Neurotoxicity of Lidocaine Involves Specific Activation of the p38 Mitogen-activated Protein Kinase, but Not Extracellular Signal–regulated or c-jun N-Terminal Kinases, and Is Mediated by Arachidonic Acid Metabolites

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Background: Pharmacologic inhibition of the p38 mitogen-activated protein kinase (MAPK) leads to a reduction in lidocaine neurotoxicity *in vitro* and *in vivo*. The current study investigated *in vitro* the hypotheses that lidocaine neurotoxicity is specific for dorsal root ganglion cells of different size or phenotype, involves time-dependent and specific activation of the p38 MAPK, that p38 MAPK inhibitors are only effective if applied with local anesthetic, and that p38 MAPK activation triggers activation of lipoxygenase pathways.

Methods: The authors used primary sensory neuron cultures and pheochromocytoma cell line cultures to detect time-dependent activation of the p38 MAPK or related pathways such as extracellular signal–regulated kinases and c-jun N-terminal kinases. Cells were divided by size or by immunoreactivity for calcitonin gene–related peptide or isolectin B4, indicative of nociceptive phenotype. The authors also investigated whether arachidonic acid pathways represent a downstream effector of the p38 MAPK in local anesthetic–induced neurotoxicity.

Results: All types of dorsal root ganglion cells were subject to neurotoxic effects of lidocaine, which were mediated by specific activation of the p38 MAPK but not extracellular signal–regulated kinases or c-jun N-terminal kinases. Neuroprotective efficacy of p38 MAPK inhibitors declined significantly when administered more than 1 h after lidocaine exposure. Activation of p38 MAPK preceded activation of arachidonic acid pathways. Neurotoxicity of lidocaine, specific activation of p38 MAPK, and neuroprotective effects of a p38 MAPK inhibitor were further confirmed in pheochromocytoma cell line cultures.

Conclusions: Specific and time-dependent activation of the p38 MAPK is involved in lidocaine-induced neurotoxicity, most likely followed by activation of lipoxygenase pathways.

LIDOCAINE is the local anesthetic (LA) of choice to achieve short-lasting nerve conduction block in regional anesthesia and analgesia. However, direct neurotoxicity is a frequent problem and may lead to permanent neurologic damage, including paraplegia.[1–3] The mechanisms of LA-induced nerve damage remain elusive to a considerable degree. Recent investigations demonstrate that lidocaine induces apoptosis in neuron cultures *in vitro*.[4–6]

Neurons in dorsal root ganglia (DRG) can be subdivided according to size or immunohistochemical phenotype, *e.g.*, by staining for calcitonin gene–related peptide (CGRP) or isolectin B4 (IB4), indicative of subsets of nociceptive neurons.[7] However, it has never been investigated whether lidocaine exhibits selective toxic effects on a specific subset of cells.

Preliminary reports suggest that a serine–threonine kinase central to stress-induced apoptosis, p38 mitogen-activated protein kinase (MAPK), is activated (phosphorylated) in cell cultures after incubation with lidocaine.[8] Conversely, its inhibition during application of toxic lidocaine doses substantially reduces the rate of apoptosis *in vitro*, and preliminary results seem to indicate improved neuronal survival *in vivo*.[6] Because p38 MAPK is closely related in structure and function to extracellular signal–regulated kinases (ERKs) 1 and 2 (also known as p44 and p42 MAPK, respectively) and c-Jun N-terminal kinases (JNKs), it is of considerable interest to establish whether activation of p38 MAPK is specific or also involves these related pathways. For example, neurotoxicity of the LA tetracaine in a neuronal cell line is largely mediated by JNK, whereas the p38 MAPK plays a subordinate role.[9]

We have shown that p38 MAPK inhibition may mitigate lidocaine-induced neurotoxicity when coinjected with lidocaine. However, it is not known whether p38 MAPK inhibition is capable of decreasing neurotoxicity when administered later, *i.e.*, after injury has taken place. Therefore, it is important to determine whether p38 MAPK administration must be concomitant with lidocaine exposure or whether it is sufficient to administer the p38 MAPK inhibitor after a neurologic deficit is diagnosed. Currently, we can extrapolate from literature that the average time to activation of the p38 MAPK is roughly 4 h.[10] However, in the setting of painful nerve injury, p38 MAPK inhibitors showed consistent and long-term neuroprotective effects by far surpassing the narrow time frame of p38 activation in neurons.[11]

Several effector mechanisms are known by which p38 MAPK activation leads to cellular death. For example, activation of the proinflammatory enzymes phospholipase A_2 (which liberates arachidonic acid[12]) and lipoxy-

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genase[13] have been described as major pathogenic pathways in p38-mediated nerve injury, but it is unclear whether this group of enzymes is also relevant in LA neurotoxicity.

Knowledge of pathogenic mechanisms will be essential to identify potential therapeutic targets for inhibitors of apoptotic pathways, with the ultimate aim of decreasing LA-induced neurotoxicity. The current study therefore investigated *in vitro*, in primary sensory neurons and pheochromocytoma cell line cultures, the hypotheses that (1) lidocaine neurotoxicity is specific for DRG cells of different size or phenotype; (2) lidocaine toxicity involves time-dependent and specific activation of the p38 MAPK; (3) p38 MAPK inhibitors are only effective if applied simultaneously with LA; and (4) p38 MAPK activation triggers activation of lipoxygenase pathways.

Materials and Methods

Drugs

Unless stated otherwise, drugs were purchased from Sigma Aldrich (Vienna, Austria, or St. Louis, MO). For the *in vitro* experiments, the pH of the 1 M lidocaine stock solution was 4.65 (in dimethylsulfoxide). The pH of the final solution added to medium was 7.38 for 40 mM lidocaine. The concentration of dimethyl sulfoxide in treated cultures was not significantly higher than in control cultures incubated with vehicle only.

Primary Sensory Neuron Culture

Dorsal root ganglia cultures were obtained as described previously.[6] Neurons were acutely harvested from adult (8–9 weeks) female Sprague-Dawley rats, which were killed by carbon dioxide narcosis according to the institutional protocol (Animal Committee of the Austrian Federal Ministry of Education, Science and Culture, Vienna, Austria). DRGs were desheathed and incubated in 5,000 U/ml collagenase for 90 min at 37°C, followed by 15 min in 0.25% trypsin–EDTA. After dissociation in Roswell Park Memorial Institute (RPMI) medium containing 10% horse–5% fetal bovine serum, neurons were plated in RPMI medium supplemented with N2 additives (1:100) and antibiotics (penicillin, 1,000 U/ml; streptomycin, 1,000 μg/ml; and amphotericin B, 25 μg/ml in 0.85% saline), all purchased from Invitrogen (Carlsbad, CA). Neurons were allowed to adhere to the glass floor of dishes coated with poly-D-lysine–laminin for 24 h. Poly-D-lysine was applied at a concentration of 0.1 mg/ml in distilled water and laminin at 7 μg/ml in RPMI solution. Cell cultures were kept at 37°C in a humidified atmosphere containing 5% CO_2.

Determination of Neurotoxic Effects of Lidocaine in DRG Cells of Different Size. In experiments aimed at determining cell type–dependent lidocaine toxicity, we treated cultures with lidocaine at a concentration of 40 mM (approximately 1%). After incubation with lidocaine for 24 h, cultures were fixed with 4% paraformaldehyde at 4°C for 30 min. After permeabilization with Tween 20 (0.5% in phosphate buffered saline) for 10 min at room temperature, monoclonal antibodies against neurofilament (N52m, 1:800) were added for 2 h at 37°C or overnight at 4°C. After two washes with phosphate-buffered saline, the cultures were treated with anti-mouse immunoglobulin G (IgG) (Alexa Fluor 488 goat anti-mouse IgG, 1:2,000; Molecular Probes, Carlsbad, CA) for 1 h at room temperature in darkness and subsequently washed twice with phosphate-buffered saline.

We determined neuron number in cultures by counting the number of neurons along two passes through the diameters of each well at 20× magnification. DRG neurons typically have a large and distinctly spherical cell body with a clearly visible nucleus and are thereby distinguished from nonneuronal cells, such as glial cells. In all experiments, control cultures were incubated with vehicle dimethyl sulfoxide corresponding to the highest concentration of lidocaine.

To assess the effect of lidocaine neurotoxicity using histograms of dissociated rat primary sensory neurons, we determined distribution of cell size in cultures, before and after incubation with lidocaine 40 mM or vehicle. For size histograms, we used Metamorph® software (Molecular Devices, Downingtown, PA), taking images of neurons in 25 defined power fields (20× magnification). Neurons were categorized by measuring two diameters (perpendicular to each other) of each neuron; the mean of these two measurements was considered the average cell diameter. Cells were divided into three arbitrary groups by size, *i.e.*, mean diameter smaller than 35 μm, between 35 and 65 μm, and larger than 65 μm, similar to previous investigations.[14]

Determination of Neurotoxic Effects of Lidocaine in DRG Cells of Different Phenotype. To determine the percentage of neurons positive for CGRP, indicative of a small-diameter peptidergic sensory neuron phenotype that transmits pain sensations and is central to the pathogenesis of neurogenic inflammation, we used rabbit polyclonal antiserum to CGRP in a dilution of 1:400 in phosphate-buffered saline–bovine serum albumin 0.3% (Biomol, Plymouth Meeting, PA). Alexa Fluor 488 goat anti-rabbit IgG, a rabbit-specific anti-IgG antibody that is conjugated to an Alexa fluorescent dye, was used as secondary antibody (1:2,000 in phosphate-buffered saline–bovine serum albumin 0.3%; Molecular Probes). Omission of primary or secondary antibodies on parallel dishes served as control to assure specificity of the antibody staining. Moreover, we used fluorescein-labeled *Bandaireae simplicifolia* IB4 for identification of the subset of small-diameter nonpeptidergic sensory neurons, which are also known to be involved in nociception. Neurons were incubated with 10 μg/ml fluoresce-

in-labeled IB4 in phosphate-buffered saline for 2 h, rinsed, and analyzed.

Enzyme-linked Immunosorbent Assay. Double-sandwich enzyme-linked immunosorbent assay for p38, phospho-p38, ERK1/2 phospho-ERK1/2, JNK, and phospho-JNK was performed using commercial kits from SuperArray Bioscience (Frederick, MD) according to the manufacturer's protocols. In brief, dissociated adult rat DRG cell cultures were prepared as described previously,[6] and cells were seeded into 96-well plates. Twenty-four hours after plating, the cultures underwent experimental treatment with 40 mM lidocaine for 4 h, and subsequently, cells were fixed with 4% paraformaldehyde to preserve any activation-specific protein modification, such as phosphorylation. Blank wells (not seeded with any cells), detection control wells (seeded with cells, incubated only with secondary but not primary antibody), and experimental control wells (seeded with cells but not experimentally treated) were included for control purposes. Moreover, each experimental condition was performed in triplicate to control for systematic variation. After fixation, cells were washed twice with 200 μl washing buffer and incubated with 100 μl quenching buffer for 20 min at room temperature (both buffers were included in the kit). Subsequently, cells were washed once with 200 μl washing buffer, incubated with 100 μl antigen retrieval buffer in a microwave oven at 30% power for 3 min, and, after cooling to room temperature, again washed once with 100 μl washing buffer. After blocking buffer (100 μl) was added for a 1-h incubation at room temperature, cells were washed with washing buffer, and 50 μl diluted primary antibody (1:100) was added to each appropriate well. To the negative control wells, only antibody dilution buffer was added. Cells were incubated for 1 h at room temperature, washed, and incubated with 100 μl diluted secondary antibody for 1 h at room temperature. Colorimetric detection was performed by addition of developing solution into each well for 10 min at room temperature. Stop solution was added to stop reaction and avoid overdevelopment, and absorbance was read at 450 nm with a reference wavelength of 620 nm on an enzyme-linked immunosorbent assay plate reader.

Antibody reading was normalized to the respective cell numbers of each well, which were determined by counting, and the phospho-protein–specific antibody ratio was normalized to the pan-protein–specific ratio for the same experimental condition.

Inhibition of p38, ERK, and JNK. To determine the relevance of putative p38, ERK, and JNK activation during LA-induced neurotoxicity, we pharmacologically inhibited these kinases using a specific inhibitor of p38 MAPK (SB203580), or its inactive analog SB202474 (Calbiochem, Darmstadt, Germany); the specific inhibitor of ERK1/2, U0126 (Calbiochem); and the specific inhibitor of JNK, SP600125 (Calbiochem). Stock solutions (2.5 mM for SB203580, 3.6 mM for SB202474, 1 mM for U0126, and 18 mM for SP600125) of the inhibitors were prepared in dimethyl sulfoxide. Lidocaine at 40 mM was incubated with or without inhibitors dissolved in medium containing N2 additives and antibiotics at a concentration of 10 μM for 24 h, and neuron number in culture was chosen as the outcome parameter. To detect a possible toxic influence of the inhibitors themselves, we incubated cultures with each of the MAPK inhibitors alone. Culture conditions in these experiments were the same as those of the main experiments.

Time Response of p38 MAPK Inhibition. To assess a potential protective effect of p38 MAPK inhibition after lidocaine exposure, the p38 MAPK inhibitor SB203580 (10 μM) was added to neuron cultures 0, 0.5, 1, 3, or 5 h after incubation with 40 mM lidocaine. Twenty-four hours after lidocaine application, cultures were fixed, stained with monoclonal antibodies against neurofilament, and subsequently evaluated with regard to neuron numbers.

Block of Lipoxygenase In Vitro. To determine whether block of arachidonic acid pathways would reduce lidocaine neurotoxicity in vitro, we incubated DRG cultures for 24 h with 20 mM lidocaine and nordihydroguaiaretic acid (NDGA; Calbiochem) at a concentration of 30 μM, which is known to block 5-, 12-, and 15-lipoxygenase. Subsequently, cultures were fixed and stained with monoclonal antibodies against neurofilament. We evaluated neurons with regard to survival, determining and comparing neuron numbers.

Time Response of Lipoxygenase Inhibition. To investigate a potential neuroprotective effect of lipoxygenase inhibition after lidocaine exposure, the unspecific lipoxygenase inhibitor NDGA was added at a concentration of 30 μM to DRG cultures 0, 0.5, 1, 2, 3, or 5 h after incubation with 40 mM lidocaine. Cultures were fixed and stained with monoclonal antibodies against neurofilament, and neuron numbers were quantitated.

Pheochromocytoma Cell Line Culture

PC12 Rat Pheochromocytoma Cell Line Culture. PC12 rat pheochromocytoma cells were used for Western blot analyses and fluorescence-activated cell-sorter analysis to confirm in a second model the main findings of experiments using primary sensory neurons. PC12 cells were seeded on 10-cm-diameter tissue culture dishes coated with rat tail collagen (20 μg/ml), and cultures were maintained in RPMI 1640 medium supplemented with 10% horse serum, 5% fetal bovine serum, antibiotics, and antimycotics (5 mg/ml penicillin, 5 mg/ml streptomycin, and 10 mg/ml amphotericin B) in humidified atmosphere containing 5% CO_2, at 37°C. Stock cultures were subcultured routinely at a cell density of 2–3 × 10^6 per dish at least once a week, and culture media were renewed every 2–3 days. When the

cultures reached confluence, they were subjected to different experimental protocols.

Western Blot Analysis of (p)p38, (p)ERK, and (p)JNK. To confirm activation of p38, we performed Western blots of p38, pp38, ERK1/2, pERK1/2, JNK, and pJNK. After treatment with 20 mM lidocaine with or without 30 μM NDGA for 1–4 h, the cells were washed twice with ice-cold phosphate-buffered saline and lysed in a buffer containing 50 mM TrisHCl (pH 7.5), 10% glycerol, 2 mM EDTA (pH 8), 150 mM NaCl, 0.25% sodium deoxycholate, dH$_2$O, 1% NP-40, protease inhibitor (Roche Diagnostics, Vienna, Austria), and phosphatase inhibitor I and II; collected with a cell scraper; and dispersed by sonication. The cell homogenate was centrifuged at 13,000 rpm for 20 min at 4°C, and the resulting supernatant was used for protein determination. Protein concentrations were determined by a modified Bradford method using bovine serum albumin as standard. After sample buffer was added to the aliquots (20 μg protein) of the lysates, they were boiled for 5 min and resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions. The resolved proteins were transferred to nitrocellulose membranes (Biorad, Vienna, Austria). For immunolabeling, the blots were blocked with 5% dry skimmed milk in phosphate-buffered saline containing 0.05% Tween 20 for 1 h at room temperature. After blocking of nonspecific binding, the membranes were incubated with rabbit anti-p38 (1:200; Santa Cruz, Santa Cruz, CA), mouse anti–phospho-p38 (1:1,000), rabbit anti-ERK1/2 (1:200; Upstate, Lake Placid, NY), mouse anti–phospho-ERK1/2 (1:1,000), rabbit anti-JNK/SAPK1 (1:800; Upstate), or mouse anti–phospho-JNK (1:400) in blocking solution overnight at 4°C. Primary antibody binding was detected with goat anti-rabbit or goat anti-mouse horseradish peroxidase–conjugated secondary antibodies (1:10,000 and 1:5,000; Biorad) and developed by chemiluminescence detection with a commercial kit (Super Signal West Pico Chemiluminescent Substrate; Pierce, Vienna, Austria). A protein standard (Precision Plus Protein Kaleidoscope Standard; Biorad) was used to assess the quality of electrophoretic transfer and visualize protein molecular weight ranges. Western blot results were quantified with QuantityOne software (Biorad). Equal protein loading was confirmed with an antibody against nuclear Ku-70 protein (Santa Cruz) as active positive control. As a second positive control for activation of MAPK pathways, nerve growth factor–treated PC12 cell extract was used.

Detection of Apoptosis by Fluorescence-activated Cell Sorter Analysis. Apoptosis was quantified by flow cytometry based on the staining of DNA with propidium iodide. PC12 cells, 8×10^5/ml, were plated on collagen-S–coated 24-well plates and incubated for 1 day until they reached a confluence of 50%. Then, culture medium (RPMI 1640: 10% horse serum, 5% fetal calf serum,

1% Pen/Strep, and 1% L-glutamine) was changed to a low-serum medium (RPMI 1640: 1.25% horse serum, 0.25% fetal calf serum, 1% Pen/Strep, and 1% L-glutamine). Cells were left untreated or stimulated with 40 mM lidocaine, 10 μM SB203580, or lidocaine and SB203580. After 4 h at 37°C, cells were harvested by transferring the supernatant with the cells to tubes, and a centrifugation step (1,390 rpm, 7 min at 4°C) followed. The pellet was resuspended in 250 μl propidium iodide staining solution (0.005% propidium iodide, 0.1% Triton-X-100 [Applichem, Darmstadt, Germany] and 0.1% trisodium-citrate-dihydrate [Merck, Darmstadt, Germany]) and transferred to fluorescence-activated cell-sorter tubes. Isolated nuclei were then analyzed on a fluorescence-activated cell-sorter scan. Apoptotic cells were characterized by morphology and by intranuclear contents of fluorescing DNA,[15] and apoptotic nuclei were identified in a hypodiploid peak, easily discriminated from the narrow diploid peak of normal nuclei.

Statistics

Sample size was based on preliminary results. The distribution of data in pharmacologic experiments was determined by Kolmogorov-Smirnov analysis. Statistical analysis used one-way analysis of variance with *post hoc* Bonferroni correction. Unless otherwise stated, summarized data are presented as mean ± SD. Statistical significance was assumed at $P < 0.05$.

Results

Lidocaine Neurotoxicity Is Not Selective for Cell Size or Specific Cell Types

Histograms of control cultures showed a roughly bimodal size distribution of the total neuronal population in the DRG (fig. 1A). Considering these size histograms, we found that incubation with 40 mM lidocaine for 24 h, despite exerting a clear neurotoxic effect, did not predominantly affect specific cell types as highlighted by analyzing size distributions, which were essentially the same as those of control cultures (fig. 1B). Specifically, in control cultures (n = 12), 65.5% of all cells had an average diameter of 0–35 μm, 30% were within the range of 35–65 μm, and 4.6% were greater than 65 μm. In lidocaine-treated cultures (n = 12), 66.3% of cells had an average diameter of 0–35 μm, 29.5% were within the range of 35–65 μm, and 6.6% were within the range of 65–95 μm in lidocaine-treated cultures. Moreover, immunohistochemistry showed that lidocaine did not preferentially affect CGRP- or IB4-positive neuron populations. In control cultures (n = 4, cell count 131.1 ± 6.1), 49% of all cells (64.3 ± 1.2) were IB4 positive, and 32% (42.0 ± 4.6) showed CGRP-positive staining. The cell count in cultures treated with lidocaine (n = 4) was 53.7
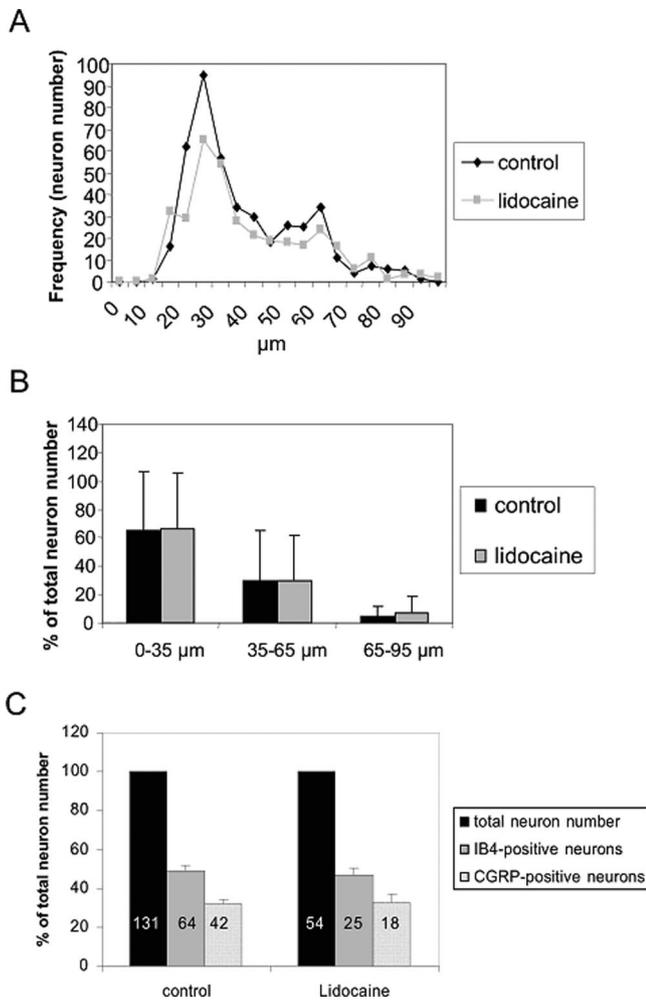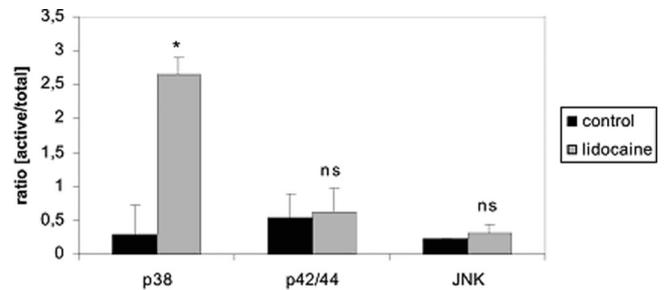
Fig. 1. (*A*) Size distribution of total dorsal root ganglion neuronal population in control cultures and cultures treated with lidocaine at 40 mM. Dorsal root ganglion neurons were pooled from all spinal cord levels, and mean somal diameters were determined. Cell size (*x-axis*) is plotted against neuron number (*y-axis*). (*B*) Cell size categories of control cultures 48 h after seeding and cultures incubated with 40 mM lidocaine for 24 h, and control cultures at the same time point. Cell size categories (*x-axis*) are plotted against relative percentage of cells. (*C*) Distribution of isolectin B4 (IB4)- and calcitonin gene-related peptide (CGRP)–positive neuron populations in control cultures and cultures treated with 40 mM lidocaine for 24 h. Absolute neuron numbers in culture are printed in columns.

Fig. 2. Enzyme-linked immunosorbent assay demonstrates specific activation of p38 mitogen-activated protein kinases in adult rat dorsal root ganglion neurons induced by treatment with lidocaine. Neurons were incubated with 40 mM lidocaine for 4 h, and the phosphorylation state of the three major mitogen-activated protein kinases (p38, extracellular signal–regulated kinase [p42/44], and c-jun N-terminal kinase [JNK]) was analyzed by enzyme-linked immunosorbent assay. p38 mitogen-activated protein kinase was activated by lidocaine, as shown by a significant increase of the respective ratio [active/total]. The same treatment had no effect on the activation of p42/44 or JNK. Data are presented as mean ± SD of three or more independent cultures. *$P < 0.05$. ns = not significant.

± 2.5. Of these cells, 47% were IB4 positive (cell count 25.0 ± 2.0), and 33% (17.7 ± 1.5) were CGRP positive (fig. 1C).

Lidocaine Specifically Activates p38 MAPK in Primary Sensory Neuron Cultures

Results of enzyme-linked immunosorbent assays showed the activation of p38 MAPK after lidocaine treatment for 4 h. Lidocaine at 40 mM induced phosphorylation of p38 MAPK, which is represented in a significant enhancement of the respective ratio [active/total] as compared with controls. The phosphorylation state of p44/42 MAPK (ERK1/2) and JNK remained unaffected (fig. 2).

Inhibition of p38 Attenuates Lidocaine Neurotoxicity within 1 h after Incubation with Lidocaine

The inhibition of p38 MAPK protected neurons from lidocaine-induced neurotoxicity. Whereas incubation with 40 mM lidocaine significantly ($P < 0.001$) reduced neuron numbers compared with controls (neuron number 249 ± 23, n = 3), addition of the active p38 MAPK inhibitor SB203580 (10 μM) resulted in increased survival of neurons. The neuron count (137 ± 15, n = 3) of cultures coincubated with lidocaine and SB203580 was significantly higher ($P < 0.05$) than that of cultures incubated with lidocaine alone (75 ± 19, n = 3) but still significantly lower than the neuron count of controls ($P < 0.001$). Coincubation with the inactive analog SB202474 (10 μM) had no protective effect on neuron survival (fig. 3A).

The p38 MAPK inhibitor SB203580 at 10 μM was able to attenuate lidocaine-induced neurotoxicity when added within the first hour after incubation with lidocaine (fig. 3B). Whereas lidocaine caused a decrease of neuron number from 64 ± 5 in control cultures (n = 4) to 25 ± 7 (n = 4, $P < 0.001$), simultaneous addition of SB203580 resulted in significantly increased survival of neurons (65 ± 11, n = 4, $P < 0.001$ *vs.* lidocaine-treated cultures). Addition of SB203580 0.5 h after incubation with lidocaine resulted in 65 ± 8 neurons (n = 4, $P < 0.001$ *vs.* lidocaine-treated cultures), and addition of SB203580 1 h after incubation with lidocaine still yielded 51 ± 14 neurons (n = 4, $P < 0.05$ *vs.* lidocaine-treated cultures). Addition of SB203580 3 h or later after incubation with lidocaine no longer had a significant protective effect on neuronal survival (n = 4, $P > 0.05$ *vs.* lidocaine-treated cultures).
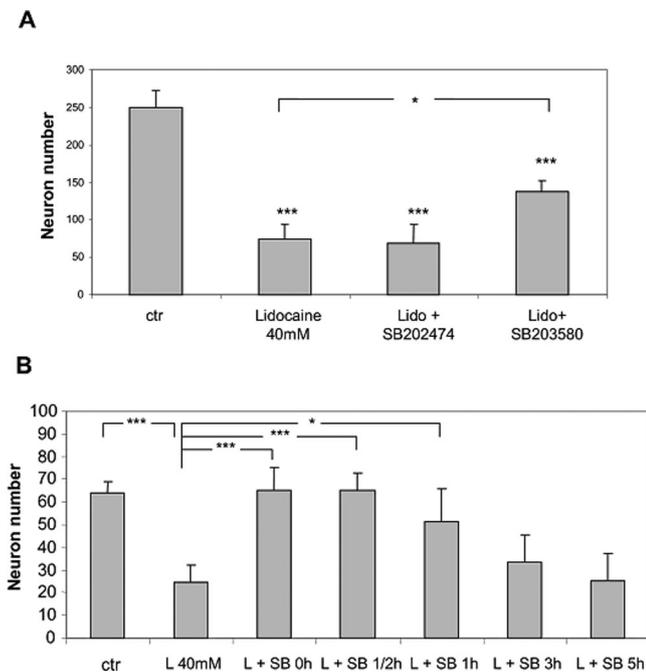
Fig. 3. (*A*) Coincubation of cultures with lidocaine at 40 mM and either the inactive p38 mitogen-activated protein kinase inhibitor, SB202474 (10 μM), or the active p38 mitogen-activated protein kinase inhibitor, SB203580 (10 μM). (*B*) Time response of the protective effect of p38 mitogen-activated protein kinase inhibition, showing that inhibition is neuroprotective when added within 1 h after incubation with lidocaine. Data are presented as mean ± SD. * $P < 0.05$, *** $P < 0.001$. n ≥ 3 cultures per group. ctr = control; L = lidocaine; SB = SB203580.

Inhibition of ERK and JNK

The inhibition of the MAPKs ERK1,2 and JNK did not show any protective effect on lidocaine-treated DRG neuron cultures (figs. 4A and B). The neuron count (42 ± 9, n = 4) of cultures coincubated with lidocaine and ERK inhibitor U0126 (10 μM) was not significantly higher

($P > 0.05$) than that of cultures incubated with lidocaine alone (36 ± 5, n = 4) and still significantly lower than the neuron count of controls (90 ± 17, n = 4, $P <$ 0.001). Also, the neuron number (14 ± 4, n = 5) of cultures coincubated with lidocaine and JNK inhibitor SP600125 (10 μM) was approximately the same as that of cultures incubated with lidocaine alone (14 ± 8, n = 5) and significantly lower than the neuron count of controls (64 ± 13, n = 5, $P < 0.001$). Moreover, single incubation of neuron cultures with 10 μM U0126 or SP600125 showed no effect on neuron survival, resulting in a neuron number (92 ± 16, n = 5 or 93 ± 8, n = 5, respectively) similar to that of controls (94 ± 6, n = 5). Western blot analyses in pheochromocytoma cell line cultures did not show relevant activation of ERK (fig. 4C) and JNK (fig. 4D) upon incubation with lidocaine.

Coincubation with NDGA Attenuates Lidocaine-induced Neurotoxicity

There was a significant effect of drug treatment in neuron cultures incubated with lidocaine and the antioxidant NDGA ($P < 0.001$; fig. 5A). Specifically, incubation with lidocaine decreased neuron number in culture significantly (34 ± 8, n = 3, $P < 0.05$) compared with controls (66 ± 5, n = 3). Addition of the cell-permeable antioxidant and lipoxygenase inhibitor NDGA resulted in a neuron count of 65 ± 14 (n = 3), which was not different from controls but significantly higher than cultures incubated with lidocaine alone ($P < 0.05$). Coincubation with the lipoxygenase inhibitor NDGA (30 μM) significantly enhanced neuron survival when added within 1 h after lidocaine exposure. Addition of NDGA after 3 h or later did not show a neuroprotective effect (fig. 5B). Although addition of NDGA attenuated lidocaine-induced neurotoxicity in cell culture experiments,

Fig. 4. (*A*) Coincubation of dorsal root ganglion cell cultures with lidocaine at 40 mM and the specific inhibitor of the extracellular signal–regulated kinase (ERK), U0126 (10 μM). (*B*) Coincubation of dorsal root ganglion cell cultures with lidocaine at 40 mM and the specific c-jun N-terminal kinase (JNK) inhibitor, SP600125 (10 μM). (*C*) Western blot analyses of ERK1,2 (constitutive) and pERK1,2 (activated) in pheochromocytoma cells after incubation with lidocaine for 1–4 h (nerve growth factor [NGF]: positive control). (*D*) Western blot analyses of JNK (constitutive) and pJNK (activated) in pheochromocytoma cells after incubation with lidocaine for 1–4 h (NGF: positive control). Data are presented as mean ± SD. *** $P < 0.001$, $P > 0.05$. n ≥ 3 cultures per group. ctr = control; ns = not significant; SB = SB203580.
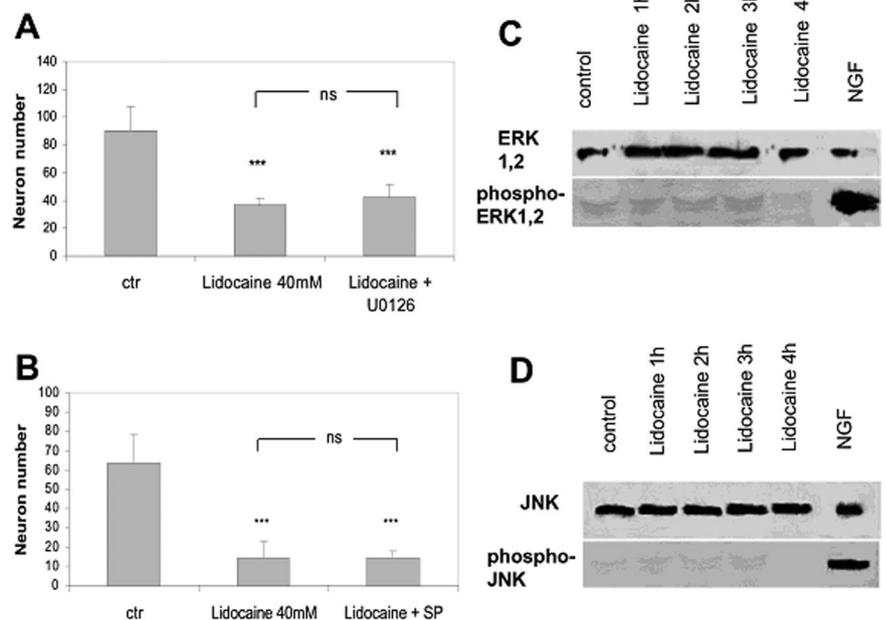
it had no effect on lidocaine-induced p38 MAPK activation in Western blot analyses (fig. 5C).

Specific p38 Activation Is Involved in Lidocaine Neurotoxicity

Western blot analyses of the three major groups of MAPKs, p38, ERK1/2, and JNK, in PC12 cells showed that specific activation of p38 MAPK is involved in lidocaine-induced neurotoxicity. Incubation of PC12 cells with 20 mM lidocaine for 1–4 h induced activation of p38 MAPK (fig. 6A), whereas it had no effect on the activation of ERK1/2 and JNK (fig. 4). Quantification of Western blot results by densitometric measurement of the respective protein bands showed threefold to fivefold enhanced intensity of activated p38 bands after treatment with lidocaine for 1–4 h (fig. 6B). The levels of total protein remained generally constant (fig. 6A).

Lidocaine-induced Apoptosis Is Attenuated by p38 MAPK Inhibition

PC12 cells were left untreated or incubated with 40 mM lidocaine, 10 μM SB203580, or lidocaine and SB203580 together for 4 h. Subsequently, apoptosis was quantified by flow cytometry using propidium iodide staining (fig. 7A). Of PC12 cells exposed to 40 mM lidocaine for 4 h, 34.2% were propidium iodide positive, whereas only 19.5% of cells exposed to 40 mM lidocaine together with 10 μM SB203580 showed DNA fragmentation. Controls and cells treated with 10 μM SB203580 alone showed less than 4% DNA fragmentation (fig. 7B).

Discussion

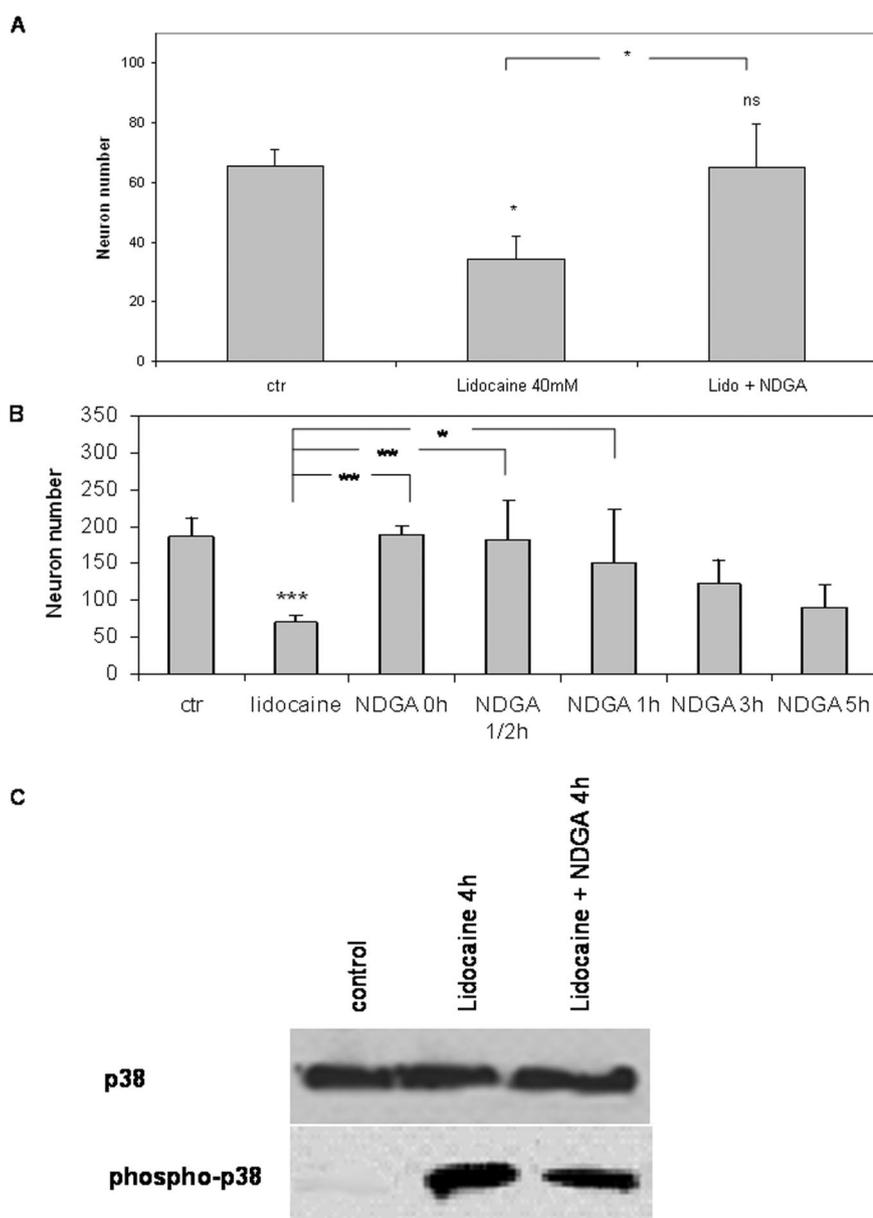The main findings of the current study are (1) lidocaine neurotoxicity shows no preferential targeting of sub-

Fig. 5. (*A*) Effect of the lipoxygenase inhibitor, nordihydroguaiaretic acid (NDGA), on lidocaine-treated dorsal root ganglion cell cultures. (*B*) Time response of the protective effect of lipoxygenase inhibition. Inhibition of lipoxygenase is neuroprotective when NDGA is added within 1 h after incubation with lidocaine. (*C*) Western blot analyses of p38 and activated p38 mitogen-activated protein kinase in pheochromocytoma cells after incubation with 40 mM lidocaine for 4 h or 40 mM lidocaine and 30 μM NDGA for 4 h, respectively. Data are presented as mean ± SD. * *P* < 0.05, ** *P* < 0.01, ***P* < 0.001. n ≥ 3 cultures per group. ctr = control; Lido = lidocaine; ns = not significant.
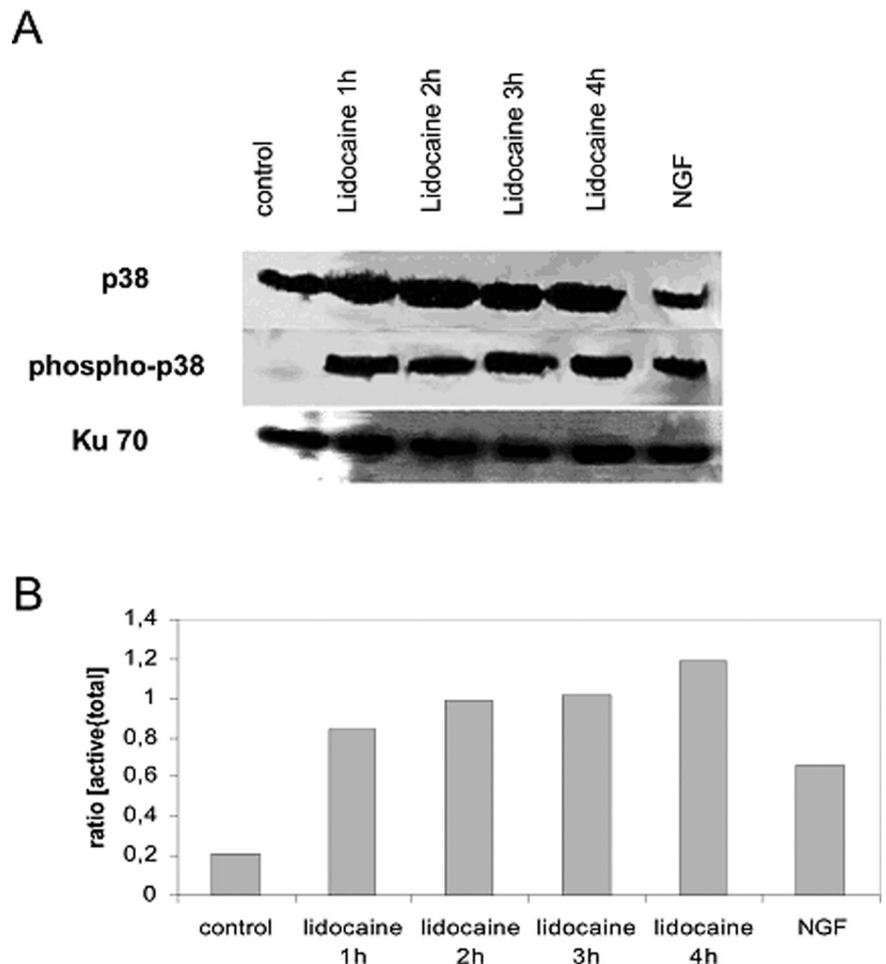
Fig. 6. (*A*) Activation of p38 mitogen-activated protein kinase in pheochromocytoma cells after incubation with 40 mM lidocaine for 1–4 h evaluated using Western blot with specific antibodies for total p38 and phospho-p38. Ku-70 served as positive control. (*B*) Activation of p38 mitogen-activated protein kinase in pheochromocytoma cells is represented as an increased ratio [active/total]. Data are presented as mean from three separate experiments. NGF = nerve growth factor.

groups of DRG neurons; (2) lidocaine specifically activates the p38 MAPK; (3) the neuroprotective efficacy of p38 MAPK inhibitors decreases sharply when applied more than 1 h after lidocaine exposure; and (4) lipoxygenase pathways most likely mediate lidocaine neurotoxicity downstream of the p38 MAPK.

The detailed pathway by which LAs act to induce neuronal cell death remains incompletely understood. Summarizing evidence from literature, it is conceivable that exposure of primary sensory neurons to LAs triggers membrane depolarization, which leads to a release of glutamate.[16] This in turn leads to opening of calcium channels, resulting in an increase in cytosolic calcium,[17] leading to a subsequent activation of the p38 MAPK. The latter is a potent activator of autodestructive pathways, such as the lipoxygenase system.[13] Furthermore, we postulate that these events occur at the neuron's axon and lead not only to an immediate neuronal injury apparent within hours, but also to a prolonged inflammatory response combined with sustained tissue damage still detectable after several days.[6]

The current findings confirm and strengthen our previous results[6] on whether lidocaine neurotoxicity displays any size or subpopulation selectivity on DRG neurons. DRG neurons are generally divided into two main subpopulations: small and large. The large neurons are thought to be involved mainly in proprioception, whereas most small neurons are involved in nociception.[7] We grouped cells arbitrarily into size categories and investigated whether lidocaine neurotoxicity would predominantly affect any of these subsets. This is of considerable clinical relevance, because a predominant effect of LAs on a distinct class of neurons is a potentially useful property. For example, bupivacaine and ropivacaine are known to exhibit some degree of differential block,[18] but such properties have not been described for lidocaine. We found that lidocaine exerts uniform cytotoxic effects on subtypes of DRG neurons.

Moreover, we showed *in vitro* that interference with proapoptotic pathways may substantially reduce extent of LA-induced neurotoxicity, and preliminary results suggest that a protective effect is also discernible *in vivo*.[6] Specifically, we note that p38 MAPK is activated in cell cultures incubated with clinically relevant doses of lidocaine. Inhibition of p38 MAPK activity has been shown to be of potential therapeutic benefit in experimental nerve trauma,[19,20] excitotoxicity,[21] and metabolic injury.[22] Interestingly, both oral and parenteral formulations of p38 MAPK inhibitors are being tested in clinical trials,[23] so that these drugs could become available for
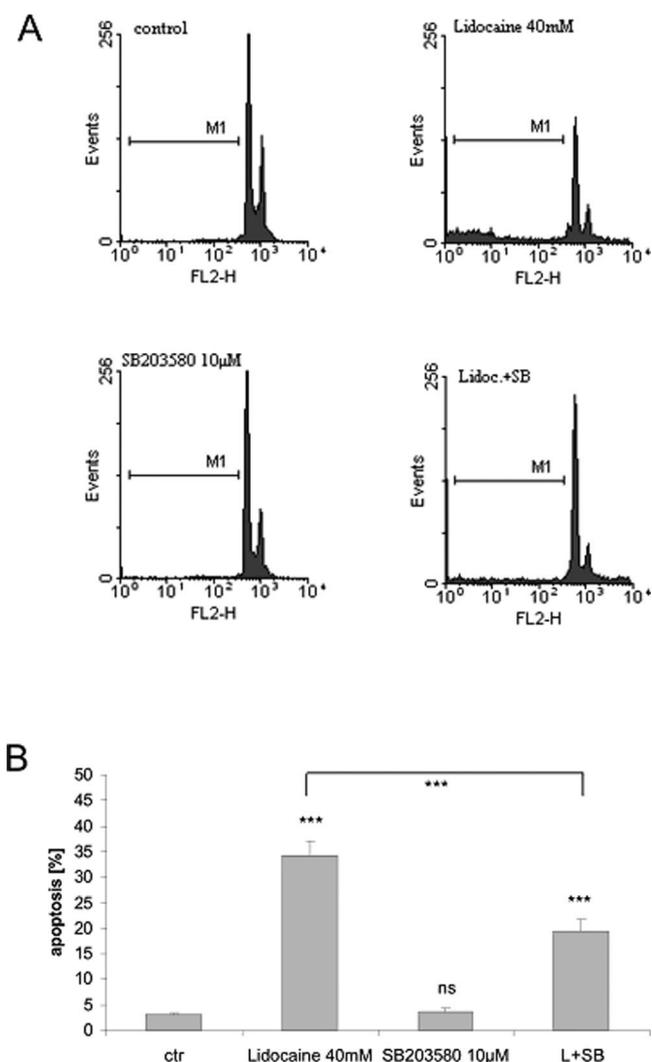
Fig. 7. (*A*) Propidium iodide staining and fluorescence-activated cell-sorter analysis after treatment with 40 mM lidocaine or without 10 μM SB203580. Fluorescence intensity (FL2-H) of detected particles is an indicator for the DNA content of the cell. The cells with subdiploid DNA content (lower FL2-H than the G_0/G_1-phase peak) represent the fraction undergoing apoptotic DNA degradation. Data are presented as mean ± SEM of three independent experiments. (*B*) Quantitation from of pheochromocytoma cells either untreated or stimulated with 40 mM lidocaine with or without 10 μM SB203580 using propidium iodide staining. *** P < 0.001. ctr = control; L = lidocaine; SB = SB203580.

therapeutic use in the foreseeable future. Although previous investigations have suggested that several subfamilies of MAPKs are activated after incubation of neuronal cultures with LAs such as tetracaine,[9] we found only evidence for activation of the p38 MAPK. Specifically, neither ERK nor JNK activation could be observed at the protein level using Western blots. Moreover, pharmacologic inhibition of ERK or JNK with the selective inhibitors U0126 and SP600125, respectively, did not show any beneficial therapeutic effect, whereas the p38 MAPK inhibitor SB203580 substantially increased the number of surviving cells in primary sensory neuron culture, suggesting specific p38 MAPK activation to be the most

relevant pathway of LA toxicity. This hypothesis is corroborated by the fact that the inactive analog, SB202174, did not attenuate neurotoxic effects, suggesting that the effects of SB203580 on p38 MAPK activation and subsequent neurotoxicity are specific. Taken together with previous results that suggest that p38 MAPK inhibition may be effective in attenuating neuronal damage incurred after regional anesthesia,[6] it is conceivable that p38 MAPK activation and subsequent apoptosis of neurons may, at least in part, account for nerve injury after lidocaine administration.

Our results further suggest that *in vitro* direct neuroprotective effects decline if the inhibitor is applied more than 1 h after lidocaine exposure. This finding is in agreement with previous findings of p38 MAPK activation (phosphorylation) in response to noxious substances over a period of 3–4 h, whereas the total amount of p38 present remains unchanged.[10] Considering that the activation kinetics of the p38 MAPK in neurons are not immediate but rather evolve over several hours, it is tempting to speculate that its inhibition may be neuroprotective even after injury has occurred. However, we found that the therapeutic efficacy of p38 MAPK inhibition declines within 2 h. If one thus assumes that the p38 MAPK inhibitor would need to reach target tissues within 2 h of injury to be effective, coinjection with the LA would seem to be the only feasible method. Ultimately, these findings could evolve into a coapplication of neuroprotective agents with LAs in certain clinical settings, much like gastroprotective agents are coapplied with nonsteroidal antiinflammatory drugs to minimize possible side effects. Potential scenarios are clinical or subclinical lesions preconditioning to neurotoxicity by LAs, such as diabetic neuropathy[24] and chemotherapy-induced neuropathy.[25] The latter conditions are known to induce activation of the p38 MAPK,[26,27] and it is conceivable that increased neurotoxicity of the LA lidocaine may be due to a toxic synergistic effect, as two stimuli converge to induce p38 MAPK, leading to the initiation of apoptosis in neurons.

We hypothesize that activation of the p38 MAPK probably triggers as a downstream event the activation of pathways known to generate proinflammatory cytokines, such as leukotrienes, by activating lipoxygenase. Our observation that coadministration of the enzyme inhibitor NDGA did not inhibit phosphorylation of p38 MAPK, but showed a strong neuroprotective effect, would indicate that its molecular target lies downstream of the p38 MAPK. This hypothesis is consistent with observations that, during spinal sensitization and hyperalgesia, inflammatory mediators from the lipoxygenase pathway such as phospholipase A_2 and cyclooxygenase 2 are potent effector molecules of the p38 MAPK.[12] Moreover, it has been demonstrated that stress-activated phosphorylation of p38 can trigger the release of lipoxygenase into the cytoplasm.[13] Finally, interruption of li-

poxygenase pathways has been shown to be neuroprotective under inflammatory conditions.[28] It should be noted, though, that our results are preliminary, because of the relatively nonspecific mode of enzyme inhibition, which may affect several key target enzymes within arachidonic acid pathways such as the 5-, 12-, and 15-lipoxygenase, which are potent mediators of an inflammatory response. Nevertheless, our results correspond well with previous observations that conjecture a link between activation of p38 MAPK and the subsequent generation of leukotrienes from arachidonic acid.[12] Lipoxygenase inhibition using NDGA seems to be effective only when performed within 1 h of lidocaine application, similar to the time course observed when the p38 MAPK inhibitor, SB23580, was used. This is consistent with previous observations that the action of p38 MAPK on (5-)lipoxygenase is almost instantaneous, occurring within minutes.[13] Therefore, inhibition of both p38 MAPK and lipoxygenase may be effective in attenuating lidocaine-induced neurotoxicity. There exist at the moment no data to indicate which of the latter enzymes shows the most potential in alleviating neurotoxicity. We assume that the further upstream (*i.e.*, p38 MAPK) the noxious pathway is blocked, the more specifically targeted the neuroprotective intervention is.

Finally, we would like to address the relative variability of neuroprotective effects of the p38 MAPK inhibitor across separate experiments involving DRG neurons. Our broad research approach included several freestanding trials, which were conducted in separate cultures freshly harvested from rat DRG. The difference in efficacy may be explained by the variability inherent in any biologic system. Moreover, the beneficial neuroprotective effects of p38 MAPK inhibition were consistent across all experiments and confirmed in a pheochromocytoma cell line culture.

In conclusion, we show that the explicit activation of p38 MAPK after exposure to clinically relevant doses of lidocaine is time dependent and specific and is probably followed by the activation of effector enzymes responsible for the synthesis of proinflammatory cytokines.

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