Bupivacaine-induced Apoptosis in the Primary Cultured Cardiomyocytes via p38 MAPKs

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**Background:** It is known that bupivacaine induce cell death in several immortalized cells. However, there is no report concerning bupivacaine-induced cell death in the primary cultured cardiomyocytes. We compared the direct cytotoxicity of local anesthetics in cardiomyocytes. Furthermore, the mechanisms of cell death were evaluated.

**Methods:** The myocardial cells of rat pups were cultured 3 days after seeding. The methyltetrazolium (MTT) assay was employed to quantify differences in cellular viability. To confirm apoptosis, Hoechst-propidium iodide staining, DNA fragmentation by electrophoresis and western blot analysis were performed. And to examine the mechanisms of cell death, intracellular calcium and expression levels of mitogen-activated protein kinases (MAPKs) family members were evaluated.

**Results:** Among the local anesthetics under 1 mM concentration for 18 h, only bupivacaine significantly decreased the MTT activity (P < 0.001). Bupivacaine induced cell death in a dose-responsive and time dependent manner. Cell death showed apoptotic characteristics, such as DNA fragmentation, chromatin condensation, decrease of precursor caspase-3 protein level, increased cleaved PARP, and cytochrome C release into the cytoplasm. Bupivacaine phosphorylated three major MAPKs, i.e. extracellular signal-regulated kinases (ERKs), p38 kinase and c-Jun N-terminal kinases (Jnk) stress-activated protein kinases. Administration of ERK inhibitor increase cell death, whereas inhibitors of p38 kinase and JNK decreased cell death (P < 0.05). In addition, the intracellular calcium level was approximately 4 times higher after the bupivacaine treatment (P < 0.001), which was inhibited by calcium chelators (P < 0.001). Calcium chelators inhibited expression of MAPKs.

**Conclusions:** In bupivacaine-induced apoptosis in cardiomyocytes, intracellular calcium increase and MAPKs family plays important roles. (Korean J Anesthesiol 2006; 50: S 48~56)

**Key Words:** apoptosis, bupivacaine, calcium, cardiomyocyte, levobupivacaine, mitogen-activated protein kinases.

INTRODUCTION

Many local anesthetics are associated with fatal cardiotoxicity related to potent depressant effect on electrical conduction in the heart and reentrant types of severe arrhythmia in patients. Recently, some local anesthetics-induced direct cytotoxicity related to the initiation or execution of apoptosis has been reported. However, regarding to bupivacaine-induced apoptosis, little is known except in the promyelocytic leukemia cells, human proximal tubular (HK-2) cells, and in the skeletal muscle tissue. Furthermore, there is no report in the cardiomyocytes, which may be important related to bupivacaine toxicity. Basically, there are two principal forms of cell death; necrosis is the major mechanism of cardiomyocyte death in cardiac diseases. However, cardiomyocyte apoptosis occurs in diverse pathologic conditions, such as myocardial infarction, congestive heart failure, hypertension, even arrhythmogenic right ventricular dysplasia (ARVD). Apoptosis is an active gene-directed cell death process, which plays a key role in myocardial damage. For example, cardiomyocytes cell death occurs in condition of myocardial in-
farction, cell death after prolonged periods of ischemia is ascribed to necrosis, reperfusion to apoptosis. Generally, myocardial ischemia/reperfusion activates the two stress-responsive mitogen-activated protein kinase (MAPK) subfamilies, namely, c-Jun N-terminal kinases (JNK) and p38 mitogen-activated protein kinases (p38 MAPKs), resulting in apoptosis. \(^{13}\)

It was also known that local anesthetics such as lidocaine cause intracellular calcium ([Ca\(^{2+}\)]\(_{i}\)) increase via Ca\(^{2+}\) influx through plasma membrane \(^{5}\) and increased intracellular calcium regulates several key steps in the apoptotic signaling pathway in cardiomyocytes, \(^{16-18}\) skeletal muscles \(^{19}\) and immortalized cell. \(^{20}\)

Taken together, the aims of this study were threefold: (1) to determine whether local anesthetics have cytotoxic effects on the primary cultured cardiomyocytes; (2) to determine whether bupivacaine-induced cell death is apoptosis, (3) to determine MAPKs and calcium have some role in bupivacaine-induced cell death.

## MATERIALS AND METHODS

The experimental protocol was approved by Ethics Committee for Animal Experimentation and was performed according to the Guidelines for Animal Experimentation of Seoul National University.

### Primary cardiomyocytes culture

All the procedure and protocol was approved by the Animal Care and Use Committee of Seoul National University College of Dentistry.

Neonatal rat hearts were obtained from 1-2 day (s) old Sprague-Dawley rats that were anesthetized with putting them in the middle of ice. The hearts from the rats were dissected, minced, and placed in an ADS buffer (116 mM NaCl, 20 mM HEPES, 9.4 mM NaH\(_2\)PO\(_4\), 5.5 mM glucose, 5.4 mM KCl, 0.4 mM MgSO\(_4\), pH 7.4). The heart was cut into 1 to 2 mm pieces and digested with 0.06% pancreatin (Sigma Chemical Co, St. Louis, MO) and 0.03% type II collagenase (Sigma) in an ADS buffer for 20 min at 37°C in a hybridization oven. The dissociated cells were collected by centrifugation and were resuspended in DMEM with 10% FBS, 100 units/ml penicillin (Gibco BRL Life Technologies, Grand Island, NY). The dissociated cells were plated in collagen-coated 100 mm culture dishes for 40 min at 37°C in a CO\(_2\) incubator. This procedure results in predominantly fibroblasts attaching to the dishes, while most cardiomyocytes remain unattached. The population of cells enriched in the cardiomyocytes by differential plating were collected and counted. The cells were seeded at a density of 7.5 × 10\(^4\) cells per collagen-coated 96 well plates or 3.5 × 10\(^4\) cells per collagen-coated 60 mm dishes. The cells were cultured in Dulbecco Modified Eagles medium (DMEM) with 10% FBS, 100 units/ml penicillin and 100\(\mu\)g/ml streptomycin. The cells were replaced with fresh medium on the second day of plating. At 3 or 4 days after plating, the cells were treated with lidocaine, ropivacaine, and bupivacaine.

### The methyl tetrazolium (MTT) assay

The cells were seeded into 96-well plates at a concentration of 7.5 × 10\(^4\) cells/well. In order to observe the cytotoxic effects of local anesthetics, the neonatal cardiomyocytes were exposed to lidocaine, ropivacaine, levobupivacaine and bupivacaine, from 1\(\mu\)M to 1,000\(\mu\)M for 18 hours with serum free media. The medium was removed after incubation. For the MTT assay, DMEM containing 10% FBS and 0.5 mg/ml MTT (Methylthiazoletetrazolium: Thyazolyl Blue: Tetrazolium bromide) was added to each of the 96 well plates for 2 h at 37°C in a CO\(_2\) incubator. After 2 h, the medium was removed and 100\(\mu\)l of dimethyl sulfoxide (DMSO) was added. The absorbance at 570 nm and 630 nm was recorded using a spectrophotometer (Bio-TEK Instruments Inc., Winooski, VT).

### DNA fragmentation on agarose gels

The characteristic formation of the oligonucleosome-sized fragments of multiples of about 200 base pairs (bp) producing the typical DNA ladders on agarose gels is the biochemical hallmark of apoptosis. After the treatments, the cells were harvested and lysed in a lysis buffer (100 mM NaCl, 10 mM Tris-HCl [pH 8.0], 25 mM EDTA, and 0.5% sodium dodecyl sulfate (SDS)), which was followed by incubation with 100\(\mu\)g proteinase K (Sigma) for 4 h at 37°C. The protein was removed through the addition of phenol and chloroform and was centrifuged at 13,000 g for 15 min. The DNA was precipitated from the supernatant with the two volumes of absolute alcohol and centrifuged at 13,000 g for 15 min. The pellet was washed twice with 70% ethanol. The DNA was dissolved in Tris-EDTA buffer containing 0.5 U of DNase-free RNase A. The DNA concentration was measured by spectrophotometry (Perkin Elmer Instruments, Shelton, CT) and 10\(\mu\)g of each DNA was electrophoretically fractionated in a 1.8% agarose gel with ethidium bromide.

2 acetoxymethyl ester (Fura-2 AM, Molecular probes, Eugene, OR). Briefly, the cells attached to the glass coverslips (di-
ameter, 12 mm) were washed twice in Krebs-Ringer-HEPES (KRH) buffer (125 mM NaCl, 1.2 mM KH₂PO₄, 1.2 mM MgCl₂, 6 mM glucose, 1 mM CaCl₂ and 25 mM HEPES, pH 7.4) and then incubated in KRH buffer containing 4 M Fura-2 AM for 30 min at 37°C in a CO₂ incubator. The loaded cells were then washed twice with KRH buffer. A coverslip with the cultured cardiomyocytes was placed on the chamber and a vacuum seal was formed between the ring and coverslip with vacuum grease. The coverslip with the cultured cells was filled with 100μl of the KRH buffer. The changes in the fluorescence intensity of the Fura-2 at excitation wavelengths of 340 nm and 380 nm and emission wavelength 510 nm were determined using by microspectrofluorometry technique (Photon Technology International, UK). The ratio of the fluorescence emitted at 510 nm in response to the alternate excitation at 340 nm and 380 nm (340 nm/380 nm ratio) was the index of [Ca²⁺].

Reactive oxygen species (ROS) measurement

Level of ROS in cells was measured using the fluorescent probe 2, 7-dichlorodihydrofluorescein diacetate (Molecular probes D-399, Oregon, USA). The procedure is based on the oxidation of 2, 7-dichlorodihydrofluorescein (H₂DCF) in the presence of ROS to the highly fluorescent, dichlorodihydrofluorescein (DCF), reflecting the rate of oxidation of the dye precursor, i.e. velocity constant. After removal of the media, cardiomyocyte cultures were loaded for 30 min with 50μM 2, 7-dichlorodihydrofluorescein diacetate (H₂DCFDA) in a 37°C, 5% CO₂ incubator and washed in Hanks Balanced Salt Solution (HBSS). And the fluorescence intensity was monitored using multiwell fluorescence plate reader. For all experiments, fluorescence was measured at 37°C with a FLx 800 multiwell fluorescence plate reader, set at 490 nm excitation and 526 nm emission wave length (Perkin Elmer LS-55). The percent change in fluorescence for each well was determined from ([(F₄₆₀-F₄₀)/F₄₀] × 100), where F₄₆₀ refers to fluorescence at approximately 60 min and F₄₀ refers to fluorescence at time 0 min.

Treatment of MAPKs inhibitors and intracellular and extracellular calcium inhibitors

After confirming non-toxic effect of chelators with the basal MTT assay of at the doses of 10μM of BAPTA-AM (Sigma Chemical Co, St. Louis, MO) and 0.5 mM EGTA (Sigma), these inhibitors were pre-treated with the primary cardiomyocytes. After the treatment of chelators, the primary cardiomyocytes were treated with 700μM of bupivacaine for 18 h.

Statistics

The data from the MTT assay is expressed as a mean ± SD in terms of the percentage. ANOVA followed by a Tukey’s post hoc test and a Dunnett’s multiple comparisons test were used to compare the differences in the MTT assay between the concentrations of each local anesthetic. A p value < 0.05 was considered significant. The dose-response data of bupivacaine were fitted to a logistic equation, yielding 50% effective dose (ED₅₀) slopes, and estimates of its 95% confidence interval (CI). The increase of intra-cellular calcium of the bupivacaine-induced apoptotic cells was compared to the control with Student’s t-test. All statistical analyses were performed using SPSS 10.0 (SPSS Inc, Chicago, IL).

RESULTS

Bupivacaine induces cell death in cardiomyocytes and apoptotic features were evident

Whether lidocaine, ropivacaine, levobupivacaine and bupivacaine induced cell death were examined at various concentrations from 1 to 1,000μM for 18 h. Among treated local anesthetics, only bupivacaine showed significant cell death at a concentration of 1 mM compared to the control and other local anesthetics (P < 0.001, Fig. 1).

![Fig. 1. Cell viability was compared among lidocaine, ropivacaine, levobupivacaine and bupivacaine in the primary cultured cardiomyocytes. The amount of cell viability was measured by the MTT assay. Among treated local anesthetics for 18 hr, only bupivacaine induced the cell death at a concentration of 1 mM. *: P < 0.001 as compared with the control.](image-url)
Fig. 2. Bupivacaine-induced cell death. It occurred in a dose (A) and time dependent manner (B) measured by the MTT assay. The results are represented as a mean ± SD of the values obtained from five independent experiments and are expressed as a percentage of the corresponding mean control value. (A) Dose-responsiveness was observed during the bupivacaine treatment for 18 hr. (B) The time-course of the cell death was after the treatment of 700 μM bupivacaine. The 50% effective dose (ED50) was calculated statistically as 696 μM (confidence interval is P < 0.05).

The bupivacaine-induced cell death showed in a dose and time dependent manner (Fig. 2). About 50% of cells were dead after treatment with 700 μM bupivacaine for 18 h. Cardiomyocyte death occurred abruptly over 550 μM after the bupivacaine treatment for 18 h (Fig. 2A) and 13 h after the exposure to 700 μM bupivacaine (Fig. 2B). The statistically calculated ED50 was 696 μM (95% C.I. 678-714 μM, Fig. 2A).

The microscopic findings were as follows. The untreated healthy cardiomyocytes showed active beating (Fig. 3A). Up to 6 h, cardiomyocytes treated with 700 μM of bupivacaine did not show any abnormal findings except losing their typical beating activity (B). However, after 9 h, they appeared to have condensed nuclei and transformed to an elongated cell shape in some cardiomyocytes (C). After 18 h almost all the cardiomyocytes lost their cellular integrity or had detached from the bottom of the culture plate (D).

Fig. 3. The microscopic morphological changes after bupivacaine treatment. The cardiomyocytes cell morphology was examined using phase-contrast microscopy in the absence (A) and presence of 700 μM bupivacaine for 6 (B), 9 (C), and 18 hr (D), respectively. The untreated healthy cardiomyocytes showed active beating (A). Up to 6 hr, the 700 μM bupivacaine treated cardiomyocytes did not show any abnormal findings except losing their typical beating activity (B). However, after 9 h, they appeared to have condensed nuclei and transformed to an elongated cell shape in some cardiomyocytes (C). After 18 h almost all the cardiomyocytes lost their cellular integrity or had detached from the bottom of the culture plate (D). In the electrophoresis, DNA fragmentation was shown 6 h
after treatment with 550μM bupivacaine, and then after 18 h, DNA laddering was prominent (Fig. 5). These nuclear findings were compatible with apoptotic cell death.

Western blot analysis also showed PARP cleavage and procaspase 3 decreases after 550μM of bupivacaine over time. We could also observe cytochrome release from mitochondria into cytoplasm at 3 h after bupivacaine treatment (Fig. 6). Taken together, bupivacaine may induce apoptosis in the primary cultured cardiomyocytes.

These finding suggest apoptotic processes were involved in bupivacaine induced apoptosis of cardiomyocytes.
Bupivacaine-induced apoptosis was significantly decreased by 2 h pre-treatment with 10μM, SB203580, which is the p38 MAPKs inhibitor (P < 0.05, Fig. 7B). However, there was no significant change in MTT assay after cotreatment bupivacaine and JNKII, which is the JNK inhibitor.

**Bupivacaine induced apoptosis by [Ca^{2+}], not by ROS generation**

The intracellular calcium level was approximately 4 times higher after the bupivacaine treatment compared to the control (P < 0.001, Fig. 8A). Extra-calcium chelators (EGTA) and intra-calcium chelators (BAPTA-AM) significantly inhibited the bupivacaine induced cell death (P < 0.001, Fig. 8). Because ROS have been reported to be generated from cells that are exposed to local anesthetics, we examined whether ROS are involved in bupivacaine-induced apoptosis. However, there was no change of ROS level between before and after bupivacaine treatment.

**Bupivacaine-induced activation of MAPKs is dependent on Ca^{2+}**

Because chelation of extracellular or intracellular Ca^{2+} decreased bupivacaine-induced apoptosis, it seemed that MAPKs and calcium in the bupivacaine-induced apoptosis closely related. The intracellular and extracellular calcium chelators increased bupivacaine-induced ERK phosphorylation and decreased p38 phosphorylation (Fig. 8) and resulted in increased cell survival.

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**DISCUSSION**

In this study, direct cytotoxic effects of lidocaine, ropivacaine, levobupivacaine and bupivacaine on the primary cultured cardiomyocytes were compared. Among the treated local anesthetics, only bupivacaine induced apoptosis in a dose-responsive and time dependent manner less than 1 mM.

Because the rank order potency of cytotoxicity correlates well with the in vivo potency and toxicity of local anesthetics and higher cardiotoxicity related to bupivacaine was expected to induce cell death more than treated other local anesthetics. Therefore, among many local anesthetics, lidocaine, ropivacaine, levobupivacaine and bupivacaine are chosen for this study. But the results in this study do not limit the clinical use of bupivacaine because the cytotoxic concentration is much more (about 200 times) higher than clinically accepted dose. But in some clinical setting, continuous infusion of bupivacaine around nerve tissue or skeletal muscles for examples, apoptosis could occur.

Apoptosis is a distinct form of cell death, which displays characteristic alterations in the cell morphology and cell fate. Nuclear condensation, nuclear fragmentation, and sequestration of the cell fragments have been documented in the apoptotic cardiomyocytes. Bupivacaine-induced cell death in the cardiomyocytes in this study, showed distinct apoptotic features, e.g. the morphological changes such as cell shrinkage and...
Fig. 8. Bupivacaine increase in [Ca\(^{2+}\)](aq) in the cardiomyocytes [Ca\(^{2+}\)](aq) was measured in the Fura-2 AM loaded cardiomyocytes in the absence (control) and the presence of 550µM bupivacaine for 18 hr. The graph represents the mean of fluorescence intensity values obtained in the ten independent cells from the three separate experiments (A). Bupivacaine-induced apoptosis was inhibited by pretreatment of intra-cellular calcium chelator (BAPTA-AM) and extra-cellular calcium chelator (EGTA) in the cardiomyocytes. The cardiomyocytes were pre- incubated with BAPTA-AM (10µM) for 2 hr (A), or EGTA (0.5 mM) for 1 hr (B) and then treated with 700µM of bupivacaine for 18 hr. Both chelators showed significant cell death inhibition (P < 0.001). Cell viability was determined by MTT assay. The results are represented as a mean ± SD of the values obtained from three independent experiments and are expressed as a percentage of the corresponding mean control value. (C) Bupivacaine-induced activation of p38 MAPKs is dependent on Ca\(^{2+}\) after incubation with 5 mMol/L EGTA for 2 min and with 40 µmol/L BAPTA for 30 minutes, cardiomyocytes were treated with 100 µmol/L DM for 20 min. p38 MAPKs from 3 independent experiments are shown.

Rounding, which are characteristic features of apoptotic death. And fluorescent DNA-binding dyes, such as Hoechst 33258 dye, are commonly used to define the nuclear chromatin morphology as a quantitative index of apoptosis within a cell culture system. In this study, nuclear fragmentation in the Hoechst-Pi staining was obvious. In addition, it was confirmed that the process of apoptosis defined based on the cellular and nuclear chromatin morphology correlated with apoptosis defined as internucleosomal DNA fragmentation assessed by gel electrophoresis. All these finding supports that bupivacaine induced cell death of cardiomyocyte is by apoptosis.

Increased intracellular calcium\(^{29}\) or ROS generation\(^{30}\) is common finding in various apoptotic process. In this study, increased intracellular calcium was definite, but increased ROS generation was not found in bupivacaine induces apoptosis of cardiomyocyte. And addition of the intracellular and extracellular Ca\(^{2+}\) chelator, which affected MAPKs phosphorylation, blocked bupivacaine-induced apoptosis. Increased calcium and p38 MAPKs and intracellular signals are involved in bupivacaine-induced apoptosis.

Specifically, phosphorylation of the MAPK family, ERK and p38 were also observed in the process. But JNK activation was not definite, and inhibition of phosphorylated JNK did not affect the cell survival. So it seems that JNK family do not
involve in bupivacaine-induced apoptosis in cultured cardiomyocyte. Inhibition of p-ERK potentiated the bupivacaine-induced apoptosis. However, inhibitors of phosphorylated p38 attenuated cardiomyocytes cell death. So p38 MAPKs is seems to be the main MAPK involved in bupivacaine-induced apoptosis in cultured cardiomyocyte.

Signal transduction via MAPKs plays a key role in a variety of cellular responses including proliferation, differentiation, and cell death. More than a dozen MAPKs pathways have been identified so far. Among the MAPKs family, three members, in particular, p42/p44-ERK kinase (extracellular signal-regulated kinase, ERK), p38 MAPKs, and JNK have been well characterized. In general, activation of the ERK signal pathway usually functions to protect cells from a variety of cellular stresses. In contrast, the JNK and p38 MAPKs signal pathways lead to cell death. Also in our study, ERKs showed cell protective effect against bupivacaine and p38 MARKs promoted apoptosis in bupivacaine treated cardiomyocyte.

The p38 MAPKs signaling pathway activated in many cell types by cellular stressors, like UV radiation, osmotic stress, and oxidative stress. Activation of p38 MAPKs is known to results in changes in transcription, protein synthesis, cell surface receptor expression, and cytoskeletal structure, ultimately affecting cell survival or leading to the programmed cell death. Our results demonstrate that bupivacaine-induced apoptosis was related to the activation of p38 MAPKs signal transduction pathway in the cardiomyocytes.

Our study demonstrated bupivacaine induce apoptosis in cultured cardiomyocyte and showed increased calcium and p38 MAPKs play key role for the first time. In promyelocytic leukemia cells HL-60, bupivacaine induced apoptosis through an indirect action of bupivacaine on mitochondria. However, in this study, intracellular calcium level increased and intra and extra cellular calcium chelators inhibited bupivacaine-induced apoptosis. Zink et al. and Komai et al. reported that cytotoxicity of bupivacaine in skeletal myocytes related to persistently higher [Ca\(^{2+}\)] levels. So, the intracellular calcium levels was examined in this study. These finding are consistent with our results.

In conclusion, among treated lidocaine, ropivacaine, levobu- 

vivacaine and bupivacaine, only bupivacaine induced apoptosis less than 1 nM concentration in the primary cultured cardiomyocytes. Calcium elevation and p38 MAPKs may play important role in the bupivacaine-induced apoptosis.

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