversibility of mitochondrial depolarization by lidocaine, average fluorescence during the first prelidocaine 5-min baseline was compared with that of the second prelidocaine 5-min baseline by paired two-sample, two-way *t* test. Fluorescence for the first and second pre-Tris 5-min baselines were compared similarly. For the oxygen consumption experiments, the slope of the percent oxygen-*versus*-time curve was calculated by linear regression for each experiment (three to four experiments for each lidocaine concentration). Analysis of variance with slope as a dependent variable and lidocaine concentration as an independent variable was then performed, and individual lidocaine and Tris concentrations were compared using the Tukey *post hoc* correction.

To analyze the effect of z-VAD-fmk and lidocaine concentration on cytochrome c-GFP release, Kaplan-Meier estimation, with exact failures and right censoring, and Mantel log-rank test were performed for each lidocaine concentration with stratification on the presence or absence of z-VAD-fmk. When z-VAD-fmk proved to have no effect, \pm z-VAD-fmk data were combined for each con-

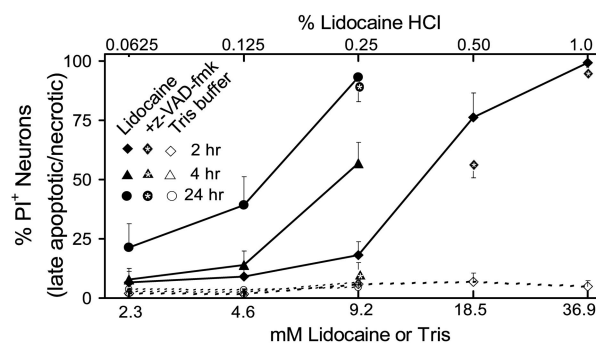


Fig. 1. Effect of continuous lidocaine exposure on neuronal death. ND7 neurons were exposed to lidocaine or Tris buffer in media at 37°C for indicated time and then analyzed by flow cytometry for cell death (necrosis and late apoptosis) using propidium iodide (PI) staining. Filled symbols = lidocaine; open symbols = Tris buffer; filled with asterisk = lidocaine + 100 μ M z-VAD-fmk (pan-caspase inhibitor). Note logarithmic x-axis with dual concentration scales in percent and millimolars of lidocaine HCl. $P < 0.01$ for all lidocaine values *versus* their equimolar Tris controls for each concentration at each time tested, except $P > 0.05$ for 2.3 mM at 2 and 4 h. z-VAD-fmk did not cause a significant change for 9.2 mM lidocaine at 24 h or 36.9 mM at 2 h, whereas its effect was significant for 9.2 mM at 4 h and 18.5 mM at 2 h. Each data point is the average of three to five experiments.

centration of lidocaine and compared with stratification on all pairs of lidocaine concentration, with Bonferroni correction for the P value of each pair.

Results

Lidocaine is Neurotoxic

ND7 neurons were exposed to lidocaine at concentrations ranging from that seen for 15 min after initial injection and mixing with CSF (2.3–9.3 mM) to the lowest undiluted concentration that gives a clinically effective spinal anesthetic (37 mM, 1%).⁶ Tris buffer, similar to lidocaine in being a weak amine base with a similar acid dissociation constant, was used as an equiosmotic and equicharged control. Lidocaine, 4.6 mM or more for 2 and 4 h and 2.3 mM or more for 24 h, caused a significant increase in PI staining, indicating either necrotic or late apoptotic neuronal death²⁶ (fig. 1). When the pan-caspase inhibitor z-VAD-fmk was present, neuronal death decreased 20–40% for conditions near the LD₅₀ (9.2–18.5 mM, 2–4 h). For conditions causing complete neuronal death (36.9 mM, 2 h; 9.2 mM, 24 h), z-VAD-fmk was not protective. Therefore, both necrotic and apoptotic pathways can be induced by lidocaine.

Lidocaine Dose Determines Apoptosis versus Necrosis, Independent of Na⁺ Channel-Blocking Properties

When neurons were exposed to 37 mM lidocaine for 30 min and then allowed to recover in medium for 120 min, at least half of the neurons lost phosphatidylserine membrane asymmetry and stained with annexin V, an

[#] National Institute of Mental Health: ImageJ software. Available at: <http://rsb.info.nih.gov/ij/docs/index.html>. Accessed March 11, 2003.

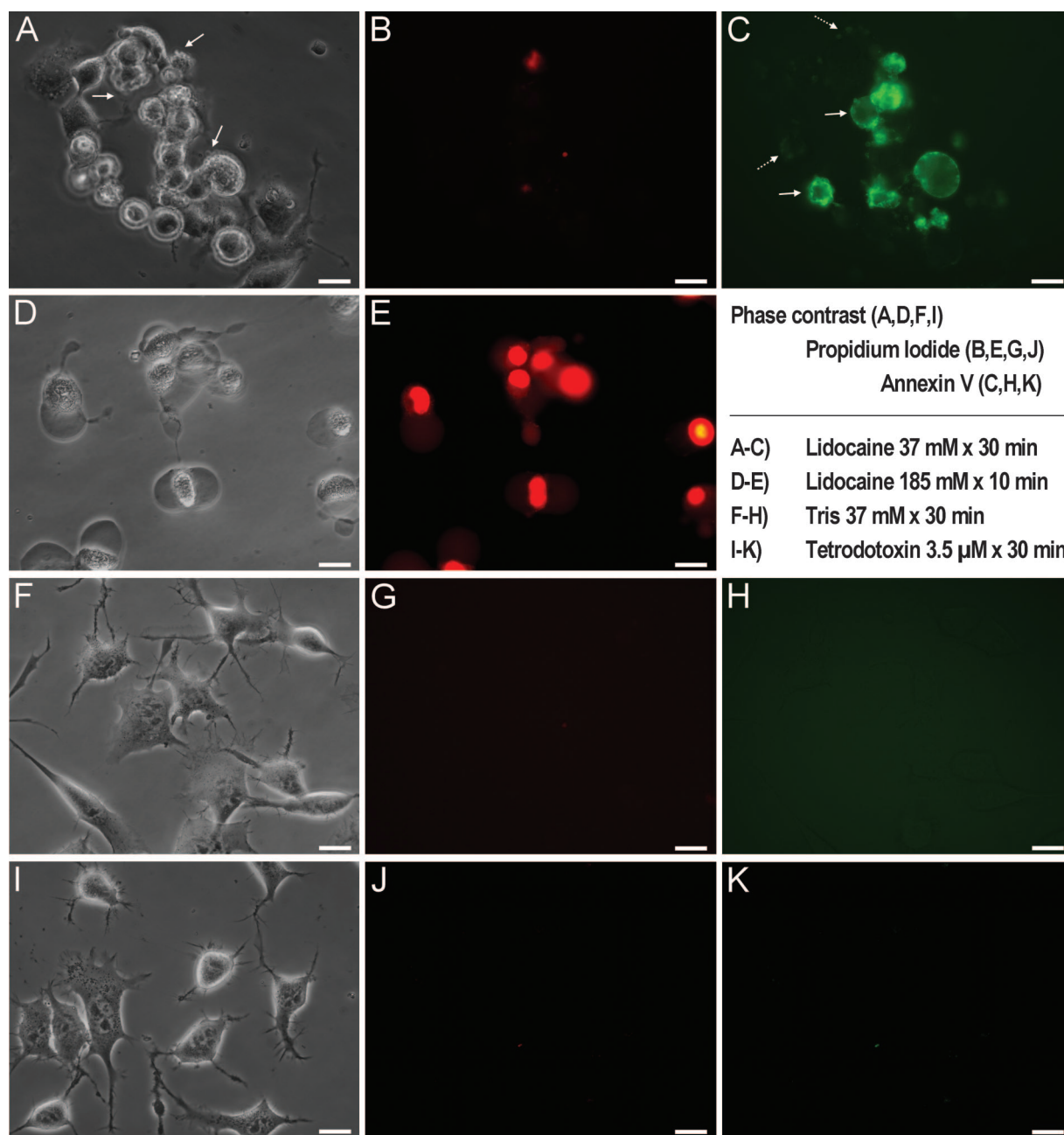


Fig. 2. Effect of 10- to 30-min lidocaine exposure followed by 120-min recovery period on neuronal viability. (A–C) Lidocaine, 37 mM \times 30 min; phase contrast, propidium iodide (PI), and annexin V images of same field. Several neurons have small blebs (arrows in phase image) and annexin V staining without PI staining, indicating apoptosis. Dashed arrows in annexin V image indicate light staining consistent with early apoptosis; solid arrows indicate darker staining consistent with greater externalization of phosphatidylserine and more advanced apoptosis. (D–E) Lidocaine, 185 mM \times 10 min; phase and PI images of same field. All neurons have large blebs and are PI⁺, consistent with necrosis. Annexin V image not done because all PI⁺ neurons will be annexin V⁺, and greater than 92 mM lidocaine inhibited annexin V staining (data not shown). (F–H) Tris buffer control, 37 mM (185 mM Tris gave identical results); phase, PI, and annexin V images of same field. (I–K) Tetrodotoxin as Na⁺ channel-blocking control, 3.5 μ M equipotent to 185 mM lidocaine; phase, PI, and annexin V images of same field. Both F and I show normal morphology with no apparent necrosis or apoptosis. Scale bar = 20 μ m. Each experiment typical of three experiments; > 7 fields/experiment.

early indicator of apoptosis,^{27,28} with minimal PI staining (fig. 2). At least one quarter of the neurons in each field showed small membrane blebs typical of apoptosis. In contrast, all neurons exposed to 185 mM lidocaine (5%, the highest concentration clinically available) for only 10 min, with 120 min recovery, stained with PI and devel-

oped large swollen blebs typical of necrosis. Neurons treated with either equimolar Tris or equipotent tetrodotoxin (an Na⁺ blocker structurally unrelated to lidocaine) did not stain with either annexin V or PI.

To confirm that the lower concentration of lidocaine activates an apoptotic pathway, neurons were exposed

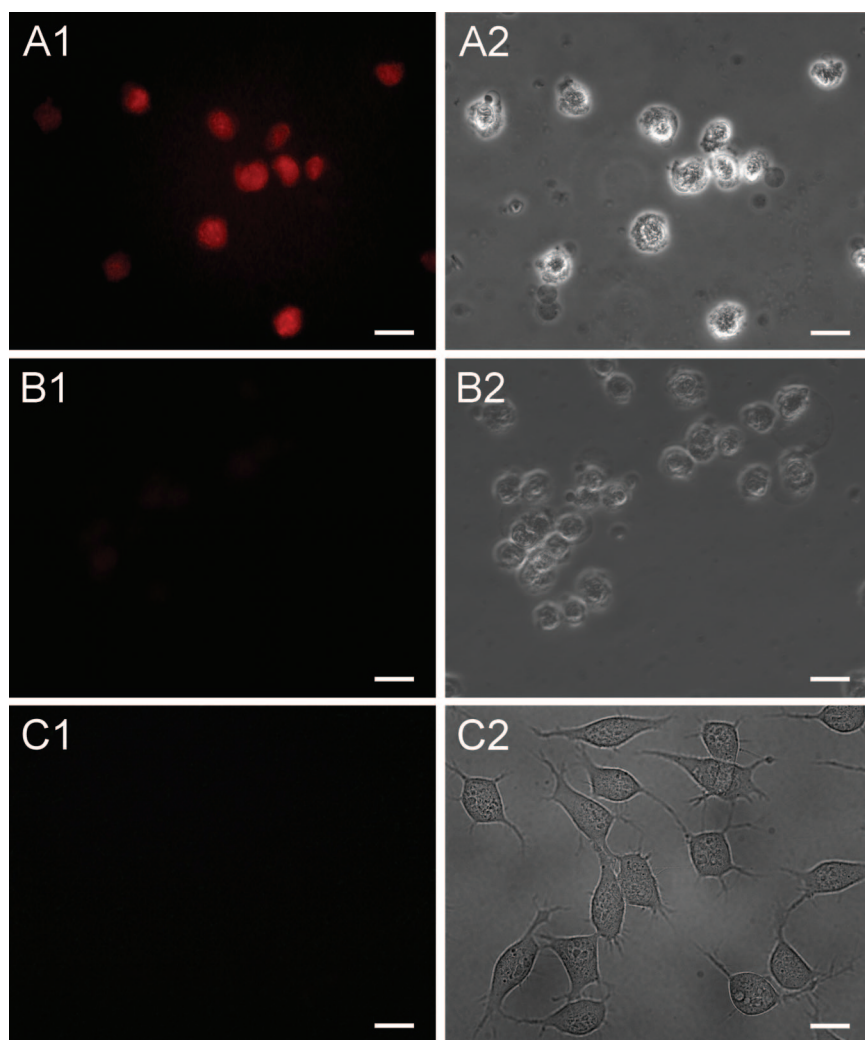


Fig. 3. Caspase activation by lidocaine. Neurons were exposed to 37 mM lidocaine (A and B) or 37 mM Tris buffer (C) for 30 min, with (B) or without (A and C) 100 μ M z-VAD-fmk and then incubated with sulforhodamine-VAD-fmk for 60 min. (1) Sulforhodamine fluorescent images, indicating caspase activation; (2) phase contrast images of the same field; each typical of three experiments; > 7 fields/experiment.

to 37 mM lidocaine for 30 min and then incubated with sulforhodamine-VAD-fmk, an *in situ* fluorescent indicator of caspase activation with broad caspase specificity.²⁹ As shown in figure 3, 37 mM lidocaine caused caspase activation in all neurons. Caspase activation was prevented by z-VAD-fmk, although blebbing still occurred. Therefore, 37 mM lidocaine activates apoptosis rather than necrosis, at least for exposures of 30 min or less. As the severity of a toxic injury decreases, apoptosis becomes more likely than necrosis.^{12,13} Hence, it is probable, although not established by these data, that the cell death observed for lidocaine at a concentration of 37 mM or less in figure 1 is apoptotic and detected by PI staining because of late changes in apoptotic plasma membrane permeability.²⁶

Lidocaine Depolarizes Mitochondria in Intact Neurons

Mitochondria play a central role in the initiation and amplification of apoptosis. A previous study reported that lidocaine decreased mitochondrial $\Delta\psi$ in intact fibroblasts.³⁴ It did not study exposures longer than a few

minutes that would be more relevant to clinical neurotoxicity and reported that lidocaine required a lipophilic counter-ion to facilitate its entry into the cytoplasm. To test the effect of lidocaine on $\Delta\psi$ in intact neurons, we used the cationic fluorophore R123, which concentrates in energized mitochondria and yields a punctate intracellular fluorescence in proportion to $\Delta\psi$.³⁰ As shown in representative single neuron curves in figure 4, lidocaine at a concentration of 18 mM or greater and 19 mM bupivacaine rapidly depolarized neuronal mitochondria in less than 5 min, with depolarization persisting for the 60 min experimental period. When the change in fluorescence was quantified at 5 and 60 min for all experiments (fig. 5), all lidocaine concentrations of 18 mM (0.5%) or greater were significantly different from their Tris controls. Similar experiments with bupivacaine demonstrated a statistically significant effect on $\Delta\psi$ only with 19 mM bupivacaine (0.625%, equipotent to 92 mM = 2.5% lidocaine) compared with its equimolar Tris control. The decrease in $\Delta\psi$ seen with 0.25 and 0.125% bupivacaine was not statistically significant compared with equimolar Tris controls, in contrast to the effect of

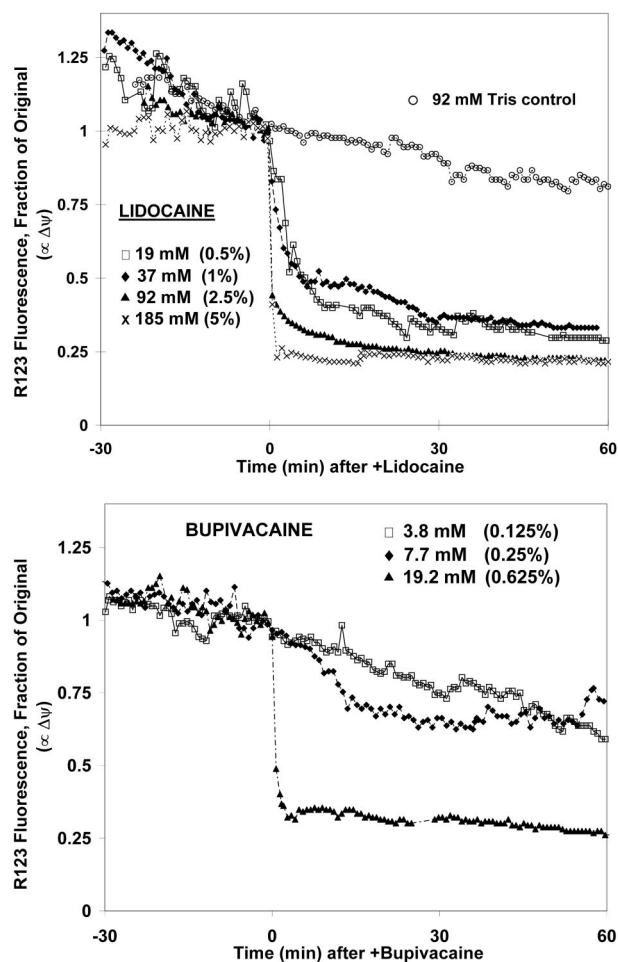


Fig. 4. Effect of lidocaine and bupivacaine on mitochondrial membrane potential ($\Delta\psi$), measured with rhodamine 123 (R123) over a mitochondria-rich area. Representative individual ND7 neuron tracings: 30 min baseline after loading with R123, then + lidocaine or bupivacaine at 0 min. Lidocaine, 3.7 mM, omitted for clarity; similar to Tris control. Slow decrease in fluorescence before lidocaine is due to slow leakage of R123 from neuron. Further decrease in fluorescence beyond leakage rate indicates decrease in $\Delta\psi$.

equipotent 1 and 0.5% lidocaine. Figure 6 shows that the rapid loss of $\Delta\psi$ with 37 mM lidocaine is reversible on removal of lidocaine and repeatable on reexposure to lidocaine. Average fluorescence for the baseline 5 min preceding the second exposure recovered to $96.7 \pm 6.4\%$ of initial for 37 mM lidocaine and to $98.4 \pm 5.7\%$ for 37 mM Tris control ($P > 0.05$ for first *vs.* second exposure baselines, three experiments each for lidocaine and Tris).

To confirm that lidocaine is a mitochondrial depolarizer, we also used a FRET assay of mitochondrial $\Delta\psi$, based on an increase in MFG fluorescence as TMRM diffuses out of the mitochondria when $\Delta\psi$ is lost, thus unquenching MFG fluorescence.³¹ This assay has the advantage that it is not affected by plasma membrane potential or the speed with which cytoplasmic rhodamine fluorophore diffuses out of the neuron. As shown

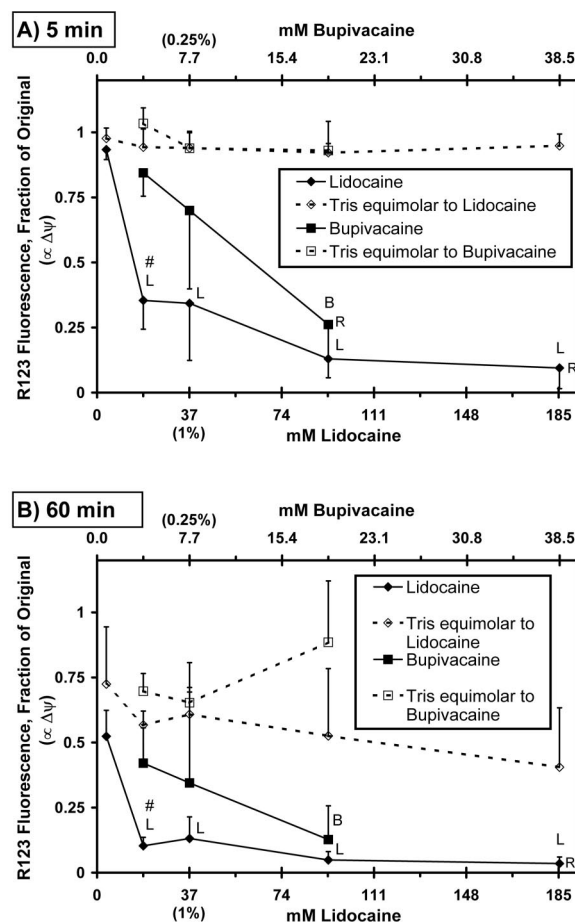


Fig. 5. Quantitative analysis of lidocaine and bupivacaine effect on mitochondrial depolarization, determined using rhodamine 123 (R123) fluorescence at 5 min (A) and 60 min (B) after lidocaine addition. Lidocaine and its equipotential Tris controls are plotted on the lower x-axis, with bupivacaine and its equipotential Tris controls plotted on the upper x-axis. The lower and upper x-axes are scaled so that equipotent concentrations of lidocaine and bupivacaine are at equal x-coordinates assuming a 1:4 ratio of hydrochloride salts ($36.9 \text{ mM} = 1\% \text{ lidocaine HCl}$ equipotent to $7.7 \text{ mM} = 0.25\% \text{ bupivacaine HCl}$). $P < 0.05$ for (L) lidocaine *versus* equipotential Tris, (B) bupivacaine *versus* equipotential Tris, (#) lidocaine *versus* equipotent bupivacaine at indicated concentration. $P < 0.05$ for (R) effect of concentration on indicated local anesthetic or Tris control. $P > 0.05$ for unmarked comparisons. Four to six experiments for each condition; $n > 13$ neurons/experiment.

in figure 7, this assay confirmed that lidocaine at a concentration of 18 mM or greater rapidly caused a complete depolarization of neuronal mitochondria in intact neurons. Compound 1799 is a potent, nonfluorescent mitochondrial uncoupler which maximally depolarizes mitochondria at the concentration tested, leading to a maximal increase in MFG fluorescence.³¹ The effect of compound 1799 was similar to that of 18–74 mM lidocaine, and addition of compound 1799 after depolarization by lidocaine caused no additional mitochondrial depolarization. Quantitative analysis of the FRET assay at 5 and 30 min, similar to that done for R123 in figure 5, confirmed that the effect of lidocaine compared with

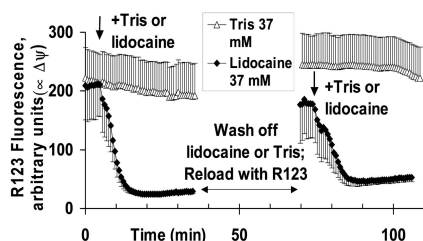


Fig. 6. Mitochondrial depolarization with 37 mM lidocaine is reversible, tested with rhodamine 123 (R123)-loaded ND7 neurons. One experiment typical of three for both lidocaine and Tris; $n > 15$ neurons/experiment.

Tris control was significant for all concentrations tested (18–74 mM, 0.5–2%) and not different from that of compound 1799 (data not shown). These data suggest that lidocaine neurotoxicity is mediated by a direct effect on mitochondria.

Lidocaine Inhibits Mitochondrial Respiration

Lidocaine could cause mitochondrial depolarization by an uncoupling effect or direct respiratory chain inhibition. Lidocaine has been reported both to inhibit respiration¹⁰ and to uncouple respiratory transport³⁵ in isolated mitochondria, although another study found that a lipophilic counter-ion was required to facilitate mitochondrial membrane transport of lidocaine.⁹ The integrated effect on oxygen consumption in whole neurons has not been reported. As shown in figure 8, lidocaine at a concentration of 37 mM or greater almost completely inhibited oxygen consumption in intact ND7 neurons. Therefore, rather than uncouple mitochondria, which would enhance oxygen consumption, lidocaine actually blocks respiratory chain function.

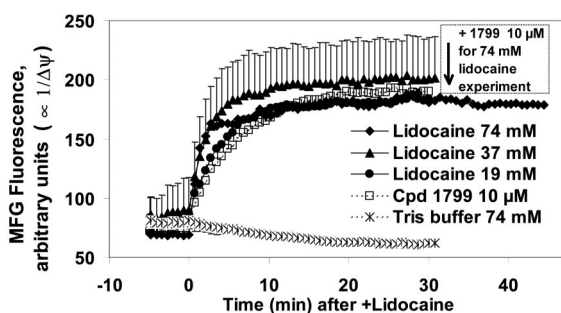


Fig. 7. Effect of lidocaine on mitochondrial membrane potential ($\Delta\psi$), determined by fluorescence resonance energy transfer between MitoFluor Green (MFG) and tetramethyl-rhodamine methyl ester. MFG fluorescence is quenched as tetramethyl-rhodamine methyl ester leaves the mitochondria, so that increased fluorescence indicates decreased $\Delta\psi$. Each curve represents one experiment typical of four experiments with > 15 neurons/experiment. SDs were similar for all concentrations of lidocaine and compound 1799 and are omitted for clarity, except for 37 mM lidocaine. There was no statistically significant difference between lidocaine concentrations at 5 and 30 min. All lidocaine concentrations differed from Tris control ($P < 0.05$) when similarly analyzed. Lack of effect of compound 1799 (a potent nonfluorescent mitochondrial uncoupler) when added at 30 min to 74 mM lidocaine indicates complete mitochondrial depolarization.

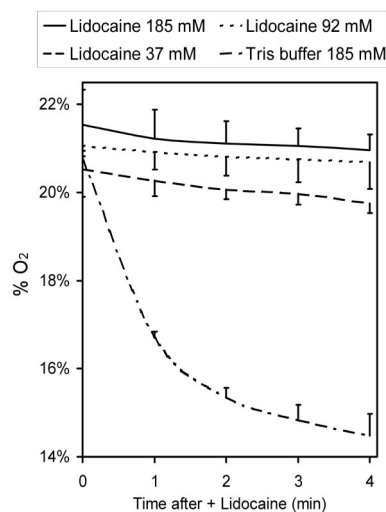


Fig. 8. Effect of lidocaine on neuronal oxygen (O_2) consumption by ND7 neurons in suspension, 10^6 /ml in 5 ml, in a closed container with no gas phase at 25°C. Each curve is an average of three or four experiments. $P < 0.0001$ for slope of Tris buffer versus slope of all lidocaine concentrations; $P > 0.05$ for all comparisons between individual lidocaine concentrations.

Lidocaine Causes Release of Cytochrome c from Mitochondria to Cytoplasm Independent of Caspase Activation

A mitochondrial injury severe enough to proceed to neuronal death generally causes the release of mitochondrial apoptosis-activating factors, including cytochrome c.¹³ ND7 neurons were transfected with cytochrome c-GFP and exposed to lidocaine. Cytochrome c-GFP colocalized with TMRM in energized mitochondria (fig. 9, A1–3). Representative confocal micrographs in figure 9 illustrate the release of cytochrome c-GFP from mitochondria to cytoplasm during lidocaine exposure, with the cytochrome c-GFP image changing from a punctate mitochondrial distribution to a more diffuse cytoplasmic distribution during exposure to 37 mM (1%) lidocaine (fig. 9, A1 and A4) and 185 mM (5%) lidocaine (fig. 9, C1–3) while retaining a mitochondrial distribution with Tris buffer (fig. 9, B1 and B2). Coincident TMRM images (fig. 9, A2 and A5) qualitatively confirmed that 37 mM lidocaine completely depolarized neuronal mitochondria. Quantitative survival curves for the release of cytochrome c-GFP during lidocaine exposure are presented in figure 10. The effect of lidocaine was dose dependent, with 50% of neurons releasing cytochrome c-GFP by 90 min for 37 mM lidocaine, decreasing to 2 min for 185 mM lidocaine. To determine whether release of mitochondrial cytochrome c-GFP was an upstream primary toxic effect of lidocaine or a downstream result of caspase activation by other means, the effect of the pan-caspase inhibitor z-VAD-fmk (100 μ M) was tested. As shown in figure 10, z-VAD-fmk had no significant effect on the time course of cytochrome c-GFP release with lidocaine. Mitochondrial injury by lidocaine is upstream of

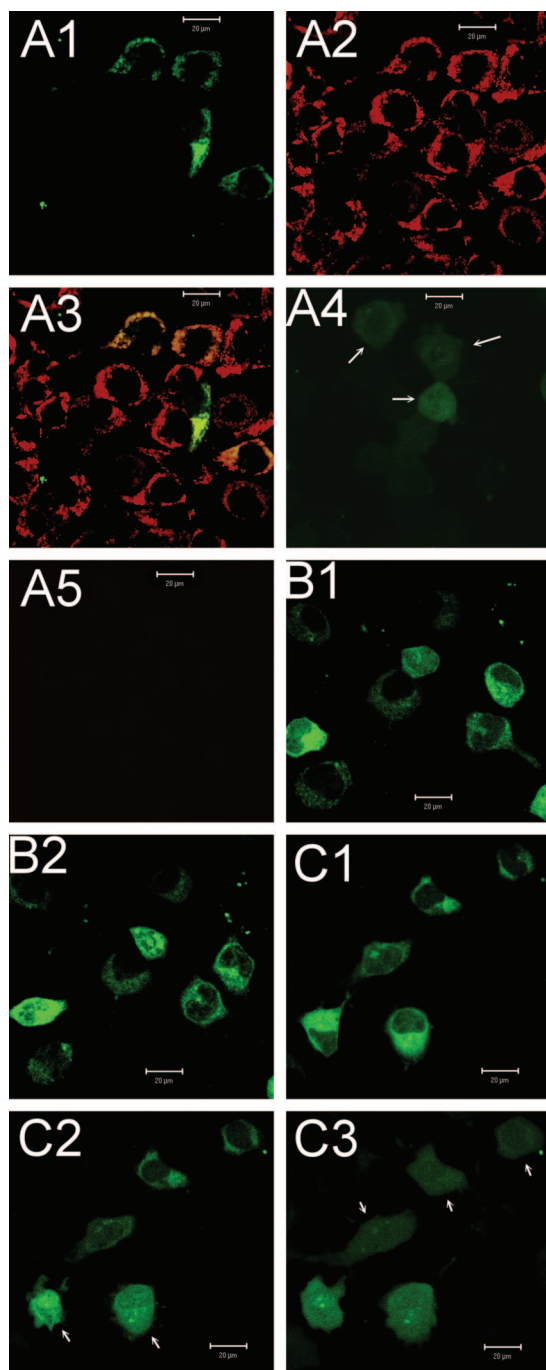


Fig. 9. Cytochrome c–green fluorescent protein (cyt c-GFP) release from mitochondria into cytoplasm in confocal fluorescence micrographs. *Arrows* indicate neurons that have released cytochrome c to the cytoplasm since the preceding cyt c-GFP image of the same field, shown by a change from punctate to diffuse, more homogeneous fluorescence image. Scale bar = 20 μ m. (A) Lidocaine, 37 mM, same field. (A1–3) Before lidocaine, A1 = cyt c-GFP, A2 = tetramethyl-rhodamine methyl ester (TMRM), A3 = TMRM image overlaid on cyt c-GFP image. Yellow-orange neurons in overlaid image (A3) show colocalization of TMRM and cyt c-GFP to mitochondria. (A4–5) 110 min exposure to 37 mM lidocaine, A4 = cyt c-GFP, A5 = TMRM. (B) Tris buffer, 37 mM, same field, cyt c-GFP. B1 = before Tris, B2 = 120-min exposure to Tris. Tris buffer, 185 mM, similarly did not cause cyt c-GFP release (not shown). (C) Lidocaine, 185 mM, same field, cyt c-GFP. C1 = before lidocaine, C2 = 2 min, C3 = 4-min exposure to lidocaine.

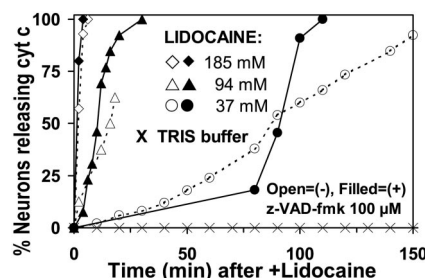


Fig. 10. Time course of cytochrome c release from mitochondria to cytoplasm with and without the pan-caspase inhibitor z-VAD-fmk 100 μ M. Symbols without z-VAD-fmk: \diamond = 185 mM lidocaine; \triangle = 94 mM lidocaine; \circ = 37 mM lidocaine; X = 37–185 mM Tris. Symbols with 100 μ M z-VAD-fmk: \blacklozenge = 185 mM lidocaine; \blacktriangle = 94 mM lidocaine; \bullet = 37 mM lidocaine. The effect of z-VAD-fmk was not significant for any lidocaine concentration. All pairwise comparisons between different lidocaine concentrations and Tris buffer control were significant ($P < 0.001$). $n = 19, 34, 61$, and 51 neurons for 185 mM, 92 mM, and 37 mM lidocaine and equimolar Tris controls, respectively, in three or more experiments for each condition.

caspase activation and is therefore likely a primary event in lidocaine neurotoxicity.

Discussion

The question of whether lidocaine is neurotoxic at concentrations injected into the subarachnoid space for spinal anesthesia has been contentious, despite case reports of such neurotoxicity.^{36–38} All local anesthetics have marked toxic effects on the growth of embryonal or cancerous neurons at less than a hundredth of the concentration used clinically for spinal anesthesia.^{39–44} Injury to peripheral nerve occurs with higher perineural lidocaine concentrations (20–100 mM) similar to those injected for spinal anesthesia.^{45–47} However, the relevance of these models to the effect of lidocaine on mature spinal neurons is unclear.

Our previous study using the ND7 cell line as a model of a terminally differentiated, central sensory neuron found that for an exposure period of 1 h, only the highest concentration tested, 185 mM lidocaine, caused necrotic cell death, whereas 18–92 mM caused no necrosis.⁷ The current study, assessing multiple markers of apoptosis as well as necrosis, establishes that lidocaine can induce both apoptosis and necrosis in mature, differentiated neurons at concentrations that are routinely injected into the subarachnoid space for spinal anesthesia. Rapid dilution of injected lidocaine *via* mixing with CSF is undoubtedly a major factor preventing irreversible neuronal injury in the majority of cases, but the potential for maldistribution with high local concentrations exists and has resulted in persistent clinical neurotoxicity.¹ Although rapid dilution of regular dose lidocaine generally limits CSF lidocaine concentrations to approximately 10 mM (approximately 0.25%) for the first 15 min after subarachnoid injection,⁶ well-mixed high-dose lidocaine

can yield 55 mM (1.5%) in CSF at 2 min after injection, and poorly mixed high-dose lidocaine can yield greater than 55 mM in CSF for at least 15 min after injection.⁴⁸

Our findings also suggest a speculative but plausible mechanism for the transient pain syndrome that often follows lidocaine spinal anesthesia without maldistribution: Lower concentrations of lidocaine and shorter exposure times inhibit mitochondrial energetics (figs. 4, 5, and 7) and manifest early activation of apoptosis with phosphatidylserine membrane asymmetry (fig. 2), while being reversible (fig. 6) and not leading to the irreversible release of cytochrome c until much longer exposures than would be maintained in the CSF after a single-injection spinal anesthetic (figs. 9 and 10).

The relevance of our *in vitro* data to lidocaine spinal neurotoxicity is supported not only by its concentration dependence being in the range of clinically achievable CSF concentrations, but also by the greater loss of $\Delta\psi$ with equipotent concentrations of lidocaine, compared with bupivacaine (figs. 4 and 5). This is consistent with animal models of spinal neurotoxicity^{49,50} and a preponderance of lidocaine over bupivacaine in clinical reports of neurotoxicity,^{1,51} although the latter is confounded by the commercial preparation of spinal lidocaine (5%) being more potent than bupivacaine (0.75%). A limitation of our model, as with most *in vitro* models, is the difference between an isolated, transformed cell line and the *in vivo* cell type it is modeling. Neoplastic cell lines cultured under optimal conditions are generally more resistant to most toxic injuries than the corresponding parent cell.⁵² Such a difference would argue that our results underestimate, rather than exaggerate, the neurotoxic potential of lidocaine.

The rapidity with which $\Delta\psi$ is lost on exposure to lidocaine even at 37 mM (figs. 4 and 7) where cytochrome c release is delayed (figs. 9 and 10) suggests that mitochondrial injury is the proximal event of lidocaine neurotoxicity, followed by downstream caspase activation. This contrasts with many other models of apoptosis (e.g., ultraviolet light,³³ staurosporine⁵³) where loss of $\Delta\psi$ does not occur until after release of cytochrome c and always precedes the exposure of phosphatidylserine on the external face of the plasma membrane. Although lidocaine does activate neuronal caspases, blockade of caspase activation did not prevent cytochrome c release (fig. 10) or membrane blebbing (fig. 3) and delayed but did not change the final extent of neuronal death at saturating lidocaine concentration or exposure time (fig. 1). Hence, there seem to be both caspase-dependent and caspase-independent pathways to cell death after mitochondrial injury with lidocaine.

The nature of the mitochondrial injury from lidocaine remains to be determined. Local anesthetics are lipophilic amines that are weakly acidic in their protonated form, with an acid dissociation constant of approximately 8, suggesting the hypothesis that they act as

mitochondrial uncouplers, collapsing the proton gradient similar to the classic uncoupler 2,4-dinitrophenol. There is experimental support for uncoupling in isolated mitochondria with bupivacaine, a more lipophilic congener of lidocaine.^{9,54} However, if this were the primary effect of lidocaine on intact neurons, lidocaine should increase oxygen consumption, but instead it almost completely blocks oxygen consumption (fig. 8). A more recent study has shown that although bupivacaine at a concentration of 1.5 mM or less does uncouple isolated muscle mitochondria, higher concentrations inhibit respiration, consistent with our findings.⁵⁵

The concentrations of lidocaine that caused neuronal death in this study, 2.3 mM and higher, are at least two orders of magnitude higher than plasma concentrations when lidocaine is administered intravenously for arrhythmia or neuroprotection. Plasma concentrations exceeding the ED₅₀ of lidocaine for Na⁺ block, approximately 50 μ M, cause seizures and cardiovascular collapse.⁵⁶ Our data are therefore most applicable to spinal anesthesia, where the highest perineural concentrations of lidocaine are encountered, and possibly to other forms of regional anesthesia where lidocaine must be injected at concentrations of 37 mM (1%) or greater to allow sufficient diffusion and Na⁺ blockade of the target neurons.

Our results add to the growing body of evidence that lidocaine and other local anesthetics have multiple effects other than Na⁺ block. At concentrations much lower than that needed for Na⁺ block, lidocaine potently affects G protein-coupled receptors, attenuating hyperactive inflammatory and coagulation responses,⁵⁷ is therapeutic against some types of neuropathic pain,^{58,59} and inhibits axonal transport.⁴² Dibucaine, although not lidocaine, inhibits Bax-induced cytochrome c release from neuronal mitochondria.⁶⁰ Even higher concentrations of lidocaine, administered as a spinal anesthetic, have been shown to have long-lasting analgesic effects in some animal pain models for days beyond dissipation of the Na⁺ block.⁶¹ Therefore, although our findings have particular implication for the neurotoxicity of lidocaine spinal anesthesia, they also suggest caution in the use of lidocaine as a specific Na⁺-blocking agent in experimental preparations.

The central role of mitochondrial injury as a key initiator, effector, or amplifier in many, if not most, models of apoptosis¹⁷⁻²¹ argues that the mitochondrial injury described here is likely to be mechanistically important in lidocaine neurotoxicity. However, further research is needed to establish that mitochondrial injury, rather than another yet uncharacterized effect of lidocaine, is the most important element in the pathway to neuronal death after lidocaine exposure.

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