Natural Science Research Univ. Tokushima (Peer-Reviewed Paper)
Vol. 24, No.1 (2010) p.1-5

Cytotoxic actions of lidocaine at sublethal concentrations: A model *in vitro* experiment using rat thymocytes

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Abstract

In order to reveal some cytotoxic features of lidocaine, the effects of lidocaine at sublethal concentrations (10 mM or less) on rat thymocytes were examined by the use of flow-cytometric technique with fluorescent probes. Treatment with lidocaine at 3-10 mM for 1-3 hr significantly increased intracellular Ca^{2+} and Zn^{2+} concentrations under normal condition. After the removal of external Ca^{2+} and Zn^{2+}, lidocaine still increased intracellular Ca^{2+} and Zn^{2+} concentrations, suggesting the lidocaine-induced release of intracellular Ca^{2+} and Zn^{2+}. Treatment with lidocaine at 0.3-1 mM for 24 hr significantly increased the populations of dead cells and shrunken cells. Lidocaine at sublethal concentrations exerts some cellular actions that are supposed to be linked to the cytotoxicity. The mechanism of lidocaine-induced cell death varies in time- and concentration-dependent manners.

1. Introduction

Local analgesics are the most commonly used group drugs in dental practice. However, it may be unlikely that dentists properly understand the toxicology of these drugs due to their frequent use and high margin of safety. About 260 million cartridges of local anesthetics are administered in the United States of America and more than half contain lidocaine (Pogrel, 2007). The concentration of lidocaine in cartridges is 2%, indicating 69.2 mM as lidocaine hydrochloride with one mol H_2O per mol. In our previous study (Nishimura *et al.*, 2006), lidocaine at 30 mM induced necrosis with some apoptotic characteristics in rat thymocytes. The agent increased the populations of dead cells, shrunken cells, and phosphatidylserine-exposed cells, but not the cells with hypodiploidal DNA. However, the incubation of cells with lidocaine at 10 mM or less for 3 h did not affect the populations. However, one may argue the possibility that lidocaine at lower concentrations induce cytotoxic action in a time-dependent manner.

In present study, we set up the hypothesis that lidocaine at concentrations of 10 mM or less exerts a variety of cellular actions that are possibly linked to the cytotoxicity because the cytotoxic action is not only concentration-dependent but also time-dependent.

2. Materials and Methods

2.1. Chemicals:

Lidocaine hydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fluorescent probes, propidium iodide, Fluo-3-AM, and FluoZin3-AM, were the products of Molecular Probes Inc. (Eugene, Oregon, USA). Dimethyl sulfoxide (DMSO) as a solvent for Fluo-3-AM and FluoZin-3-AM was purchased from Sigma Chemical. Final concentration of DMSO in cell suspension was 0.1 % or less. The incubation with DMSO at 0.3 % or less did not affect the viability of rat thymocytes during experiments. Other chemicals were also purchased from Woko Pure Chemicals.

2.2. Animals and cell preparation:

The procedure to prepare cell suspension was similar to that previously reported (Chikahisa and Oyama, 1992; Chikahisa et

al., 1996). Briefly, thymus glands dissected from ether-anesthetized rats (Wistar strain) were sliced at a thickness of 400-500 μm with razor under an ice-cold condition (1-4ºC). The slices were triturated by gently shaking in chilled Tyrode's solution (in mM: NaCl 150, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 5, HEPES 5, with an appropriate amount of NaOH to adjust pH to 7.3-7.4) or RPMI 1640 to dissociate thymocytes. Thereafter, the solution containing the cells was passed through a mesh (a diameter of 10 μm) to prepare the cell suspension (about 5×10^5 cells/mL). Lidocaine was added to cell suspension (2 ml cell suspension). To examine the effects of lidocaine on Fluo-3 and FluoZin-3 fluorescence, the cells were incubated with the agent at 36-37°C for 1 hr under room air condition. To test the effect of prolonged exposure of lidocaine, the cells were cultured in 24-well Falcon tissue culture plates (2 ml in each well) at 36-37ºC in a CO_2 incubator (Sanyo, Tokyo).

Thymocyte is used for a model experiment of cell death induced by chemical compounds because of following reasons. First, the cell membranes are remained intact because single cells are prepared without any enzymatic treatment. Second, the process of apoptosis is defined in thymocytes (Winoto, 1997; Quaglino and Ronchetti, 2001). Third, several types of hormones, biological compounds, and chemicals induce apoptosis in thymocytes (Quaglino and Ronchetti, 2001). Finally, the cells spontaneously undergo apoptosis with apparent changes in cell size and cellular parameters (Tiso et al., 1995; Rinner et al., 1996; Rennecke et al., 2000; Ortiz et al., 2001).

2.3. Fluorescence measurements of cellular and membrane parameters:

The methods for measurements of cellular and membrane parameters using a flow cytometer equipped with an argon laser (CytoACE-150, JASCO, Tokyo, Japan) and fluorescent probes were similar to those previously described (Chikahisa and Oyama, 1992; Chikahisa et al., 1996). The fluorescence was analyzed by JASCO software (Ver.3XX, JASCO). As to chemicals used in this study, there was no fluorescence detected under our experimental condition. To assess cell lethality, propidium iodide was added to cell suspension to achieve a final concentration of 5 μM. Since propidium stains dead cells, the measurement of propidium fluorescence from cells provides a clue to estimate the lethality. The fluorescence was measured at 2 min after the application of propidium iodide by a flow cytometer. Excitation wavelength for propidium was 488 nm and emission was detected at 600 ± 20 nm. FluoZin-3-AM (Gee et al., 2002) and Fluo-3-AM (Kao et al., 1989; Minta et al., 1989) are indicators for Zn^{2+} and Ca^{2+}, respectively. The cells were respectively incubated with 500 nM FluoZin-3-AM or 500 nM Fluo-3-AM for 60 min before any fluorescence measurements to estimate the changes in intracellular Zn^{2+} and Ca^{2+} concentration of rat thymocytes with intact membranes. FluoZin-3 or Fluo-3 fluorescence was measured from the cells that were not stained with 5 μM propidium iodide. Excitation wavelength for FluoZin-3 and Fluo-3 was 488 nm and emission was detected at 530 ± 15 nm.

3. Results and Discussion

3.1. Cytotoxic actions of lidocaine at sublethal milimolar concentrations

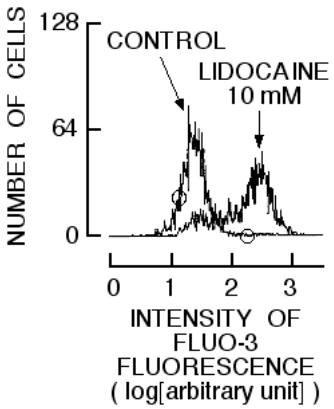

Figure 1
Effect of 10 mM lidocaine on histogram of Fluo-3 fluorescence monitored from 2000 cells.

As shown in Figure 1, 10 mM lidocaine shifted the histogram of Fluo-3 fluorescence to a direction of higher intensity, indicating an increased concentration of intracellular Ca^{2+} when the cells were incubated with the agent for 1 h. The augmentation of Fluo-3 fluorescence by lidocaine was observed when

the concentration was 3 mM while it was not the case for 1 mM (Figure 2).

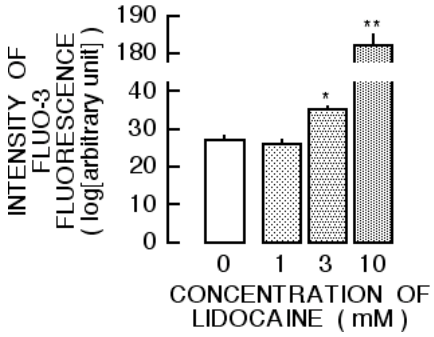

Figure 2
*Dose-dependent change in lidocaine-induced augmentation of Fluo-3 fluorescence. Mean and SD of 4 experiments. * P<0.05, ** P<0.01*

Removal of external Ca^{2+} greatly attenuated the increase in intensity of Fluo-3 fluorescence by 10 mM lidocaine (Figure 3). However, the increase by lidocaine was statistically significant when the concentration was 3 mM or more.

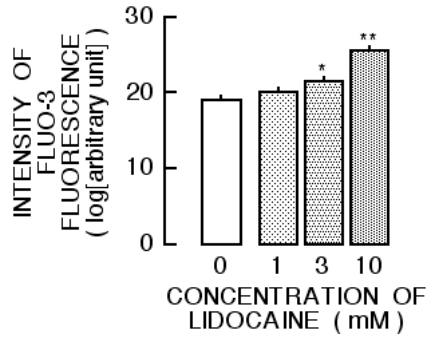

Figure 3
Dose-dependent effect of lidocaine on Fluo-3 fluorescence under external Ca^{2+}-free condition.

Lidocaine at 10 mM also shifted the histogram of FluoZin-3 fluorescence to a direction of higher intensity (Figure 4), indicating an increased concentration of intracellular Zn^{2+}. Significant increase in FluoZin-3 fluorescence was observed in the cases of 3 mM and 10 mM lidocaine (Figure 5).

Under external Ca^{2+}- and Zn^{2+}-free condition, the augmentation of FluoZin-3 fluorescence by 3 mM and 10 mM lidocaine was observed. It can be concluded that lidocaine at 3 mM or more affect intracellular circumstance of Ca^{2+} and Zn^{2+}.

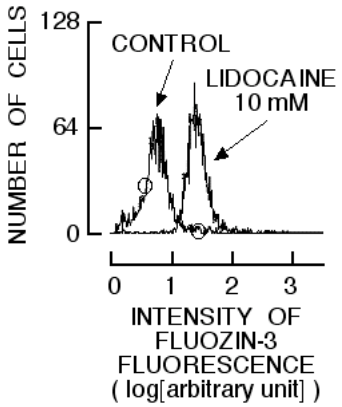

Figure 4
Effect of 10 mM lidocaine on histogram of FluoZin-3 fluorescence monitored from 2000 cells.

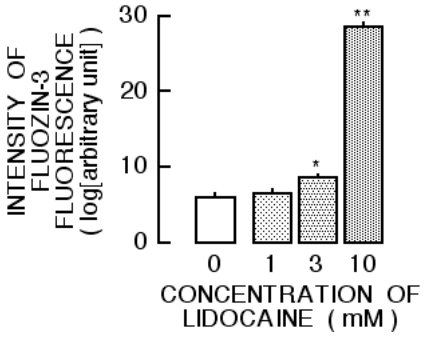

Figure 5
*Dose-dependent change in lidocaine-induced augmentation of FluoZin-3 fluorescence. Mean and SD of 4 experiments. * P<0.05, ** P<0.01*

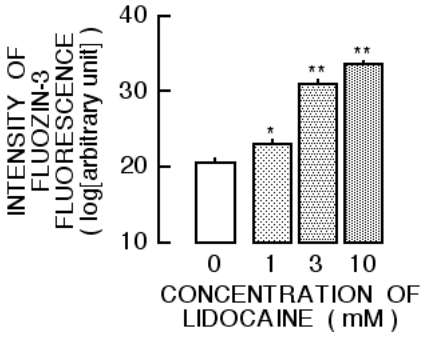

Figure 6
Dose-dependent effect of lidocaine on FluoZin-3 fluorescence under external Zn^{2+}-free condition.

3.2. Cytotoxic actions of prolonged incubation with micromolar lidocaine:

The incubation of cells with 1 mM lidocaine for 24 h increased the population of cells stained with propidium, presumably dead cells or the cells with compromised membranes (Figure 6).

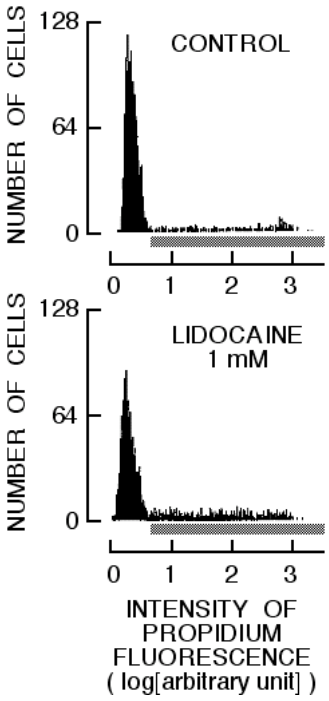

Figure 7
Histogram of propidium fluorescence monitored from control and lidocaine-treated cells.

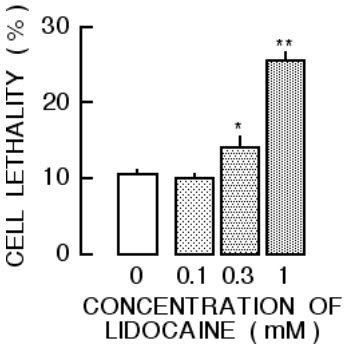

Figure8
Dose-dependent change in population of cells exerting propidium fluorescence by lidocaine.

Significant increase in population of cells exerting propidium fluorescence was also observed when the concentration was 0.3 mM (Figures 7 and 8). However, it was not the case for 0.1 mM lidocaine.

Population of cells with decreased intensity of forward scatter, shrunken cells as shown in Figures 9 and 10, was significantly increased by 1 mM lidocaine. It was also the case for 0.3 mM lidocaine. Thus, it can be concluded that lidocaine at 0.3 mM or more affect the cells when the cells are incubated with lidocaine for 24 h.

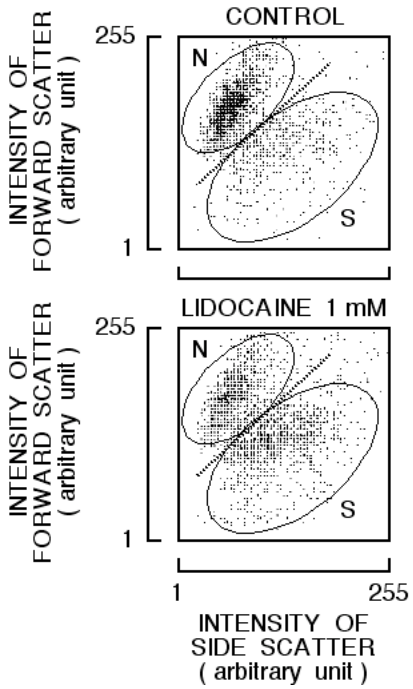

Figure 9
Cytograms of control and lidocaine-treated cells.

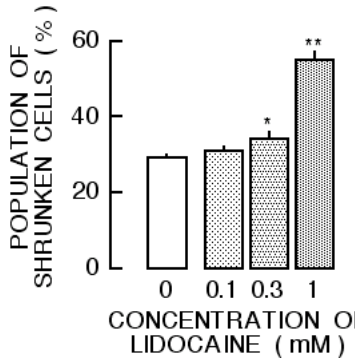

Figure 10
Dose-dependent change in population of shrunken cells by lidocaine.

The incubation of cells with 1 mM, but not 0.3 mM, lidocaine for 24 h significantly

increased the population of cells with hypodiploidal DNA from 25.8 ± 1.2 (control) to 42.0 ± 2.7 (Figure 11).

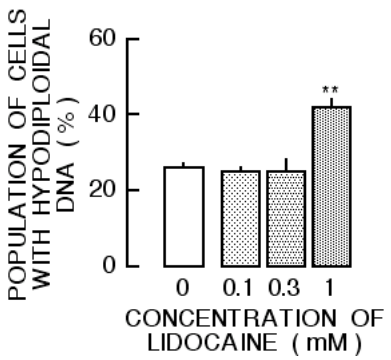

Figure 11
Dose-dependent change in population of cells containing hypodiploid DNA by lidocaine.

4. Conclusion

Lidocaine at sublethal concentrations exerts some cellular actions that are supposed to be linked to the cytotoxicity. The mechanism of lidocaine-induced cell death varies in time- and concentration-dependent manners.

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(Article History)
Received: December 17, 2009
Revised: December 23, 2009
Accepted: December 25, 2009