

Lidocaine Induces Apoptosis via the Mitochondrial Pathway Independently of Death Receptor Signaling

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Background: Local anesthetics, especially lidocaine, can lead to persistent cauda equina syndrome after spinal anesthesia. Recently, lidocaine has been reported to trigger apoptosis, although the underlying mechanisms remain unknown. To elucidate the pathway of lidocaine-induced apoptosis, the authors used genetically modified cells with overexpression or deficiencies of key regulators of apoptosis.

Methods: Human Jurkat T-lymphoma cells overexpressing the antiapoptotic protein B-cell lymphoma 2 as well as cells deficient of caspase 9, caspase 8, or Fas-associated protein with death domain were exposed to lidocaine and compared with parental cells. The authors evaluated cell viability, mitochondrial alterations, cytochrome *c* release, caspase activation, and early apoptosis. Apoptosis was in addition investigated in neuroblastoma cells.

Results: In Jurkat cells, lidocaine reduced viability, associated with a loss of the mitochondrial membrane potential. At low concentrations (3–6 mM) of lidocaine, caspase 3 was activated and release of cytochrome *c* was detected, whereas at higher concentrations (10 mM), no caspase activation was found. Apoptosis by lidocaine was strongly reduced by B-cell lymphoma-2 protein overexpression or caspase-9 deficiency, whereas cells lacking the death receptor pathway components caspase 8 and Fas-associated protein with death domain were not protected and displayed similar apoptotic alterations as the parental cells. Lidocaine also induced apoptotic caspase activation in neuroblastoma cells.

Conclusions: Apoptosis is triggered by concentrations of lidocaine occurring intrathecally after spinal anesthesia, whereas higher concentrations induce necrosis. The data indicate that death receptors are not involved in lidocaine-induced apoptosis. In contrast, the observation that B-cell lymphoma-2 protein overexpression or the lack of caspase 9 abolished apoptosis clearly implicates the intrinsic mitochondrial death pathway in lidocaine-induced apoptosis.

LIDOCAINE can lead to cauda equina syndrome and transient neurologic symptoms after spinal anesthesia.^{1,2} In animal studies, a concentration- and time-dependent cytotoxicity of many local anesthetics including lidocaine has been demonstrated.^{3–9} The mechanism of this toxicity is unrelated to the primary action of all local anesthetics, the blockade of the voltage-gated sodium

channel.⁶ *In vitro* lidocaine has been shown to induce apoptosis, a major form of programmed cell death,^{10,11} but the underlying mechanism remains unknown. Mitochondrial injury has been described as one possible cause of cytotoxic effects of lidocaine in neural hybrid cell cultures¹² and dorsal root ganglia cells,¹³ but could not be linked to a specific pathway of apoptosis.

Apoptosis is largely controlled by a family of aspartate-specific cysteine proteases, called *caspases*, that function as initiators and executioners of the apoptotic process.¹⁴ Caspases are activated by two major signaling routes, the extrinsic death receptor and the intrinsic mitochondrial pathway, which both depend on the formation of large multiprotein complexes.¹⁵ Initiator caspase 8 is the key mediator of the extrinsic pathway.¹⁶ In a simplified model (fig. 1), binding of death ligands, such as tumor necrosis factor or CD95L, to their respective death receptors leads to receptor oligomerization. This event then results in the recruitment of the adapter Fas-associated protein with death domain (FADD) and caspase 8 into a death-inducing signaling complex (DISC). In the DISC, the caspase 8 is activated by dimerization and autoproteolytic cleavage and subsequently activates caspase 3, resulting in the further cleavage of several cellular targets that are responsible for the morphologic alterations of cell death.

The intrinsic pathway, in contrast, is regulated at mitochondria, which release cytochrome *c* and other proapoptotic factors during different forms of cellular stress.^{17–19} The release of cytochrome *c* is controlled by proteins of the B-cell lymphoma-2 (Bcl-2) protein family, which are characterized by so-called Bcl-2 homology (BH) domains. Bcl-2 protein family members are classified into two major groups: first, the antiapoptotic members, including the Bcl-2 protein, which inhibits mitochondrial membrane permeabilization and subsequent release of apoptosis-inducing proteins from the mitochondrion^{20,21}; and second, the proapoptotic members, which are further subdivided into the multidomain proteins, *e.g.*, the Bcl-2-associated X protein (Bax) or the Bcl-2-homologous antagonist/killer (Bak), and the BH3-only proteins.^{20,21} Upon apoptosis induction, BH3-only proteins activate Bax and Bak, which subsequently undergo a conformational change, leading to their assembly into pore-forming multimers at the outer mitochondrial membrane and cytochrome *c* release.²² In the cytosol, cytochrome *c* together with caspase 9 induces the formation of the apoptosome, thereby triggering the caspase cascade and subsequent apoptosis.

In the current study, we investigated the role of the

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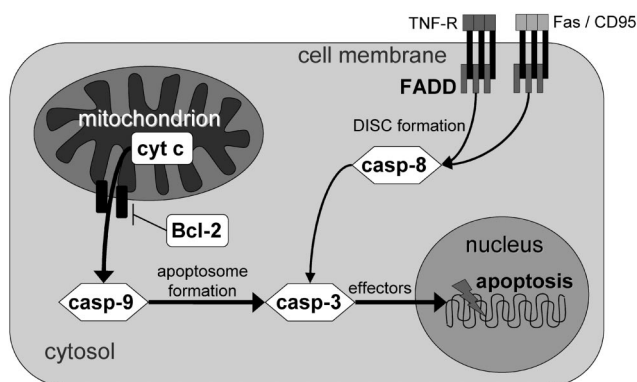


Fig. 1. The two major pathways of apoptosis. The intrinsic or mitochondrial pathway of apoptosis (*left*) involves mitochondrial dysfunction, release of cytochrome *c* (cyt *c*), and the subsequent activation of caspase 9 (casp-9) at the apoptosome. The antiapoptotic protein B-cell lymphoma 2 (Bcl-2) inhibits the release of cytochrome *c* from the mitochondrion. The extrinsic or death receptor pathway (*right*) is initiated by binding of death ligands to their cognate death receptors and subsequent recruitment of the adapter protein Fas-associated protein with death domain (FADD) and caspase 8 (casp-8) into the death-inducing signaling complex (DISC). Both apoptosis pathways converge at the activation of effector caspase 3 (casp-3), which cleaves several cellular proteins, finally leading to the typical alterations of apoptosis such as DNA fragmentation. Fas = fibroblast-associated receptor; TNF-R = tumor necrosis factor receptor.

cellular antiapoptotic protein Bcl-2 on lidocaine-induced apoptosis. In addition, we investigated the consequences of caspase 9, caspase 8, or FADD deficiency on lidocaine-induced apoptosis by determining the amount of apoptotic cells induced by lidocaine. Finally, we evaluated apoptosis induction at similar lidocaine concentrations and the protective effect of a pancaspase inhibitor in neuroblastoma cells. We hypothesized that lidocaine can induce apoptosis by specific activation of the mitochondrial pathway and does not interfere with the death receptor-mediated pathway in a human cell culture model.

Materials and Methods

Reagents

Unless stated otherwise, reagents were purchased from Sigma Aldrich (St. Louis, MO). Lidocaine was obtained as a hydrochloride salt in the commercially available 20% solution (AstraZeneca, London, United Kingdom). Phosphate-buffered saline (PBS) without calcium and magnesium was purchased from Gibco, Invitrogen (Carlsbad, CA). The fluorogenic caspase-3 substrate *N*-acetyl-Asp-Glu-Val-Asp-aminomethyl-coumarin (Ac-DEVD-AMC) was obtained from BIOMOL International (Plymouth Meeting, PA). The fluorescent probe annexin-V-FITC conjugate was obtained from BD Biosciences (San Diego, CA). The fluorescent dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraacetylbenzimidazolyl-carbocyanine iodide (JC-1) and the pancaspase inhibitor *N*-(2-quinoly)valyl-aspartyl-(2,6-difluorophenoxy)methylketone (Q-VD) were purchased from Calbiochem (San Diego, CA).

The following primary antibodies were used: mouse monoclonal anti-human Bcl-2 (Novocastra, Newcastle, United Kingdom), goat polyclonal anti-caspase 3 (R&D Systems, Minneapolis, MN), rabbit polyclonal anti-human caspase 9 (New England BioLabs Inc., Beverly, MA), mouse monoclonal anti-human caspase 8 (Cell Signaling Technology, Beverly, MA), polyclonal rabbit anti-human β -actin, mouse monoclonal anti-human cytochrome *c*, and mouse monoclonal anti-Tom 20 (clone 29; BD Biosciences Pharmingen, Heidelberg, Germany). As secondary antibodies, goat anti-rabbit from Jackson Immunolab (Dianova, Hamburg, Germany) and goat anti-mouse and rabbit anti-goat antibodies (Southern Biotechnology Associates, Birmingham, AL) conjugated to horseradish peroxidase were used.

Cell Culture

Jurkat cells stably overexpressing Bcl-2 and the corresponding wild-type cells (clone J16) have been described before.²³ Caspase 9-deficient (clone JMR) and -proficient Jurkat cells have been characterized before.^{24,25} FADD- and caspase 8-deficient Jurkat cells and the parental cell line (clone A3) were kindly provided by John Blenis, Ph.D. (Department of Cell Biology, Harvard Medical School, Boston, Massachusetts).²⁶ Human SHEP neuroblastoma cells have been characterized before.^{27,28} All cells lines were grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, and 50 μ g/ml each of penicillin and streptomycin. All cells were cultured under equal conditions, including a humidified atmosphere containing 5% carbon dioxide at 37°C.

Exposure to Lidocaine and Experimental Protocol

Before the experiments, cells were cultured overnight in complete medium at a concentration of 4×10^5 cells/ml to allow logarithmic growth. Subsequently, Jurkat cells were seeded at the appropriate density depending on assay protocol. Cells were cultured with medium alone as negative control, the proapoptotic kinase inhibitor staurosporine as positive control, or indicated concentrations of lidocaine. The pancaspase inhibitor Q-VD (10 μ M) was added to cell cultures 1 h before the experiments.

Cell Viability Assay

For detection of viability, the cells were adjusted to a density of 5×10^5 /ml and a sample volume of 5 ml before incubation. After incubation with lidocaine for 24 h, cells were resuspended, and 10- μ l samples of each condition were stained with 90 μ l trypan blue solution. Cell viability was assessed immediately by estimating the ratio of stained and unstained cells in four fields of a Neubauer counting chamber by light microscopy.

Caspase-3 Activity Assay

For detection of caspase activity, cells were adjusted at a density of 5×10^5 /ml in a sample volume of 15 ml and

incubated with lidocaine for 24 h. Cells were then harvested and lysed for 20 min in a high-salt buffer containing 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, and 1% NP-40 as well as 1 μ M pepstatin, 0.1 μ M phenylmethylsulfonylfluoride, 0.15 μ M aprotinin, and 1 μ M leupeptin as protease inhibitors. Lysates were centrifuged at 10,000g at 4°C for 15 min, and the supernatants were harvested. Samples were adjusted to an equal protein concentration of 1 μ g/ μ l with lysis buffer in a volume of 50 μ l and measured in 150 μ l substrate buffer (50 mM HEPES, pH 7.3, 100 mM NaCl, 10% sucrose, 0.1% 3-[(3-cholamidopropyl)demethyl-ammonio]-1-propanesulfonate, 10 mM dithiothreitol) supplemented with 50 μ M of the fluorogenic caspase substrate Ac-DEVD-AMC. Caspase-3 activity was recorded every 10 min by measuring the release of AMC at 440 nm in a Lambda Fluoro 320 Plus fluorometer (Biotek, Bad Friedrichshall, Germany) at 37°C until values of the positive control reached a plateau.

Mitochondrial Membrane Potential Assay

The mitochondrial transmembrane potential ($\Delta\Psi_m$) was analyzed using the $\Delta\Psi_m$ -specific stain JC-1.²⁹ In brief, after 24 h of treatment, 5×10^5 cells per sample were washed twice with cold PBS and stained for 20 min in the dark with 10 μ g/ml JC-1 in PBS. Cells were then washed twice, resuspended in 250 μ l PBS, and immediately analyzed by fluorescence-activated cell-sorting (FACS) analysis. The loss of $\Delta\Psi_m$ was detected by an increased ratio of green and red fluorescence intensity of stained mitochondria.³⁰

Western Blot Analysis

Cell extracts were prepared as described above and measured for protein content using the bicinchoninic acid assay (Pierce, Rockford, IL). Equal amounts of protein (20 μ g per lane) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Amersham Pharmacia, Piscataway, NJ) as described.³¹ Blotting was performed at 1 mA/cm² for 1 h in a transblot SD cell (Bio-Rad, Munich, Germany). The membrane was blocked for 2 h with 0.05% Tween-20 in PBS containing 4% bovine serum albumin and incubated with the primary antibodies overnight at 4°C. After washing with 0.05% Tween-20 in PBS, the respective horseradish peroxidase-coupled secondary antibodies were applied for 1 h at room temperature. Finally, the membrane was washed in PBS with 0.05% Tween-20, and protein bands were detected using the enhanced chemiluminescence system (Amersham Buchler, Braunschweig, Germany).

Measurement of Cytochrome c Release

Approximately 3×10^7 cells were permeabilized for 15 min at 4°C in a buffer containing 50 μ g/ml digitonin, 250 mM sucrose, 20 mM HEPES, pH 7.4, 1.5 mM MgCl₂, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 2 μ g/ml of each of the

protease inhibitors aprotinin, pepstatin, and leupeptin. Samples were resuspended 20 times through a 21-gauge injection needle to permeabilize the cell membranes. Cells were centrifuged at 1,000g for 5 min at 4°C to remove cell nuclei. The supernatant was transferred to a fresh tube and centrifuged at 10,000g for 15 min at 4°C. For recovery of the mitochondrial fraction, the remaining pellets containing mitochondria were lysed using the high-salt cell lysis buffer as described above and centrifuged at 10,000g for 15 min at 4°C. The resulting supernatants containing the cytosolic or mitochondrial fractions were adjusted for equal protein concentrations and loaded onto a sodium dodecyl sulfate polyacrylamide gel. Detection of the translocase of outer mitochondrial membrane 20 (Tom 20) and cytochrome c release was accomplished by immunoblot analysis as described above.

Detection of Early Apoptosis

The fraction of cells in an early state of apoptosis was determined by staining cells with annexin-V and propidium iodide (PI). Annexin-V binds to phosphatidylserine that exposed onto the outer leaflet of the plasma membrane in early apoptosis, whereas PI is excluded by cells with intact plasma membranes. PI uptake is therefore a sign of necrosis, whereas cells positive for annexin-V but negative for PI are generally defined as early apoptotic.³² Briefly, to determine early apoptosis, cells were washed twice with cold PBS and resuspended at a concentration of 1×10^6 cells/ml in annexin-V binding buffer (10 mM N-[2-hydroxyethyl] piperazin-N'-3[propanesulfonic acid]/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂). Cells were then incubated for 15 min at room temperature with 5 μ l annexin-V and 10 μ l PI (20 μ g/ml) and measured with a FACScalibur (Becton Dickinson, Heidelberg, Germany) using CellQuest Pro software. For each measurement, at least 10,000 cells were analyzed.

Statistical Analysis

All experiments were performed at least three times. Results are expressed as mean \pm SD. All calculations were performed with the SPSS program version 12.0 (SPSS Inc., Chicago, IL). Comparisons between groups were made by Mann-Whitney U test and corrected for multiple comparisons (Bonferroni) where appropriate. $P < 0.05$ was considered significant.

Results

Dose-dependent Effect of Lidocaine on Jurkat Wild-type Cells

Lidocaine-induced cell death was determined by incubating Jurkat wild-type cells with increasing concentrations of lidocaine (3 mM \approx 0.08%, 6 mM \approx 0.18%, and 10 mM \approx 0.27%) for 24 h. In a first set of experiments, cell death was measured by trypan blue staining, demonstrating that lido-

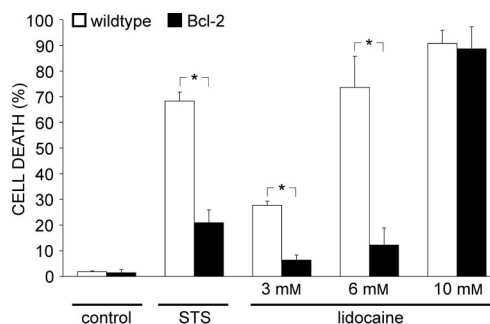


Fig. 2. Lidocaine-induced cell death in B-cell lymphoma-2 protein (Bcl-2)-overexpressing cells compared with parental wild-type cells. Jurkat wild-type cells and Jurkat cells overexpressing Bcl-2 were treated with the indicated concentrations of lidocaine or 1.25 μ M staurosporine (STS) as a positive control. After 24 h, cell death was quantified by trypan blue staining. Note that Bcl-2 overexpression strongly protected against cell death in response to treatment with 3 mM ($\approx 0.08\%$) and 6 mM ($\approx 0.16\%$) lidocaine, whereas no protection by Bcl-2 was observed after incubation with 10 mM ($\approx 0.27\%$) lidocaine. * $P < 0.05$. Error bars indicate SD.

caine as well as the broad protein kinase inhibitor staurosporine, which was used as a positive control, led to dose-dependent induction of cell death (fig. 2). Furthermore, treatment with both agents resulted in the loss of mitochondrial membrane potential ($\Delta\Psi_m$), which is one the first detectable signs of apoptosis. Flow cytometric analysis revealed a loss of $\Delta\Psi_m$ in 39.1 ± 12.2 , 84.6 ± 15.5 , and $98.8 \pm 1.2\%$ of cells treated with 3, 6, and 10 mM lidocaine, respectively (fig. 3). Because activation of caspase 3 is a manifest sign of the execution phase of apoptosis, the activation of caspase 3 was investigated in substrate assays using the fluorogenic substrate Ac-DEVD-AMC. Treatment with 3 and 6 mM lidocaine increased caspase-3 activity by 9.0- and 11.6-fold compared with untreated cells (fig. 4). Results of Western blot analysis are presented in figure 5. The activation of caspase 3 was confirmed detecting the

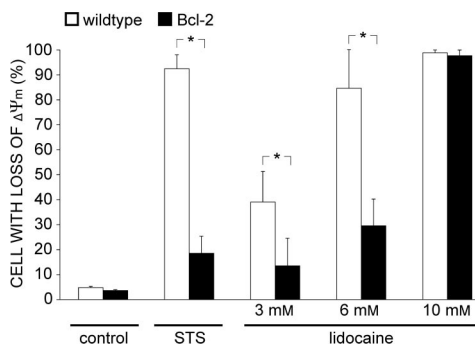


Fig. 3. Lidocaine induces a loss of the mitochondrial membrane potential, which is preserved by B-cell lymphoma-2 protein (Bcl-2). Jurkat wild-type and cells overexpressing Bcl-2 were treated for 24 h with 1.25 μ M staurosporine (STS) or the indicated concentration of lidocaine. The mitochondrial membrane potential ($\Delta\Psi_m$) was measured by staining with the potentiometric dye JC-1 and subsequent flow cytometry. The fraction of wild-type cells with reduced $\Delta\Psi_m$ was dose-dependently increased after lidocaine treatment, whereas $\Delta\Psi_m$ remained stable in Bcl-2-overexpressing cells. In contrast, Bcl-2 did not preserve $\Delta\Psi_m$ after treatment with 10 mM ($\approx 0.27\%$) lidocaine. * $P < 0.05$. Error bars indicate SD.

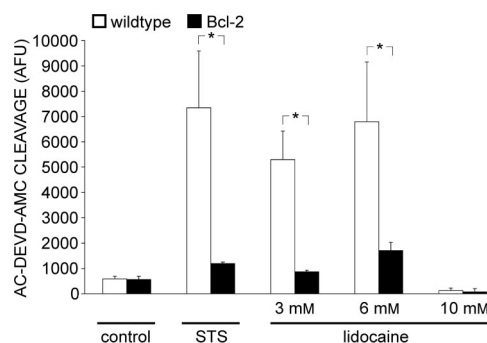


Fig. 4. Activation of caspase 3 by lidocaine in Jurkat wild-type and B-cell lymphoma-2 protein (Bcl-2)-overexpressing cells. Caspase-3 activity was measured in cell lysates after 12 h of treatment with 1.25 μ M staurosporine (STS) or the indicated concentrations of lidocaine using the fluorogenic caspase substrate Ac-DEVD-AMC. Overexpression of Bcl-2 strongly prevented caspase-3 activation in response to 3 mM ($\approx 0.08\%$) and 6 mM ($\approx 0.16\%$) lidocaine. Note that after treatment with 10 mM ($\approx 0.27\%$) lidocaine, no activation of caspase 3 was detected. AFU = arbitrary fluorescence units. * $P < 0.05$. Error bars indicate SD.

processing and hence the proteolytic activation of caspase 3 in cells treated for 12 h with 3 and 6 mM lidocaine (fig. 5A). A decrease of procaspase 9 was also detected, indicating an activation of caspase 9 by lidocaine (fig. 5B). Whereas low concentrations of 3–6 mM lidocaine induced these apoptotic alterations, caspase activation was not detected after treatment with 10 mM (figs. 4 and 5A), suggesting that treatment with higher concentrations of lidocaine was associated with necrotic cell death.

In addition to caspase activation, lidocaine induced the proapoptotic release of cytochrome *c* from mitochondria. Cell fractionation revealed the release of mitochondrial cytochrome *c* into the cytosol, which was strongly pronounced after treatment with 3 and 6 mM, but less evident at 10 mM lidocaine (fig. 5E). Another apoptotic feature is the exposure of phosphatidylserine onto the outer leaflet of a still preserved plasma membrane, which can be measured by double staining with annexin-V and PI. Apoptotic cells that were positive for annexin-V but negative for PI uptake were found after treatment with 3 and 6 mM lidocaine within 2 h to more than 24 h. The highest fraction of early apoptotic cells ($20.7 \pm 2.9\%$) was detected after 12 h of treatment with 6 mM lidocaine and comparable to the result obtained with staurosporine (fig. 6). Treatment with 10 mM lidocaine did not increase early apoptosis but resulted in elevated necrosis, as indicated by $93.8 \pm 5.2\%$ of cells double positive for annexin-V and PI after 12 h (fig. 6).

Lidocaine-induced Apoptosis Is Inhibited by Bcl-2

The mitochondrial release of cytochrome *c* can be inhibited by antiapoptotic Bcl-2 proteins. Indeed, Bcl-2-overexpressing Jurkat cells displayed significantly increased survival after treatment with staurosporine or 3 and 6 mM lidocaine compared with parental cells. In contrast, no differences were found after treatment with

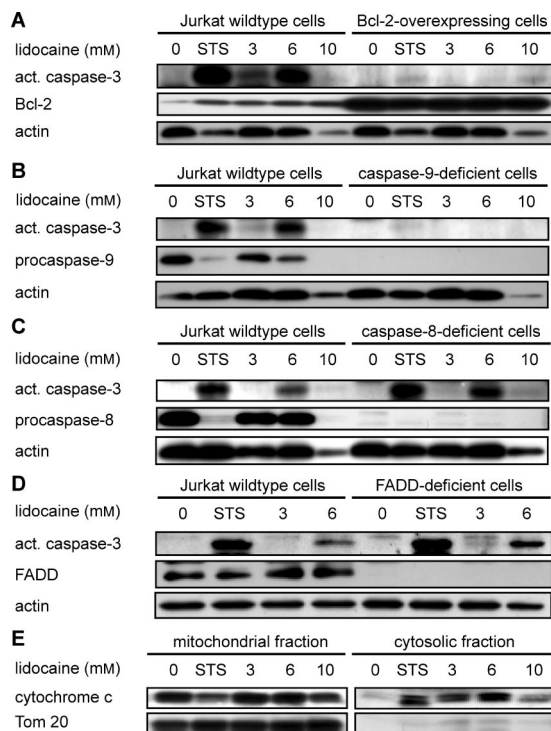


Fig. 5. (A–D) Western blot analysis of caspase activation in different Jurkat cell clones after treatment with lidocaine. (A) Jurkat wild-type cells, Jurkat cells overexpressing the B-cell lymphoma 2 protein (Bcl-2), and (B) cells deficient of either caspase 9, (C) caspase 8, or (D) Fas-associated protein with death domain (FADD) were treated with 1.25 μ M staurosporine (STS) or the indicated concentrations of lidocaine. After 12 h, cell lysates were prepared and subjected to Western blot analysis using antibodies against caspase 8, caspase 9, Bcl-2, or the cleaved active form of caspase 3 (act. caspase-3). Analysis of actin expression served as a loading control. Caspase-3 activation was detected after treatment with 3 mM ($\approx 0.08\%$) and 6 mM ($\approx 0.16\%$) lidocaine in Jurkat wild-type cells, but not in Bcl-2-overexpressing (A) or in caspase 9-deficient cells (B). In contrast, caspase-3 activation was not compromised in Jurkat cells deficient of caspase 8 (C) or FADD (D) as compared with wild-type cells. Treatment with 10 mM ($\approx 0.27\%$) lidocaine led to no detectable caspases-3 activation, indicating necrotic cell death. (E) Lidocaine treatment triggers the mitochondrial release of cytochrome c release. Parental Jurkat cells were treated with 1.25 μ M STS or the indicated concentrations of lidocaine. After 12 h, mitochondrial and cytosolic fractions were prepared and analyzed for cytochrome c content using Western blot analysis. Detection of the translocase of outer mitochondrial membrane 20 (Tom 20) confirmed the purity of the mitochondrial and cytosolic fractions. Release of mitochondrial cytochrome c into the cytosol was strongly pronounced after treatment with 3 and 6 mM, but less evident at 10 mM lidocaine.

10 mM lidocaine, resulting in approximately 90% of cell death in both the parental and the Bcl-2-overexpressing cell line (fig. 2). Bcl-2 overexpression did not only preserve cell viability but also retained the mitochondrial membrane potential after treatment with 3 and 6 mM lidocaine, whereas 10 mM lidocaine reduced $\Delta\Psi_m$ in almost all cells ($97.7 \pm 2.3\%$; fig. 3). Caspase-3 proteolytic activity after exposure to 3 and 6 mM lidocaine was also reduced compared with wild-type cells, whereas no caspase activity was detected in either cell line after incubation with 10 mM lidocaine (fig. 4). Furthermore,

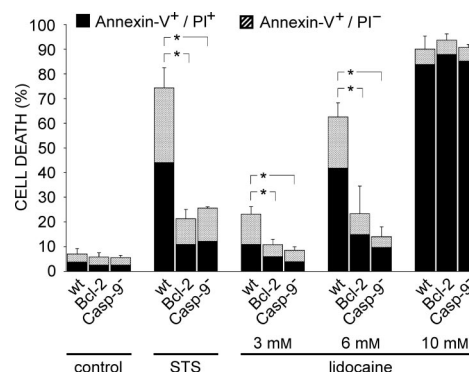


Fig. 6. Lidocaine-induced apoptosis is abolished by a blockade of the mitochondrial pathway. Wild-type (wt), B-cell lymphoma-2 protein-overexpressing (Bcl-2), or caspase 9-deficient (casp-9⁻) cells were treated for 12 h with staurosporine (STS) or lidocaine in concentrations indicated. Early apoptosis was measured by flow cytometric staining with annexin-V to detect phosphatidylserine exposure. Counterstaining with propidium iodide (PI) was used to detect dead cells undergoing primary or secondary necrosis. Inhibition of the mitochondrial pathway by Bcl-2 overexpression or caspase-9 deficiency reduced the fraction of early apoptotic (annexin-V-positive, PI-negative) cells after treatment with 3 and 6 mM lidocaine and 1.25 μ M STS. The fraction of cells undergoing a late phase of cell death (annexin-V-positive and PI-positive cells) was also significantly reduced ($P < 0.05$). Inhibition of the mitochondrial pathway, however, did not prevent cell death induced by 10 mM lidocaine, which was largely necrotic and observed in more than 90% of the cells. * $P < 0.05$. Error bars indicate SD.

Western blot analysis confirmed reduced caspase-3 activation in Bcl-2-overexpressing cells compared with the parental cells in response to staurosporine and lidocaine (fig. 5A). After treatment with 3 and 6 mM lidocaine for 12 h, overexpression of Bcl-2 reduced the fraction of early apoptotic cells as well as the overall fraction of dead cells. In Bcl-2-overexpressing cells exposed to 10 mM lidocaine, the fraction of early apoptotic cells was not higher than in untreated controls (fig. 6), whereas the overall fraction of dead cells reached $97.0 \pm 2.5\%$, again indicating a nonapoptotic form of cell death.

Caspase 9-deficient Cells Are Protected against Lidocaine-induced Apoptosis

Caspase 9 is the essential initiator caspase in mitochondrial death pathway. Similar to Bcl-2-overexpressing cells, caspase 9-deficient Jurkat cells showed a strongly reduced activation of caspase 3 (fig. 5B). The fraction of early apoptotic cells and the overall occurrence of cell death were significantly lower than in the parental caspase 9-proficient cells (fig. 6). Treatment with 10 mM lidocaine did not increase the proportion of early apoptotic cells but led to a fraction of dead cells comparable to wild-type cells, whereas no caspase-3 activation was detected (figs. 5B and 6). The degree of protection against lidocaine-induced apoptosis in caspase 9-deficient cells was comparable to the effect of Bcl-2 overexpression.

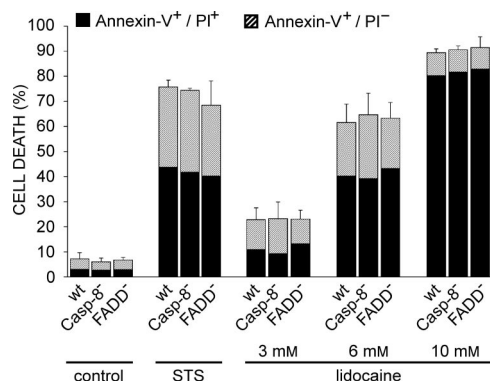


Fig. 7. Lidocaine-induced apoptosis is not mediated by death receptor signaling. Wild-type (wt), caspase 8-deficient (casp-8⁻), or Fas-associated protein with death domain (FADD)-deficient Jurkat cells (FADD⁻) were treated for 12 h with staurosporine (STS) or lidocaine in concentrations indicated. Early apoptosis was measured by flow cytometric staining with annexin-V to detect phosphatidylserine exposure. Counterstaining with propidium iodide (PI) was used to detect dead cells undergoing primary or secondary necrosis. The results show that inhibition of the death receptor pathway by caspase-8 or FADD deficiency altered neither early apoptosis (annexin-V-positive but PI-negative cells) nor later states of cell death (annexin-V-positive and PI-positive cells) as compared with wild-type cells. Treatment with 10 mM lidocaine did not induce significant apoptosis but resulted in more than 90% of necrotic cell death in all Jurkat cell lines. Error bars indicate SD.

Lidocaine-induced Apoptosis Is Unaltered by Deficiency of Caspase 8 or FADD

The death receptor pathway is triggered by the recruitment of the adapter protein FADD and caspase 8. In caspase 8-deficient Jurkat cells, no change in fraction of early apoptotic cells or overall cell death occurred after treatment with lidocaine at 3, 6, and 10 mM compared with wild-type cells (fig. 7). Similarly, Western blot analysis revealed an equal amount of active caspase 3 when compared with the parental cells (fig. 5C). Therefore, deficiency of the crucial initiator caspase 8 did not lead to a detectable difference in lidocaine-induced apoptosis or overall cell death. Similar to caspase 8-deficient cells, also FADD-deficient Jurkat cells were not protected against the proapoptotic effects of lidocaine. Compared with the parental cells, in FADD-deficient cells, no change in early apoptosis or overall cell death was observed after treatment with 3, 6, and 10 mM lidocaine (fig. 7). Also, Western blot analysis revealed an equal amount of activated caspase 3 (fig. 5D) when compared with wild-type cells. Therefore, a defective death receptor pathway does not comprise the proapoptotic effects of lidocaine.

Dose-dependent Effect of Lidocaine on Neuroblastoma Cells

To investigate whether lidocaine exerts similar effects in neuronal cells, we further used SHEP neuroblastoma cells. In these cells, lidocaine induced apoptosis at similar concentrations. Addition of the pancaspase inhibitor Q-VD protected the cells exposed to lidocaine at concentrations

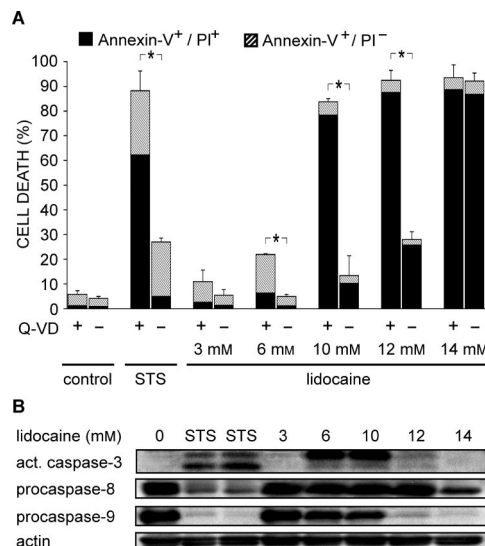


Fig. 8. Lidocaine-induced apoptosis and caspase activation in neuroblastoma cells. (A) Human SHEP neuroblastoma cells were incubated with 0.125 μ M staurosporine (STS) or the indicated concentrations of lidocaine in the presence (+) or the absence (–) of the pancaspase inhibitor Q-VD (10 μ M). After 12 h, cell death was measured by annexin-V and propidium iodide (PI) staining. The results show that caspase inhibition strongly ameliorated cell death after exposure to lidocaine (6–12 mM), but not at higher concentrations that predominantly induced necrosis. * $P < 0.05$. Error bars indicate SD. (B) SHEP neuroblastoma cells were incubated for 12 h with lidocaine and two concentrations of STS (left lane, 0.5 μ M; right lane, 0.25 μ M). The proteolytically processed active form of caspase 3 (act. caspase-3) was detected after treatment with 6–12 mM lidocaine. At these concentrations, lidocaine also diminished procaspase 9, whereas procaspase 8 remained largely unchanged.

of up to 12 mM \approx 0.32% (fig. 8A), indicating that also in neuroblastoma cells, lidocaine triggered caspase-dependent apoptosis. At higher concentrations (14 mM \approx 0.38%), treatment with Q-VD did not enhance cell viability, suggesting that at these concentrations, lidocaine induced necrosis. Western blot analysis revealed that exposure of neuroblastoma cells to lidocaine led to dose-dependent caspase-3 activation and a concomitant loss of the proform of caspase 9 (fig. 8B). In contrast, a slight decrease of the proform of caspase 8 was observed at concentration inducing necrosis (14 mM). Therefore, these results indicate that caspase activation and apoptosis in response to lidocaine are not restricted to Jurkat T-lymphoma cells, but also mediated *via* the mitochondrial pathway in neuroblastoma cells. Moreover, also in neuroblastoma cells, high concentrations of lidocaine induce a switch in the form of cell death from apoptosis to caspase-independent necrosis.

Discussion

Our results indicate that lidocaine in concentrations as measured intrathecally after spinal anesthesia³³ induces apoptosis that can be inhibited by overexpression of the cellular antiapoptotic protein Bcl-2 or by caspase-9 defi-

ciency. Furthermore, at higher concentrations, lidocaine leads to nonapoptotic cell death, which is not ameliorated by overexpression of Bcl-2 or caspase-9 deficiency or addition of a pancaspase inhibitor. Lack of crucial components of the death receptor pathway, namely caspase 8 and FADD, had no effect on apoptosis induction by lidocaine.

Although a Cochrane analysis discouraged the intrathecal use of lidocaine,² it is still widely used for short-lasting regional anesthesia. Recent publications demonstrated that lidocaine and other local anesthetics can induce apoptosis in neuronal and nonneuronal cells.^{10–12,34–40} Nevertheless, the mechanism by which lidocaine induces apoptosis is poorly understood. To delineate the molecular pathway of lidocaine-triggered apoptosis, we used human cells with genetic alterations of essential regulators of the two major apoptotic pathways, including Bcl-2 and FADD as well as caspases 8 and 9. This is a methodologic advantage compared with studies using only pancaspase inhibitors,^{12,37} which mitigate apoptotic pathways but do not always improve cell survival.⁴¹ Furthermore, the selectivity of pharmaceutical inhibitors is often questionable, whereas the selectivity of genetic engineering with overexpression or absence of one distinct protein is well defined.

We demonstrate that lidocaine induces apoptosis at concentrations that have been measured in the cerebrospinal fluid after spinal anesthesia.³³ Although these concentrations occur only transiently after single-shot spinal anesthesia and not over 12–24 h as in our experiments, maldistribution of lidocaine especially after continuous spinal anesthesia has been thought to cause neurologic dysfunction in patients.⁴²

At first glance, it may be surprising that the mechanism of toxicity switches from apoptosis to necrosis within a narrow range of concentrations. Nevertheless, this observation is not unusual, because strong noxious injuries more likely result in necrotic rather than apoptotic cell death.⁴³ Therefore, at higher concentrations, lidocaine triggers necrosis which may be caused by alterations in ion fluxes, loss of membrane integrity, or other cellular events. Furthermore, because local anesthetics in equal concentrations interfere with the mitochondrial energy production,⁴⁴ one may speculate that higher concentrations deplete cellular adenosine triphosphate, which is required for the execution of apoptosis.

The current results with genetically engineered cells clearly demonstrate that the mitochondrial pathway is responsible for lidocaine-induced apoptosis. First, the protective effect of the antiapoptotic protein Bcl-2 proved the role of mitochondria for apoptosis induction, whereas a loss of $\Delta\Psi_m$ is not only restricted to apoptosis but also occurs during necrosis. This is, for example, evidenced by the fact that high concentrations of lidocaine did not induce apoptotic caspase activation, although a loss of $\Delta\Psi_m$ was observed (fig. 3). Therefore the mitochondrial membrane depolarization observed in

studies with neuronal hybrid cells¹² and dorsal root ganglia cells^{13,39} does not provide unequivocal evidence for the occurrence of apoptosis in response to lidocaine treatment. In accord with our results, Bcl-2 overexpression was reported to decrease the toxic effects of ropivacaine in human keratinocytes, although in this study apoptosis was only detected by semiquantitative analysis of the loss of procaspase 3.³⁴ Second, our results with cells deficient for distinct initiator caspases also identified the mitochondrial pathway as responsible for lidocaine-induced apoptosis and excluded an involvement of the death receptor pathway.

Beyond the results of Johnson *et al.*¹² demonstrating a reduced neurotoxicity with a broad pancaspase inhibitor, we deciphered the pathways of lidocaine-induced apoptosis by using cells with deficiencies in the two crucial initiator caspases, including caspases 8 and 9, which are indispensable for death receptor and mitochondrial apoptosis pathways, respectively. Experiments with these cells clearly demonstrate that lidocaine-induced apoptosis is mediated by the mitochondrial pathway independently of death receptors. Furthermore, the selective decrease of procaspase 9 in neuroblastoma cells also suggests that neuronal apoptosis is mediated *via* the mitochondrial pathway.

Some studies have attributed the cytotoxicity of local anesthetics to an unspecific membrane effect of a detergent.^{38,45} However, our finding that distinct alterations of apoptotic proteins such as Bcl-2 and caspase 9 considerably decrease apoptosis argue against a detergent-like effect of lidocaine as the principal cause of apoptosis induction. Nevertheless, it is conceivable that higher concentrations of lidocaine, which induce necrosis, might be caused by such a more unspecific effect.

Nevertheless, cellular stress may trigger the death receptor pathway by induction of death ligand expression. Recently, it has been proposed that general anesthetics can lead to neuronal apoptosis in the developing rat brain *via* the intrinsic and extrinsic apoptotic pathways.⁴⁶ In contrast to general anesthetics, we have deciphered that lidocaine as a local anesthetic induces apoptosis clearly independent of the extrinsic pathway.

In conclusion, our results demonstrate that lidocaine induces apoptosis at concentrations that have been measured intrathecally after spinal anesthesia, whereas higher concentrations predominantly induce necrosis. Lidocaine-induced apoptosis is mediated *via* the mitochondrial pathway of apoptosis and is independent of caspase 8 and FADD, mediators of the death receptor pathway.

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