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ORIGINAL ARTICLE

Lidocaine depolarizes the mitochondrial membrane potential by intracellular alkalization in rat dorsal root ganglion neurons

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Abstract
Purpose The mitochondrial membrane potential ($\Delta\Psi m$) is an important factor for apoptosis, and it is produced by the proton electrochemical gradient ($\Delta\mu H^+$). Therefore, the intracellular proton concentration (pH_{in}) is an important factor for modifying the $\Delta\Psi m$. However, the effects of lidocaine on pH_{in} are unclear. To investigate mitochondrial responses to lidocaine, therefore, we simultaneously measured pH_{in} with $\Delta\Psi m$, flavin adenine dinucleotide (FAD), and reduced form of nicotinamide adenine dinucleotide (NADH) fluorescence, and calculated the FAD/NADH ratio (redox ratio), the superoxide production in mitochondria.
Methods Morphological change and early apoptosis were observed by annexin-V FITC staining under fluorescent microscope. The ratiometric fluorescent probe JC-1 and HPTS were used for the simultaneous measurements of $\Delta\Psi m$ with pH_{in} in rat dorsal root ganglion (DRG) neurons. FAD and NADH autofluorescence were simultaneously measured, and the FAD/NADH fluorescence ratio (redox ratio) was calculated. The superoxide was measured by mitosox-red fluorescent probe for mitochondrial superoxide. Lidocaine was evaluated at 1, 5, and 10 mM.
Results Morphological change and early apoptosis were observed after 10 mM lidocaine administration. Lidocaine depolarized $\Delta\Psi m$ with increased pH_{in} in a dose-dependent manner. In low-pH saline (pH 6), in the presence of both the weak acids (acetate and propionate), lidocaine failed to depolarize $\Delta\Psi m$ and increase pH_{in}. On the other hand, lidocaine decreased the redox ratio in the cell and increased the levels of superoxide in a dose-dependent manner.
Conclusion These results demonstrated that lidocaine depolarizes $\Delta\Psi m$ by intracellular alkalization. These results may indicate one of the mechanisms responsible for lidocaine-induced neurotoxicity.

Keywords Lidocaine · Mitochondrial membrane potential · Intracellular alkalization

Introduction

Lidocaine is commonly used for regional anesthesia and postoperative pain relief. It is well known that local anesthetics, including lidocaine, induce neurotoxicity [1–3], although the exact mechanisms are still uncertain. Sakura et al. and Tokenami et al. reported that intrathecally administered lidocaine induced histological morphological changes such as cell swelling, atrophy, edema, and axonal degeneration, as well as the appearance of myelin ovoids and the loss of axons with macrophage infiltration in the cauda equina [1, 2]. Kasaba et al. [3] reported that lidocaine induced morphological changes such as cell swelling with bullae or blebs and the collapse of growth cones in isolated lymnaea neurons. However, the mechanisms of lidocaine-induced morphological change are still unclear. It has been recently demonstrated that the one of the mechanisms of the neurotoxicity induced by lidocaine is apoptosis through the mitochondrial pathway [4, 5].

A decrease in mitochondrial membrane potential ($\Delta\Psi m$) that is produced by the proton electrochemical gradient between mitochondrial matrix and cytoplasm ($\Delta\mu H^+$) is associated with apoptosis [6–9]. Because the $\Delta\Psi m$ is modified by intracellular proton concentration (pH_{in}), such

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cellular changes regulate ΔΨm [10, 11]. Because lidocaine is a tertiary amine, it can modify pH_{in}. In its base form, lidocaine can readily diffuse through the membrane bilayer and bind intracellular protons, raising pH_{in}. The lidocaine pK_a of 7.8 means that at pH 7.4, 40% of the molecules are in the base (uncharged form) and able to bind H^+. In this process, the pH_{in} may be modified, which presumably affects the ΔΨm. However, the effect of lidocaine on pH_{in} has not been investigated yet, and thus the relationship between pH_{in} and ΔΨm is obscure.

The purpose of this study was to determine if lidocaine causes alterations in pH_{in} and ΔΨm, and if these changes are associated with detectable cellular features associated with apoptosis.

Materials and methods

Cell preparation

All animal experiments were approved by the Animal Care Committee of the University of Miyazaki. The dorsal root ganglion (DRG) neurons were dissected from Wistar rats (aged 7–17 days, either sex) under sevoflurane anesthesia and incubated at 37°C for 30 min in Ca^{2+}- and Mg^{2+}-free saline containing 1 mg/ml collagenase type 1 (Wako, Osaka, Japan) and 1 mg/ml trypsin (Sigma, St. Louis, MO, USA). The DRG neurons were mechanically dissociated with repolished Pasteur pipettes in Dulbecco's modified Eagle's medium (DMEM; Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (GIBCO, Grand Island, NY, USA). The dissociated neurons were plated on a coverslip coated with poly-L-lysine (Sigma) and cultured at 37°C in DMEM supplemented with serum, 50 μ/ml penicillin, and 50 mg/ml streptomycin (Sigma) in a humidified incubator containing 5% CO_2 in air. After 3 days in culture, the neuron was used for the experiment [12]. The ionic composition of the external solution was (in mM): NaCl 140, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, HEPES 5, and D-glucose 10. Lidocaine-HCl (Wako, Tokyo, Japan) was diluted into the external solution. Except in the modified pH trials, the pH of the external solution was adjusted to 7.4. In clinical practice in Japan, 2% lidocaine (75 mM) is used in local or epidural anesthesia; therefore, we chose a maximum concentration of 10 mM. All experiments were performed at 37°C under bubbling air containing 5% CO_2.

Imaging of morphological change and determination of early apoptosis by annexin-V

The phosphatidylserine that is translocated from the inner part of the cell membrane to the outer layer in early apoptosis was stained by annexin-V conjugated with fluorescein isothiocyanate (FITC; Roche Applied Science, Mannheim, Germany) [13, 14]. Neurons were loaded for 20–30 min with annexin-V conjugated with FITC (20 μl/ml) at 37°C. Fluorescent images were acquired with an AQA-COSMOS fluorescent imaging system (Hamamatsu Photonics, Tokyo, Japan). A 488-nm wavelength was used for excitation in conjunction with a 505-nm dichroic mirror. Emitted light was separated by 535 nm.

Imaging of mitochondrial membrane potential by JC-1

The ΔΨm was assessed with 5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolocarbocyanine iodide (JC-1) (Molecular Probes, Eugene, OR, USA) based on methods described elsewhere [15, 16]. Neurons were loaded for 20–30 min with 2 μM JC-1 at 37°C. A 3-ml perfusion chamber was fitted to a fluorescent microscope (Nikon TE-300, Tokyo, Japan), and the cells were perfused at 3 ml/min while images were acquired with an AQA-COSMOS fluorescent imaging system (Hamamatsu Photonics). A 500-nm wavelength was used for excitation in conjunction with a 505-nm dichroic mirror. Emitted light was separated by 535- and 590-nm filters; the two wavelengths corresponded to the peak fluorescence from the monomer and aggregate signals, respectively. The two wavelength images were calculated into the ratio image and its corresponding value by computer.

HPTS-intracellular pH (pH_{in}) imaging

For the measurement of pH_{in}, a ratiometric fluorescent indicator, 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) (Molecular Probes), was used [17]. HPTS (1 mM) with pipette solution (140 mM KCl, 1 mM $CaCl_2$, 2 mM ATP-Mg, and 0.1 mM GTP–Tris, pH 7.4) was injected into the cells by microelectrodes (resistance, 10 MΩ) for 10 min by current injection. Measurements were made by exciting this indicator dye at 450 and 405 nm (450/405 nm ratio) with continuous recording at 510 nm.

In vivo calibration of pH_{in}

Two fluorescence ratios with HPTS were converted into the pH_{in} using the calibration curve for each neuron in vivo [18]. The calibration curve for the pH_{in} was constructed by plotting the fluorescence ratio versus the pH of the calibration solutions (140 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 10 mM HEPES, and 10 μM nigericin, adjusted to each pH level with KOH). To equilibrate the pH_{in} with the extra (pH_{out}), 20 min before the experiment, 10 μM of the K^+/H^+ exchanger nigericin (Sigma, St. Louis, MO, USA) was added, and then the neurons were exposed to pH calibration

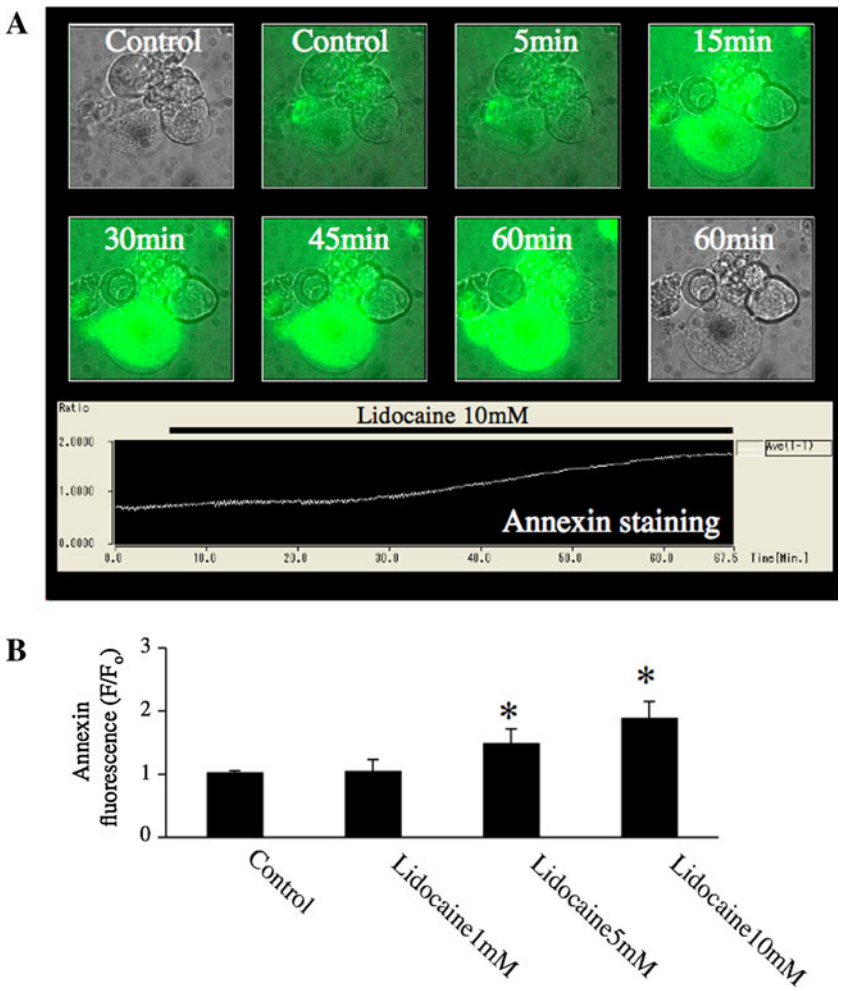

Fig. 1 **a** Morphological change before and after lidocaine perfusion. Lidocaine (10 mM) was perfused for 5 min. The fluorescent intensity of annexin-V conjugated with fluorescein isothiocyanate (FITC) was measured before (*Control*) and after lidocaine treatment. **b** Fluorescent intensity ratio versus the control value (F/F_0) of annexin-V conjugated with FITC. Results are presented as means ± SD, $n = 5$. $^*P < 0.05$ in comparison to the control values

solutions with nigericin. Fluorescent image pairs were taken, and then the neurons were exposed to each pH saline solution (pH 6.0, 6.5, 7.0, 7.5, and 8.0) for 10 min. Image pairs were taken again at the end of each exposure.

The experimental procedure for simultaneously imaging JC-1 ($\Delta\Psi$m) and HPTS (pH_{in})

In each imaging experiment, neurons were divided into three trials: normal pH, modified pH, and neutralization trials by weak acid (acetate and propionate).

In the modified pH trials, to observe the effect of pH_{in} on $\Delta\Psi$m, pH_{in} was changed by the nigericin-containing pH calibration solutions, and $\Delta\Psi$m was measured in each pH_{in}. In the normal pH trials, lidocaine (1, 5, and 10 mM) was perfused into the culture dish for 5 min after measuring the baseline values. We also investigated the effects of lidocaine when the pH of the external solution was changed to 6. In the neutralization trials, 10 mM lidocaine with 10 mM acetate was perfused for 5 min to neutralize pH_{in}, and then 10 mM lidocaine alone was perfused for 5 min and washed into the external solution. In each trial, the baseline values were measured 2 min before lidocaine perfusion.

FAD/NADH fluorescence ratio (redox ratio)

NADH was excited at 366 nm, and the emission was monitored at 470 nm. FAD was excited at 450 nm, and the emission was monitored at 520 nm. The autofluorescence ratio between FAD and NADH was calculated and is exhibited as "redox ratio" [19]. The data were analyzed with the AQA COSMOS software (Hamamatu Photonics).

Mitochondrial superoxide measurement by mitosox-red

For the measurement of superoxide in mitochondria, the fluorescent probe mitosox-red (Molecular Probes) was used [20]. Mitosox-red was mixed with the defined medium containing the neuron to a final concentration of 1 μM.

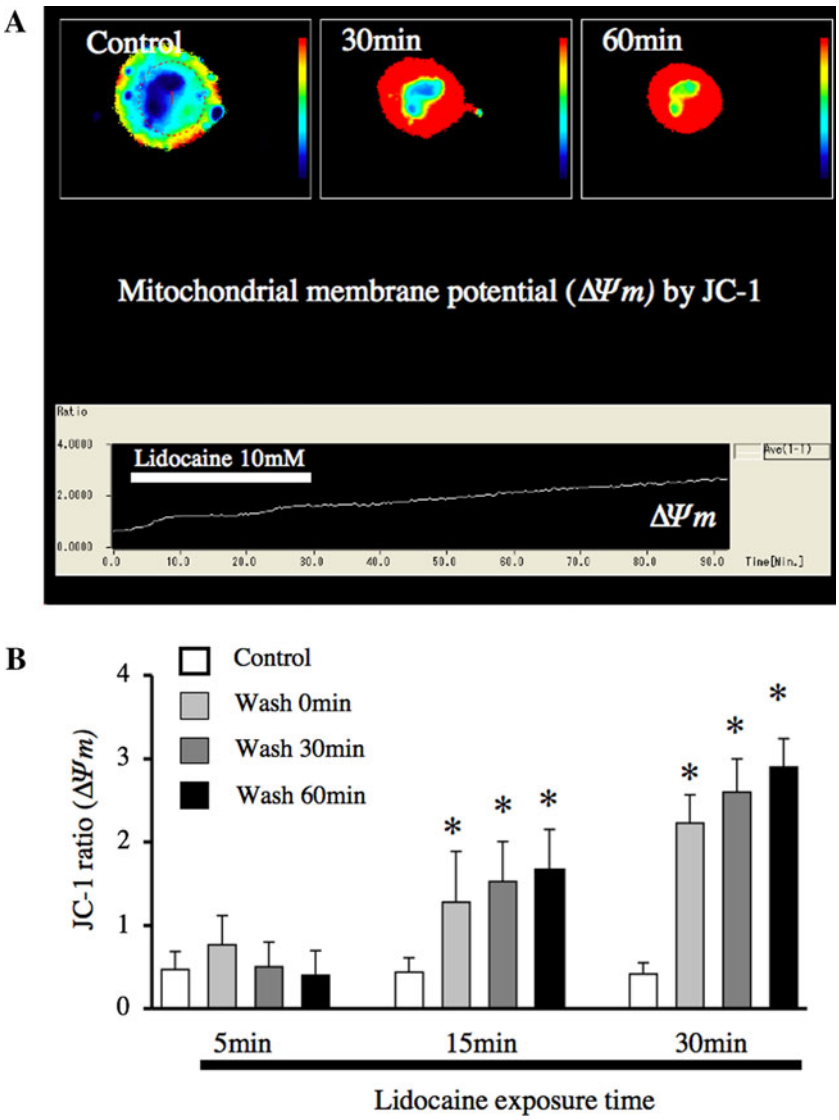

Fig. 2 **a** Mitochondrial membrane potential change ($\Delta\Psi$m) before and after lidocaine perfusion. Lidocaine (10 mM) was perfused for 2–30 min. **b** $\Delta\Psi$m was measured at 1 min (control), 30 min (immediately after lidocaine perfusion), and 60 min (after washout of lidocaine). Results are presented as means ± SD, $n = 8$. *$P < 0.05$ in comparison to the baseline values

After incubation at 20°C for 15 min, this defined medium was removed. Measurements were made by exciting this indicator dye at 510 nm, recording at 580 nm, and calculating intensity alterations (F/F_0).

Statistical analysis

The results are expressed as the mean ± standard deviation (SD). The results of repeated measurements in each dose, in each group of trials, were analyzed by repeated measurement of one-way analysis of variance (ANOVA), followed by the Scheffe's test. Between trials was analyzed by Student's paired t test. Stat view (version 4.5; Abacus, Berkeley, CA, USA) was used for these analyses. A value of $P < 0.05$ was considered to be statistically significant.

Results

Lidocaine-induced morphological change and early apoptosis

Figure 1 shows morphological changes and early apoptosis after 10 mM lidocaine administration. Figure 1b shows that the fluorescent intensity ratio versus the control value (F/F_0) of annexin-V conjugated with FITC was increased in a concentration-dependent manner.

Irreversible $\Delta\Psi$m depolarization by lidocaine

Figure 2a shows the experimental tracking of $\Delta\Psi$m before and after 10 mM lidocaine administration. Following lidocaine perfusion, $\Delta\Psi$m was significantly depolarized by

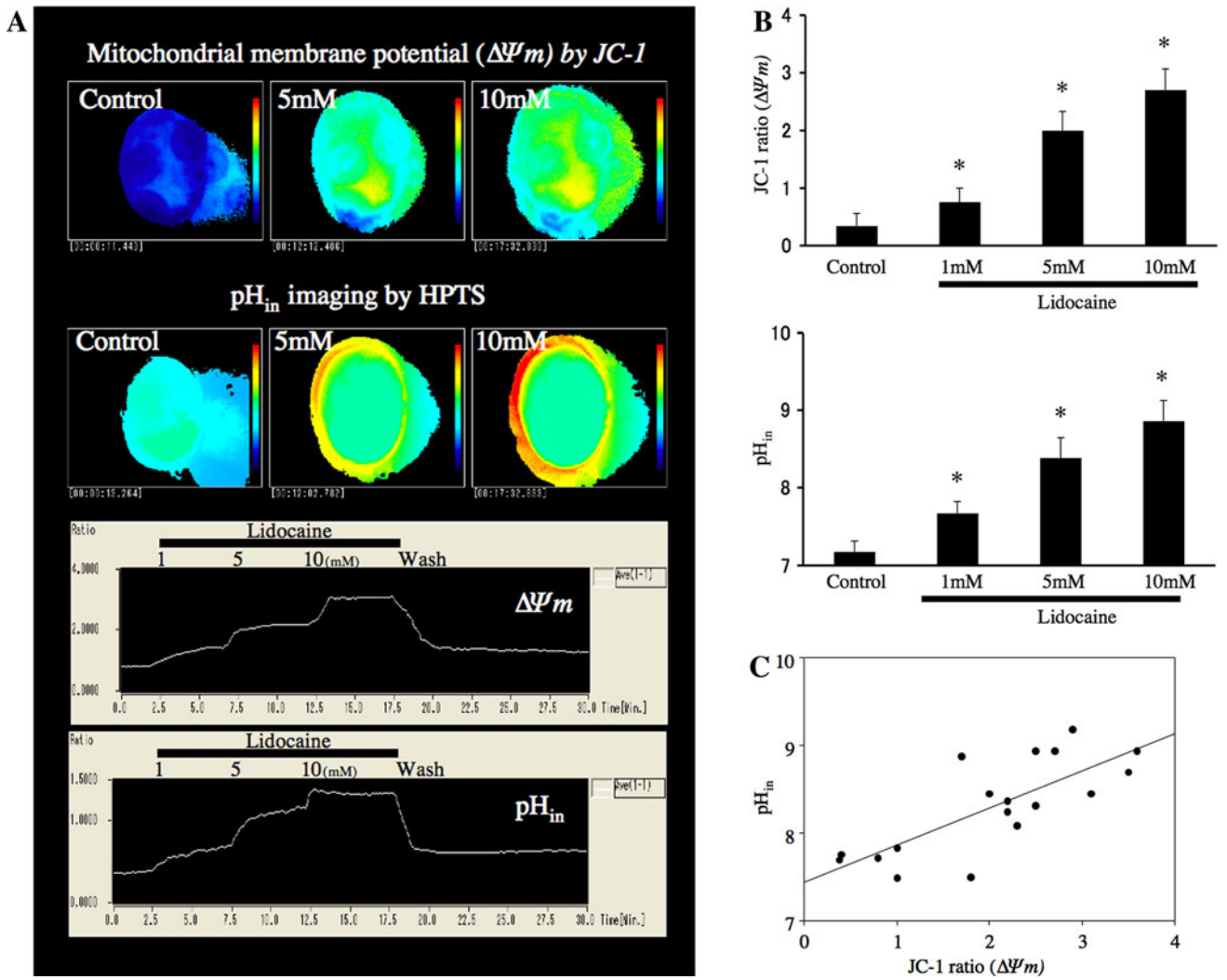

Fig. 3 **a** Simultaneously imaging ΔΨm and pH_{in} by 5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolocarbocyanine iodide (*JC-1*) and 8-hydroxypyrene-1,3,6-trisulfonic acid (*HPTS*). The external solution was changed to each dose of lidocaine-containing solution for 5 min. *Upper trace* and *graph* exhibit ΔΨm; *lower trace* and *graph* exhibit pH_{in}. **b** Data of ΔΨm and pH_{in}. Results are presented as mean ± SD, $n = 8$.*$P < 0.05$ in comparison to the baseline values; $^{\dagger}P < 0.05$ in comparison to the 10 mM lidocaine perfusion. **c** Correlation of change in ΔΨm and pH_{in} 5 min later with each concentration of lidocaine

lidocaine. The ΔΨm depolarized during the exposure period, so within 5 min of lidocaine (10 mM) exposure, the ΔΨm recovered to baseline; however, with more than 15 min exposure, recovery did not take place (Fig. 2b).

Simultaneously imaging ΔΨm and pH_{in}

Normal pH trials

In the ΔΨm imaging by JC-1, lidocaine significantly depolarized the ΔΨm (JC-1 fluorescence ratio) in a dose-dependent manner from 0.5 ± 0.2 at baseline to 0.9 ± 0.4 at 1 mM, to 2.2 ± 0.5 at 5 mM, and to 3 ± 0.5 at 10 mM (Fig. 3a,b). In the pH_{in} imaging by HPTS, lidocaine significantly increased pH_{in} in a dose-dependent manner from 7.2 ± 0.2 at baseline to 7.7 ± 0.2 at 1 mM, to 8.4 ± 0.3 at 5 mM, and to 8.9 ± 0.4 at 10 mM (Fig. 3a,b). A significant relationship between ΔΨm and pH_{in} was observed (Fig. 3c). The correlation coefficient was 0.766 ($P < 0.0001$).

Modified pH trials

In the modified pH trials, pH_{in} was changed by nigericin-containing pH calibration solutions, and ΔΨm was depolarized with pH_{in} increase (Fig. 4). A significant relationship between ΔΨm and pH_{in} was observed (Fig. 4b). The correlation coefficient was 0.892 ($P < 0.0001$).

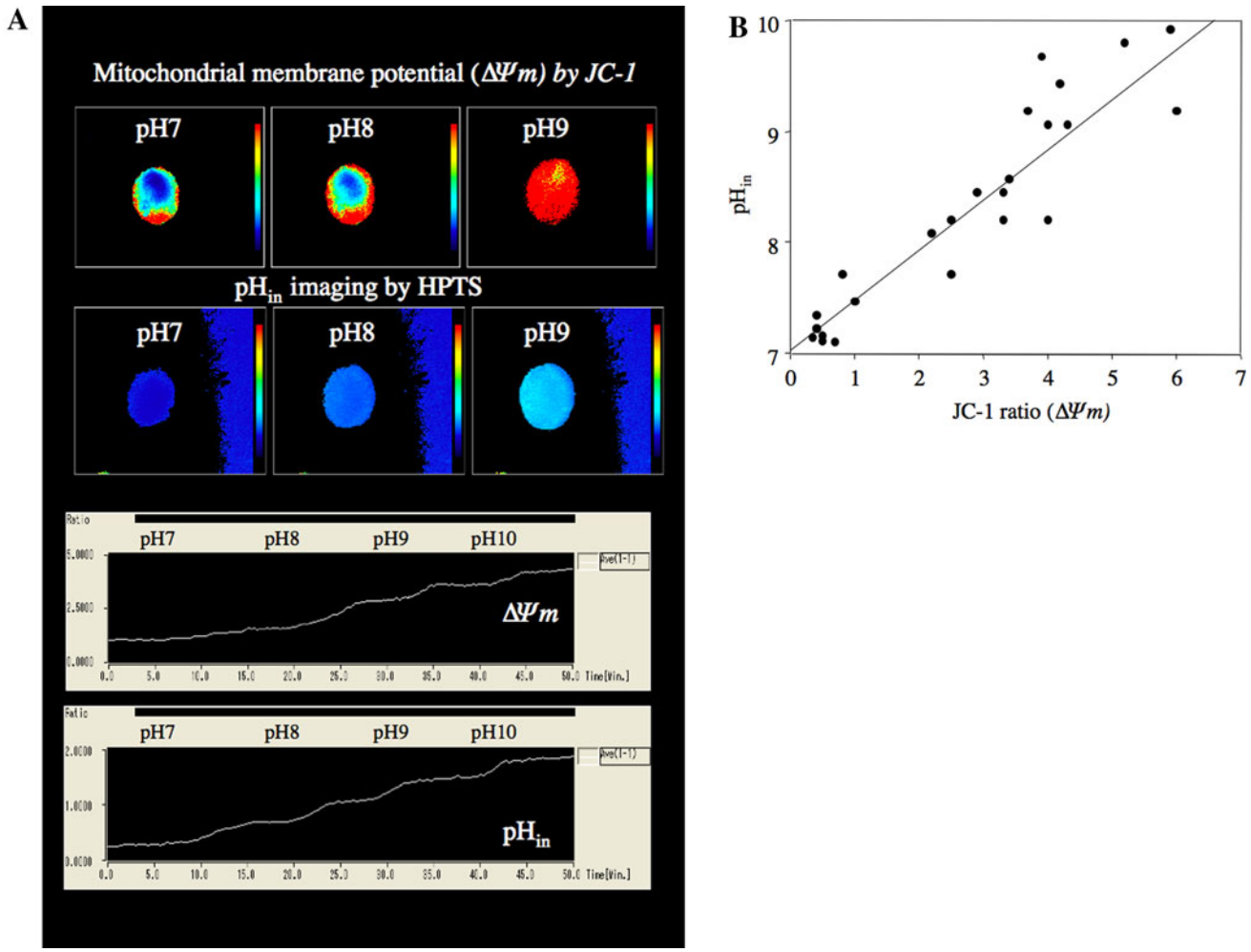

Fig. 4 **a** Simultaneously imaging of ΔΨm and pH_{in} by JC-1 and HPTS. The external solution was changed to each pH of nigericin-containing pH calibration solution for 10 min. *Upper trace* and *graph* exhibit ΔΨm; *lower trace* and *graph* exhibit pH_{in}. **b** Correlation of change in ΔΨm and pH_{in} 10 min later with each pH of pH calibration solutions

ΔΨm and pH_{in} in the neutralization trials

In the neutralization trials, 10 mM lidocaine with 10 mM acetate (Fig. 5a) and 10 mM lidocaine with 10 mM propionate perfusion significantly hyperpolarized the ΔΨm more than 10 mM lidocaine alone: in 10 mM lidocaine, ΔΨm was 2.5 ± 0.7; in 10 mM lidocaine with 10 mM acetate perfusion, ΔΨm was 1.1 ± 0.5; and in 10 mM lidocaine with 10 mM propionate perfusion, ΔΨm was 1.2 ± 0.5 (Fig. 5b). Lidocaine at low pH (pH 6.0) depolarized ΔΨm only slightly: the ΔΨm was 1.4 ± 0.6 (Fig. 5b).

FAD/NADH fluorescence ratio (redox ratio)

Lidocaine decreased the FAD/NADH fluorescence ratio in a dose-dependent manner: FAD/NADH fluorescence ratio changed from 0.89 ± 0.11 at control to 0.74 ± 0.14 at lidocaine 1 mM, to 0.51 ± 0.2 at lidocaine 5 mM, and to 0.36 ± 0.25 at lidocaine 10 mM, respectively (Fig. 6a,b). Rotenone (100 μM) a mitochondrial respiratory chain complex inhibitor, also decreased the FAD/NADH fluorescence ratio to 0.46 ± 0.17.

Superoxide measurement

Lidocaine increased both mitochondrial superoxides in a dose-dependent manner (Fig. 7a, b).

Discussion

The present study demonstrated lidocaine-induced morphological changes such as cell swelling with bullae or blebs that were similar to the morphological changes in apoptosis. The phosphatidylserine translocated from the inner part of the cell membrane to the outer layer was also

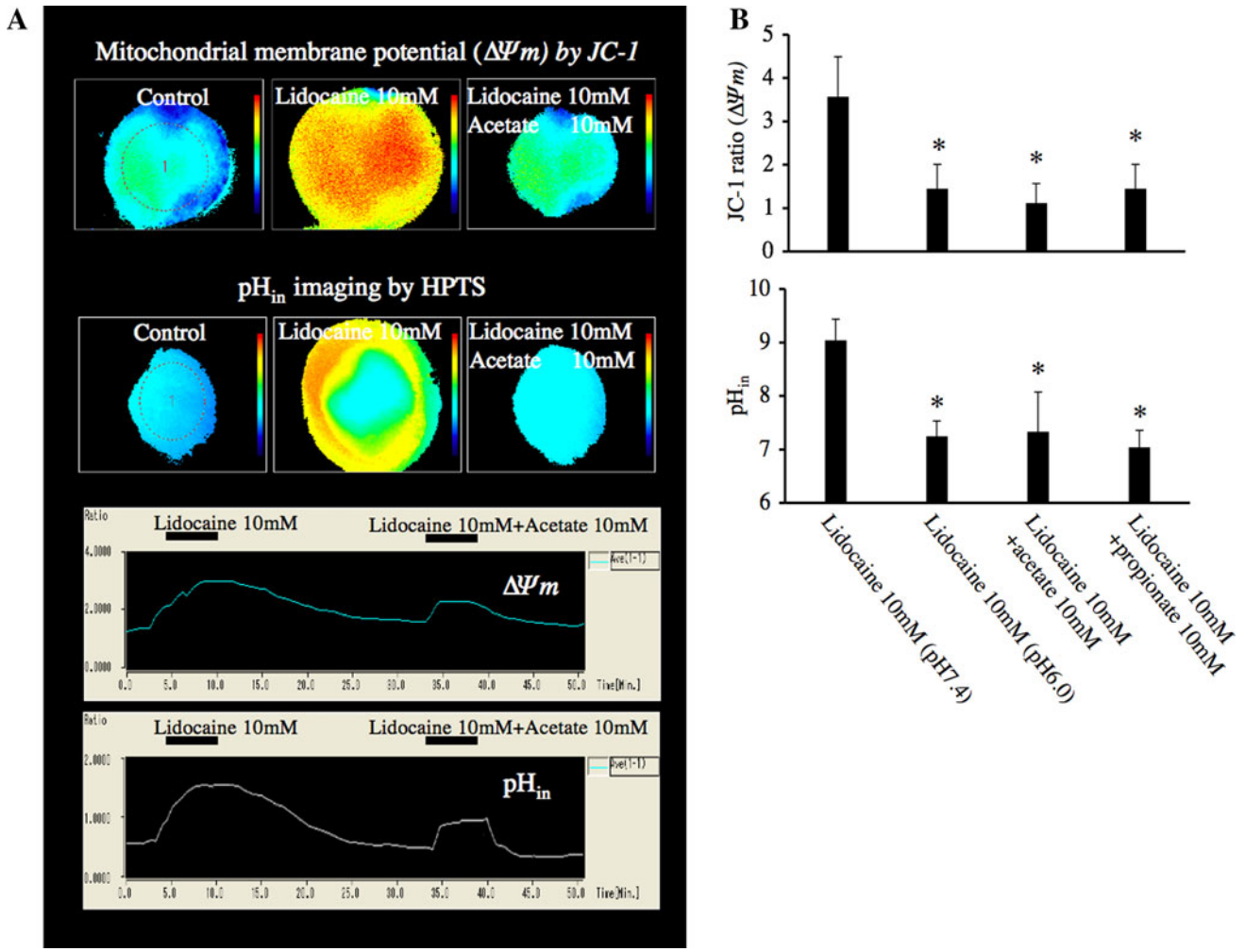

Fig. 5 **a** ΔΨm and pH_{in} in the neutralization trials. *Upper trace* and *graph* exhibit ΔΨm; *lower trace* and *graph* exhibit pH_{in}. **b** Data of ΔΨm and pH_{in}. In the neutralization trials, lidocaine with acetate (10 mM, pH 8) ($n = 8$) or lidocaine with propionate (10 mM, pH 8) ($n = 6$) was perfused for 5 min. Also, lidocaine diluted in low-pH saline (10 mM, pH 6) was perfused for 5 min ($n = 7$). Results are presented as mean ± SD;*$P < 0.05$ versus lidocaine 10 mM perfusion group

stained by the annexin-V FITC, indicating that the early stage of apoptosis was induced by lidocaine. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Jiang et al. [21] reported that externalization of PS is associated with release of cytochrome *c* from mitochondria at early periods of apoptosis. Annexin V is a Ca^{2+}-dependent phospholipid-binding protein that has a high affinity for PS and binds to cells with exposed PS. Because externalization of PS occurs in the earlier stages of apoptosis, FITC annexin V staining can identify apoptosis at an earlier stage [13, 14, 22, 23].

The present study also demonstrated that lidocaine depolarized ΔΨm in a time- and dose-dependent manner, and that ΔΨm is related to increase in pH_{in} caused by lidocaine. In the modified pH trials, the mitochondrial membrane potential showed more depolarized potential at a higher pH than at a lower pH, and a significant relationship between ΔΨm and pH_{in} was observed. In the neutralization trials, acetate and propionates neutralized the increase in pH_{in}, and it also significantly suppressed the ΔΨm depolarization by lidocaine. These results indicated that lidocaine depolarizes ΔΨm by intracellular alkalization. Moreover, lidocaine increases in NADH fluorescence and decreases in FAD fluorescence and redox ratio. Lidocaine also increased the mitochondrial superoxide production, and these results supported the idea that lidocaine inhibits the electron transport chain.

According to the equation of Sanchez et al. [24], at pH 7.4, about half of the lidocaine is a base, because the base form of lidocaine can pass across the cell membrane. After diffusion into the cell, lidocaine molecules will bind protons, making the inside of the cell more alkaline [25–27].

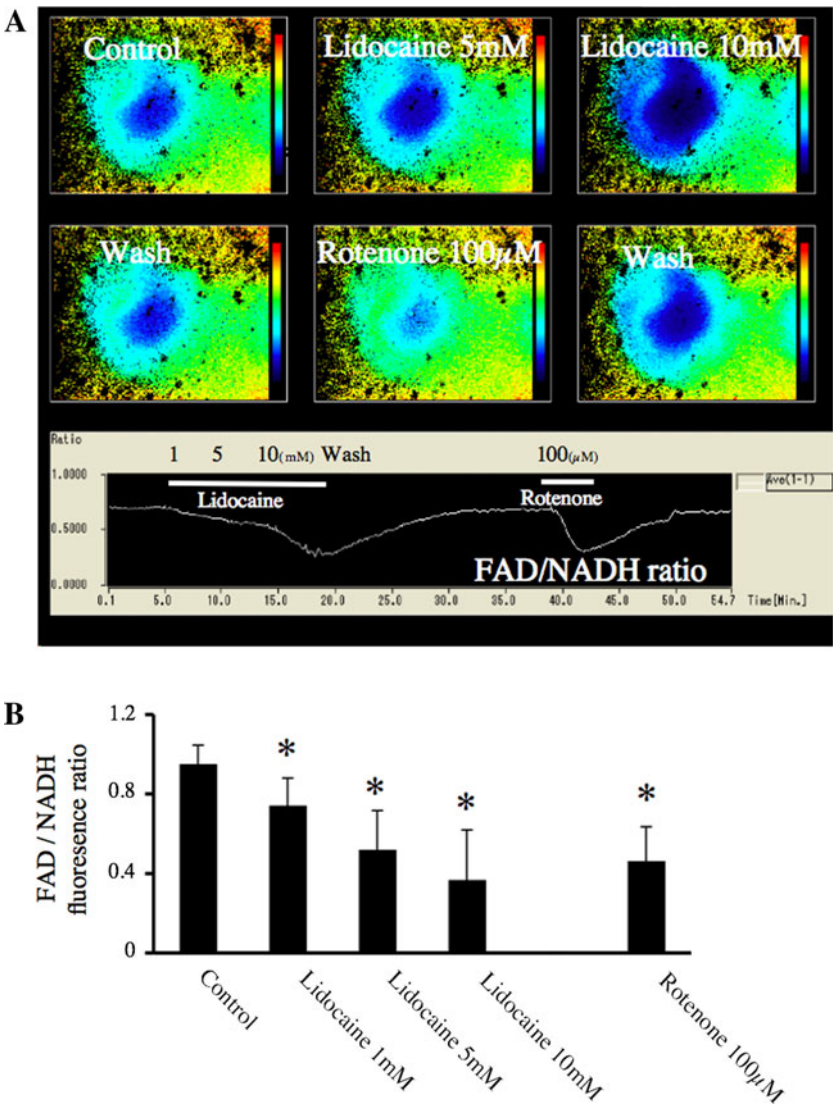

Fig. 6 **a** FAD/NADH ratio (redox ratio) change before and after lidocaine and rotenone perfusion. The external solution was changed to each dose of lidocaine and rotenone containing saline for 5 min. **b** FAD/NADH ratio (redox ratio) in each dose of lidocaine ($n = 9$) and rotenone ($n = 8$). Results are presented as mean ± SD;*$P < 0.05$ versus baseline (control)

At pH 6, most of the lidocaine is in a charged (protonated) form; therefore, at pH 6, lidocaine did not change pH_{in} and ΔΨm. Because ΔΨm is generated by the proton gradient between the cytoplasm and mitochondrial matrix, it is not surprising that increased pH_{in} (decreased $[H^+]_{in}$) decreases the ΔΨm [28–30]. Khaled et al. [31] reported that the intracellular pH rose above pH 7.8, inducing mitochondrial membrane potential depolarization. Similarly, we observed a proportional decrease in depolarized ΔΨm with increasing pH. By binding protons, lidocaine decreased the proton gradient between the mitochondrial matrix and the cytoplasm, resulting in depression of ΔΨm. Therefore, intracellular alkalization induced by the "proton-trapping" of lidocaine seems to attenuate the proton gradient between the mitochondrial matrix and cytoplasm and to induce ΔΨm depolarization.

If "intracellular alkalization," that is, the binding of intracellular protons by lidocaine, depolarizes the ΔΨm by reducing the proton gradient, then supplying protons by providing a weak acid should prevent proton depletion. We found that providing weak acids that could diffuse into the cell (acetate and propionate) prevented the decrease in protons (rise in pH) caused by the diffusion of lidocaine into the cells. The lidocaine-induced ΔΨm depolarization was suppressed, suggesting that one of the mechanisms of lidocaine-induced ΔΨm depolarization is indeed intracellular alkalization.

The FAD/NADH ratio (redox ratio) and superoxide production are markers of electron transport chain activity [32, 33]. Fujimoto et al. [34] reported that cytosolic alkalization decreased the supply of electron donors, including NADH and $FADH_2$, and the supply of endogenous mitochondrial ROS, which induces the depolarization of ΔΨm, to the respiratory chain. Rotenone, an inhibitor of NADH oxidase, inhibits NADH oxidation, which increases NADH and decreases the redox ratio [35, 36]. In this study, we

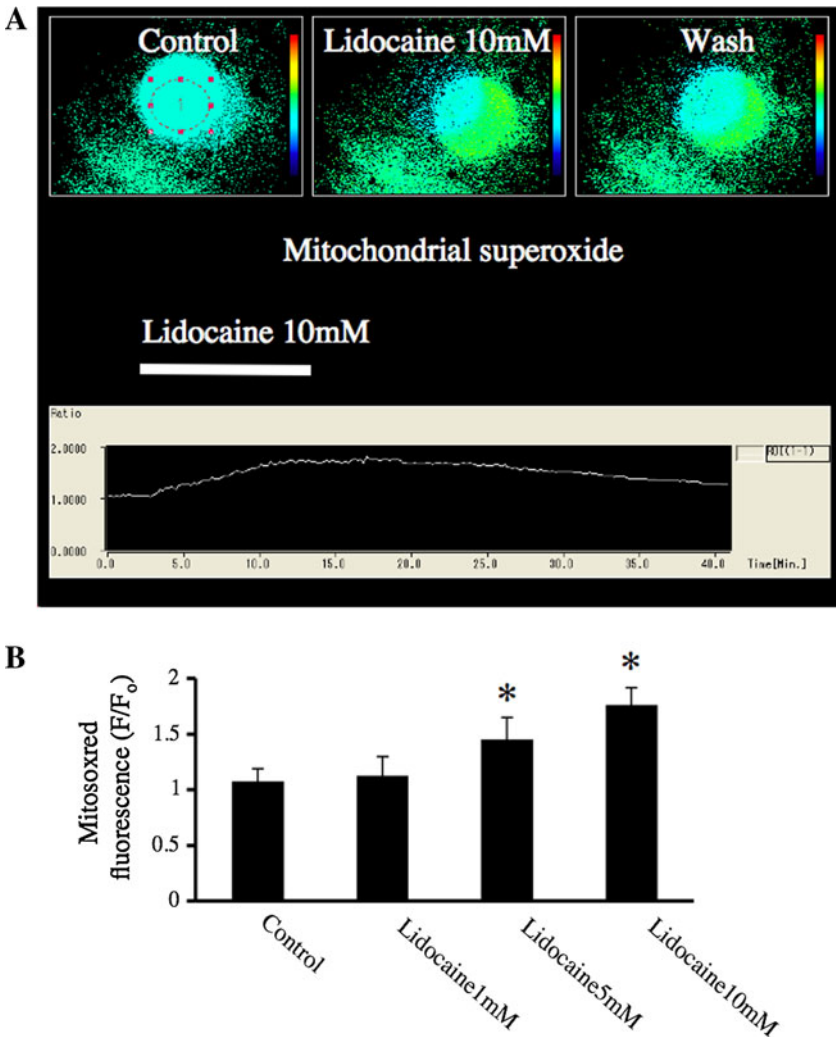

Fig. 7 **a** Mitochondrial superoxide change before and after lidocaine perfusion. The external solution was changed to each dose of lidocaine for 5 min. **b** Mitochondrial superoxide in each dose of lidocaine. Results are presented as mean ± SD, $n = 6$;*$P < 0.05$ versus baseline (control)

demonstrated the first evidence that lidocaine increases NADH fluorescence and decreases the redox ratio the same as rotenone. It is, therefore, suggested that lidocaine inhibited the electron transport chain and increased superoxide, resulting in the inhibition of the electron transport chain.

There is another mechanism for ΔΨm depolarization is opening of the permeability transition pore (PTP) in mitochondria. The PTP is affected by cytosolic free calcium and is blocked by cyclosporin A. Irwin et al. [37] reported that bupivacaine opened the PTP and induced ΔΨm depolarization of rat skeletal muscle. They also investigated the effect of bupivacaine on oxygen utilization and NADH level and observed that bupivacaine has biphasic effects by its concentration. They reported that in the low concentration, less than 0.5 mM, bupivacaine increased oxygen utilization and decreased NADH level. On the other hand, at a high concentration greater than 1 mM, bupivacaine induced a decrease in oxygen utilization and an increase in NADH level at a high concentration similar to ours.

It has been demonstrated that ΔΨm depolarization is one of the triggers of apoptosis through the mitochondrial pathway, such as the point of release of cytochrome *c* [38]. Chen et al. [39] reported that an alkaline condition (pH 7.4 to 8.0) induced cell apoptosis by activating caspase-8 and caspase-3 in the ASTC-a-1 cells. Suzuki et al. [40] reported that ammonia (NH4Cl) induced a pH increase from pH 5 to 7, inducing apoptosis in GSM06 cells. Because the elevation of intracellular pH enhances the conversion of NH_3 to NH_4^+, it seems reasonable to assume that at higher ambient pH, relatively high concentrations of NH_3 diffuse across the cell membrane more readily and enhance cytochrome *c* release from the mitochondria, thereby accelerating the apoptotic pathway. This theory is quite similar to our idea that lidocaine increases intracellular pH and enhances the conversion of a base form to a charged form.

To measure ΔΨm, JC-1 was used in this experiment; however, no interference or fluorescence change was observed in conjunction with the pH (between pH 7 and 9) of diluted solutions and lidocaine. Lidocaine at 10 mM

lidocaine and acetate or propionate changed the osmolarity by 4%; however, no change was observed in the pH_{in} or $\Delta\Psi m$.

In this study, we did not observe cytosolic free calcium and direct membrane toxicity of lidocaine; therefore, it is not certain whether lidocaine-induced $\Delta\Psi m$ depolarization is caused by only the "intracellular alkalization" or is caused by another mechanism such as cytosolic calcium and membrane toxicity. In any case, further investigation about the effects for PTP, cytosolic free calcium, and direct membrane toxicity by lidocaine, are required.

In conclusion, lidocaine depolarizes the mitochondrial membrane potential by intracellular alkalization that may induce morphological change, apoptosis, and cell toxicity. Our results may suggest that the mitochondrial depolarization induced by the long-term exposure of lidocaine will produce mitochondrial irreversible damage and that this will be one means by which lidocaine induces apoptosis through the mitochondrial pathway.

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