The combined effects of hyperthermia at 44 °C and local anesthetics on apoptosis in human histiocytic lymphoma U937 cells were investigated. When the cells were exposed to hyperthermia for 10 min marginal DNA fragmentation and nuclear fragmentation were observed. The order of the concentration required for maximum induction was the reverse order of the lipophilicity (prilocaine > lidocaine > bupivacaine). Western blotting revealed that in hyperthermia there was initial release of Ca²⁺ from the intracellular store sites as indicated by increased expression of the type 1 inositol-1,4,5-trisphosphate receptor. However, the combination with lidocaine did not induce any further enhancement. Lidocaine enhanced the decrease in ATP content and the increase in intracellular Ca²⁺ concentration in individual cells induced by hyperthermia. In addition, superoxide formation, decrease in the mitochondrial membrane potential, and activation of intracellular caspase-3 were found in the cells treated with hyperthermia and lidocaine. All of these were suppressed in part in the presence of the intracellular Ca²⁺ ion chelator BAPTA-AM (bis-(O-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl). The present results indicate that local anesthetics at optimal concentrations enhance hyperthermia-induced apoptosis via Ca²⁺- and mitochondria-dependent pathways. Initial release of Ca²⁺ from intracellular store sites caused by hyperthermia and followed by the subsequent increase in the intracellular Ca²⁺ concentration and the additional activation of the mitochondrial caspase-dependent pathway (partly regulated by intracellular Ca²⁺ concentration) plays a crucial role in the enhancement of apoptosis induced by the combination of hyperthermia and lidocaine.

The effectiveness of hyperthermia combined with radiotherapy (2–6) or chemotherapy (7) compared with radiotherapy alone. However, the uniform and precise delivery of heat to tumors still remains a challenge. In many circumstances the tumor cell killing is insufficient. Drugs that have been discussed as overcoming this difficulty are heat sensitizers. An ideal sensitizer would be nontoxic at normothermia but could become cytotoxic at hyperthermic temperatures. Local anesthetics belong to a class of clinically useful compounds that exert a pharmacological effect by blocking nerve impulse propagation. The involvement of cell membranes as the site for these drug actions has been widely accepted, and many reports showing modification of cell killing due to hyperthermia by LAs have been published (e.g. potentiation by procaine in murine L5178Y lymphoma cells (8) and by lidocaine in murine FM3A mammary carcinoma cells (9), potentiation of survival of tumor-bearing mice after hyperthermia combined with lidocaine (10), enhancement of hyperthermia-induced tumor regression by lidocaine in murine tumor models (11, 12), and enhancement of cytotoxic effects of hyperthermia by dibucaine, tetracaine, and procaine in hepatoma tissue culture cells (13)). However, little is known about the modification and mechanism of apoptosis when hyperthermia is combined with LAs.

Here we will present our recent findings that subtoxic levels of LAs enhanced hyperthermia-induced apoptosis via the Ca²⁺- and mitochondria-dependent pathways in human lymphoma U937 cells. Evidence for the cardinal roles of initial release of Ca²⁺ from intracellular store sites due to hyperthermia and the subsequent increase of [Ca²⁺], and the additional activation of the mitochondrial caspase-dependent pathway in enhancement of apoptosis induced by the combination of hyperthermia and lidocaine will also be presented. In addition the potential usefulness of LAs as sensitizers of heat-induced apoptosis in cancer therapy will be discussed.

EXPERIMENTAL PROCEDURES

Cells and Hyperthermic Treatment—The human histiocytic lymphoma cell line (U937) was obtained from the Japanese Cancer Research Resource Bank. Cells were grown in RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) at 37 °C in humidified air with 5% CO₂. The cells in log phase (doubling time is 23.5 h) were used for the experiments after confirmation that they were free from any mycoplasma contamination.

We added LAs 5 min before heating, and hyperthermic treatments were performed by the immersion of plastic culture tubes containing the cell suspensions (3 ml) in a water bath (NTT-1200, Eyela, Tokyo, Japan) at 44.0 °C (± 0.05 °C). The temperature of the culture medium was

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Received for publication, August 22, 2001, and in revised form, January 10, 2002
Published, JBC Papers in Press, February 22, 2002, DOI 10.1074/jbc.M108084200

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monitored with a digital thermometer (#7636, YOKOGAWA, Tokyo, Japan) coupled with a 0.8-mm thermocouple.

**Determination of DNA Fragmentation**—The amount of DNA extracted from cells that had undergone DNA fragmentation was assayed using the method of Sellins and Cohen (14) with a few modifications (15). The cells were lysed in 0.2% Triton X-100 (1 ml EDTA, 0.2% Triton X-100, pH 7.5) and centrifuged at 13,000 × g for 10 min. Subsequently, each DNA sample in the supernatant and the pellet were precipitated in 12.5% trichloroacetic acid at 4°C and quantified using a diphenylamine reagent after hydrolysis in 5% trichloroacetic acid at 90°C for 20 min. The percentage of fragmented DNA in each sample was calculated as the absorbance at 10 μM of Photofluor-G1D2 substrate solution in RPMI 1640 at 37°C divided by the total DNA for that sample (supernatant plus pellet). Significance was assessed by a two-way analysis of variance followed by a Fisher's PLSD test and was assumed for p values <0.05.

**Observation of DNA Ladder Formation**—DNA was extracted from the treated cells using a Sepa Gene DNA extraction kit (Sanko Junyaku Co., Ltd., Tokyo, Japan) and digested with Rnase A (50 μg/ml, Sigma) in PBS. After drying staining was performed with 3% Giemsa (Sigma) coupled with a 0.8-mm thermocouple.

**Morphological Observation**—To identify the apoptotic cells after exposure to hyperthermia, the cells harvested after 6 h of incubation at 37°C on Dulbecco's modified Eagle medium (DMEM) were fixed with methanol and acetic acid (3:1) and spread on glass slides. After drying staining was performed with 3% Giemsa solution (pH 6.8) for 15 min. The apoptotic cells were determined by counting a total of 1,000 cells per sample in randomly selected areas.

**Flow Cytometry**—Because annexin V is a human protein with a molecular mass of 26 kDa and a high affinity for phosphatidylserine on the cell membrane, a FITC-labeled annexin V kit (Immunotech, Marseilles, France) was used to detect phosphatidylserine expression on the cell membrane as an end point of early apoptosis (18). The samples were washed in cooled PBS at 4°C and centrifuged at 500 × g for 5 min. The resulting pellets were adjusted to 10^6 cells/ml with the binding buffer from a FITC-labeled annexin V kit. FITC-labeled annexin V (5 μl) and PI (5 μl) were added to the suspension for 10 min. The samples of cells stained with Giemsa were examined under an oil-immersion microscope (Eupic XL, Beckman-Coulter, Miami, FL).

**Determination of Intracellular Concentration of Calcium Ion in Single Cells**—The cells were collected by centrifugation and washed with HR (NaCl, 118 m M; KCl, 4.7 m M; CaCl₂, 2.5 m M; MgCl₂, 1.13 m M; Na₂HPO₄, 22.3 m M; glucose, 5.5 m M, and HEPES, 10 m M). The buffer was supplemented with 0.5% bovine serum albumin (Sigma), minimal Eagle's essential amino acids (Flow Laboratories, Irvine, UK), and 2 mM l-glutamine. Approximately 3 × 10^5 cells in 3 ml of HR were loaded with 5 m M Fura-2/AM (Dojindo Lab., Kumamoto, Japan) for 30 min at 37°C. The cells were washed twice with PBS and were exposed to 40 nM DiOC₆(3) and UVB illumination (16, 17). The enhancement of DNA ladder formation after treatment with hyperthermia and lidocaine, alone or in combination, was evaluated using a diphenylamine reagent after hydrolysis in 5% trichloroacetic acid at 4°C for 20 min. Subsequently, each DNA sample in the supernatant and the pellet were precipitated in 12.5% trichloroacetic acid at 4°C for 20 min. The apparent enhancement of DNA fragmentation by LAs was obtained as an increase in the nuclei, especially nuclear fragmentation, was observed using a Bio-Rad protein system. Western blot analyses of IP3R (1, 3) and α-actin were performed using specific polyclonal or monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).

**Measurement of Intracellular Superoxide Anion Radicals**—Superoxide anion levels were measured using the method employed by Gorman et al. (23). We used the dye, hydroethidine (Molecular Probes, Eugene, OR) which is oxidized by superoxide anion radicals within the cell, which fluoresces when it intercalates into DNA. Briefly, the cells (10^6 cells/ml) were incubated with 2 μM hydroethidine for 15 min at 37°C. After a second washing levels of the intracellular superoxide anion radicals were assessed using flow cytometry.

**Single Cell Analysis of Intracellular Caspase Activity**—The cell-permeable fluorogenic substrate (PhiPhiLux-G1D2) was used to monitor the intracellular caspase-3 activity according to the manufacturer’s recommendations (OncoImmunin, Inc., Gaithersburg, MD). Briefly, the sample (10^6 cells/ml) was centrifuged, and the cell pellet was resuspended with 10 μM Photofluor-G1D2 substrate solution in RPMI 1640 and then supplemented with 10% fetal bovine serum. After incubation for 1 h at 37°C in the dark the samples were washed once and diluted with 0.5 ml of ice-cold flow cytometry dilution buffer. The fraction of cells showing high caspase-3 activities was measured by flow cytometry (24).

**Measurement of Intracellular ATP Level**—Intracellular ATP level was measured using a CheckLite plus 250 kit (Kikkoman, Tokyo, Japan). After treatment the cells were centrifuged at 1,000 rpm for 2 min, and the filtrate was removed. A cell suspension (2 × 10^6 cells/200 μl) was added to the lysis buffer (200 μl) and was kept for 5 min at 25°C. Afterward the absorbency of ATP, which was included in 100 μl of the cell suspension, was measured by the administration of 100 μl of the emission reagent. The intracellular ATP concentration was calculated using the standard curve (from 2 × 10^−3 M to 2 × 10^−7 M) of ATP. Significance was assessed by a two-way analysis of variance followed by a Fisher’s PLSD test and was assumed for p values <0.05.

**RESULTS**

**Effects of LAs on Hyperthermia-induced Apoptosis**—To study the ability of LAs to potentiate hyperthermia-induced apoptosis, DNA fragmentation was examined when U937 cells were treated with hyperthermia combined with three kinds of amide-type LAs (lidocaine, bupivacaine, or prilocaine), which have different lipophilicities. Fig. 1 shows the effects of graded concentrations of LAs on the DNA fragmentation when the cells were incubated for 6 h at 37°C after treatment with or without hyperthermia at 44°C for 10 min. The apparent enhancement of hyperthermia-induced DNA fragmentation by LAs was observed at concentrations of more than 0.5 mM. The order of concentration of LAs for the maximum DNA fragmentation was prilocaine > lidocaine > bupivacaine, and this was the reverse order of lipophilicity and potency as anesthetic agents (25). When the cells were treated with LAs at 37°C no DNA fragmentation induced by prilocaine was observed. In contrast, lidocaine and bupivacaine increased DNA fragmentation at concentrations more than 2 and 1.5 mM, respectively. In addition, the DNA fragmentation induced by bupivacaine decreased at a concentration of 1.5 mM. Because lidocaine is more effective with less toxicity among the LAs in this study, it was used for the following experiments.

The enhancement of DNA ladder formation after treatment with hyperthermia and lidocaine was observed (Fig. 2). When the samples of cells stained with Giemsa were examined under a light microscope a marked enhancement of apoptotic change in the nuclei, especially nuclear fragmentation, was observed in the samples treated with hyperthermia and lidocaine, although hyperthermia alone induced marginal changes (Fig. 3, A–D). The percentage of cells containing fragmented nuclei after the combined treatments was 3 × higher than those of the samples treated with hyperthermia (Fig. 3E). These results of DNA ladder formation and cell morphology such as chromatin condensation and nuclear fragmentation consistently revealed that lidocaine enhanced apoptosis induced by hyperthermia in U937 cells.

**Kinetics of Early Apoptosis and Secondary Necrosis**—Flow cytometry using annexin V/FITC and PI double staining revealed that after the combined treatment of hyperthermia and lidocaine, the cells with externalized phosphatidylserine significantly increased depending on the incubation time. This phos-
phatidyserine externalization is an early sign of apoptosis (Fig. 4). At 6 h, the percentage of early apoptosis, i.e. annexin V+/PI− cells, induced by the combined treatment (52.0 ± 5.0, mean ± S.D., n = 3) was significantly higher than that in the cells treated with hyperthermia alone (16.3 ± 6.3). In contrast, the percentage of secondary necrosis, i.e. annexin V+/PI+ cells, was small and was less than 20% even in the cells treated with hyperthermia and lidocaine.

Analysis of the Ca²⁺-dependent Pathway—Because U937 cells have been reported to possess a Ca²⁺-dependent apoptosis pathway (16–18), we carried out digital imaging of Fura-2 fluorescence to examine the change in [Ca²⁺]i immediately after the treatment of hyperthermia combined with or without lidocaine. The average [Ca²⁺]i was 83.8 ± 38.7 nM in the control cells (mean ± S.D., n = 500), 77.3 ± 44.2 nM in the cells treated with lidocaine alone, 100.5 ± 61.6 nM in the cells treated with hyperthermia alone, and 140.4 ± 119.8 nM in the cells treated with hyperthermia and lidocaine.

As shown in Fig. 5, E–H (histograms of [Ca²⁺]i in 500 randomly selected cells) the distribution in the non-treated control cells and in the cells treated with lidocaine alone was relatively uniform. In contrast, when the cells were treated with hyperthermia, especially when combined with lidocaine, the number of cells containing higher [Ca²⁺]i increased markedly. Because the [Ca²⁺]i in ≥95% of control cells was less than 135 nM, the fraction of cells showing above 135 nM was calculated and evaluated for statistical significance. No significant difference in the fraction of cells containing above 135 nM between the control (3.3 ± 5.9%, mean ± S.D., n = 5) and the cells treated with lidocaine (6.0 ± 7.1%) was observed. When the cells were treated with hyperthermia the fraction of cells showing higher [Ca²⁺]i (16.7 ± 7.1%) increased significantly compared with the
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control (p < 0.05). Furthermore, when the cells were treated with hyperthermia combined with lidocaine the fraction of cells (31.0 ± 20.3%) increased significantly compared with the cells treated with hyperthermia (p < 0.01). These results indicate that lidocaine further increased the hyperthermia-induced increase in [Ca\(^{2+}\)].

To explore reasons why the [Ca\(^{2+}\)] increased when the cells were treated with hyperthermia combined with the presence or absence of lidocaine, the expression of IP\(_3\)R proteins (types 1 and 3), which is known to be related to intracellular Ca\(^{2+}\) homeostasis (26, 27), was examined. Western blotting of whole cell extracts with IP\(_3\)R-specific antibody showed that proteins of IP\(_3\)R1 were slightly expressed in the U937 cells and therefore the expression was increased with hyperthermia alone immediately after the treatment (Fig. 6). However, no further increase was observed in the cells treated with hyperthermia and lidocaine. In contrast, no expression of IP\(_3\)R3 was detected in the control cells and in the cells treated with hyperthermia combined with the presence or absence of lidocaine. The increase in [Ca\(^{2+}\)], induced by hyperthermia caused by the elevation of IP\(_3\)R1 expression was revealed. This finding suggests that the intracellular release of Ca\(^{2+}\) from store sites induced by hyperthermia is a reason for the initial increase in [Ca\(^{2+}\)].
E, histograms of \([\text{Ca}^{2+}]_i\) in the presence or absence of lidocaine (1 mM) cells were stained with Fura-2/AM as described under "Experimental Procedures," and \([\text{Ca}^{2+}]_i\) were then measured. A–D, digital images of Fura-2 fluorescence. E–H, histograms of \([\text{Ca}^{2+}]_i\).

**Analysis of the Mitochondrial Caspase-dependent Pathway—**To examine whether the mitochondrial caspase-dependent pathway for apoptosis is related to the enhancement of hyperthermia-induced apoptosis by lidocaine or not, we measured the MMP change, the intracellular superoxide anion radical level, and the intracellular caspase-3 activities. When the cells were treated with hyperthermia in the presence of lidocaine and incubated at 37 °C for 6 h, cells with low MMP (Fig. 7), a high level of superoxide anion radicals, and high activities of caspase-3 (Fig. 8) increased significantly. The percentage of cells with high superoxide anion radical levels was 1.8 ± 0.4% in the control cells (mean ± S.D., n = 3), 3.9 ± 2.5% in the cells treated with lidocaine alone, 4.4 ± 0.5% in the cells treated with hyperthermia, and 57.4 ± 15.4% in the cells treated with hyperthermia and lidocaine. These results confirmed our hypothesis that the activation of mitochondria-caspase pathway is involved in the enhancement of hyperthermia-induced apoptosis by lidocaine.

Because apoptosis has been known to depend strongly on the intracellular concentration of ATP (28, 29) and our result shows that mitochondria are related to the enhancement of hyperthermia-induced apoptosis by lidocaine, the ATP concentration in cells treated with various concentrations of lidocaine with or without hyperthermia was measured 3 h after treatment (Fig. 9). An increasing concentration of lidocaine induced a gradual decrease in ATP concentration at 37 °C. However, hyperthermia reduced ATP concentration to a level similar to that attained when lidocaine alone was used and significantly decreased it furthermore to 11.2 ± 3.4% (mean ± S.D., n = 4) from 100% of the control when 5 mM lidocaine was added.

**Effects of BAPTA-AM on DNA Fragmentation—**To examine the role of \([\text{Ca}^{2+}]_i\), on the enhancement of hyperthermia-induced apoptosis by lidocaine, a cytosolic free \(\text{Ca}^{2+}\) chelator, BAPTA-AM, was utilized. When cells were treated and incubated for 6 h in the presence of BAPTA-AM at a concentration of 50 μM, the inhibition rates (IRs) by BAPTA-AM on DNA fragmentation, on the fraction of cells expressing superoxide formation, on cells with low MMP, and on cells with activated caspase-3 were obtained. IR was calculated as: IR (%) = \(\left(\frac{F_{\text{HL}} - F_{\text{HLB}}}{F_{\text{HL}}}\right) \times 100\), where \(F_{\text{HL}}\) is percent in the cells treated with hyperthermia and lidocaine and \(F_{\text{HLB}}\) is percent in the cells treated with hyperthermia and lidocaine in the presence of BAPTA-AM. BAPTA-AM significantly reduced these end points of apoptosis induced by hyperthermia and lidocaine. The IR on DNA fragmentation, on the fraction of cells expressing superoxide formation, on cells with low MMP, and on cells with activated caspase-3 are 60.9 ± 5.1% (mean ± S.D., n = 5), 50.9 ± 5.2% (mean ± S.D., n = 5), 42.9 ± 8.6% (mean ± S.D., n = 4), and 38.0 ± 17.6% (mean ± S.D., n = 3), respectively (Fig. 10).

In contrast, the reduced ATP concentration in cells treated with hyperthermia and lidocaine further decreased by 75.7 ± 5.2% in the presence of BAPTA-AM.
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DISCUSSION

LAs have been reported to enhance cell killing induced by hyperthermia either in vitro or in vivo (10, 12, 30). Previous studies have demonstrated that LAs interact extensively with the cell membrane. For example, LAs induce disorders among the lipid part of the cell membrane, fluidizing and expanding it by nonspecific binding (13, 31). LAs raise the intracellular calcium ion level (32, 33), inhibit Ca\(^{2+}\)/Mg\(^{2+}\)-ATPase activity (34), and reduce the mitochondrial oxidative energy metabolism (35). However, the mechanism by which LAs potentiate hyperthermic damage to cells is not clearly understood, and little is known about the modification of hyperthermia-induced apoptosis by LAs. The present results reveal that the mechanism of enhancement of hyperthermia-induced apoptosis by lidocaine involves increasing the initial release of Ca\(^{2+}\) from intracellular store sites caused by hyperthermia and a subsequent increase in [Ca\(^{2+}\)\], and the results also reveal that

**FIG. 7.** Loss of MMP after hyperthermia combined with lidocaine. After treatment with hyperthermia (44 °C, 10 min) in the presence or absence of 1 mM lidocaine cells were incubated for 6 h at 37 °C. They were stained with 40 nM DiOC\(_6\)(3) as described under “Experimental Procedures,” and then MMP was measured by flow cytometry. A, 37 °C; B, 37 °C with 1 mM lidocaine; C, 44 °C; D, 44 °C with 1 mM lidocaine. E, percentage of cells with low MMP. Bars in figures represent mean ± S.D. (n = 3).

**FIG. 8.** Increase of caspase-3 activity after hyperthermia combined with lidocaine. After treatment with hyperthermia (44 °C, 10 min) in the presence or absence of 1 mM lidocaine, cells were incubated for 6 h at 37 °C. They were stained with PhiPhiLux-G1D2 as described under “Experimental Procedures,” and then caspase activity was measured by flow cytometry. A, 37 °C; B, 37 °C with 1 mM lidocaine; C, 44 °C; D, 44 °C with 1 mM lidocaine. E, percentage of cells showing high caspase-3 activities. Bars in figures represent mean ± S.D. (n = 3).
activation of a mitochondria-caspase pathway is accompanied by a loss of mitochondrial membrane potential, superoxide production, and activation of caspase-3 (See Fig. 11).

The cytotoxic effects of hyperthermia as measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (13) were enhanced by subtoxic levels of anesthetics, and the potency of these effects was related to their respective lipophilicity (13, 25). Fluidization of the hydrophobic core of the membrane may contribute to anesthetic potentiation of heat-induced cell death (13). In addition, bupivacaine (because of its high lipophilicity) penetrated the cell membrane, reached the mitochondria to induce deep ultrastructural modifications, and lowered the MMP (35). In this study, the order of the concentration of LAs for the maximum DNA fragmentation at 44 °C was in the reverse order of their lipophilicity. LAs with high lipophilicity may cause much more sensitization of cells to hyperthermia by interacting with and destabilizing membranes so that fluidity is increased, because higher lipophilicity makes it easy for LAs to integrate in the cell membrane. Therefore, LAs with higher lipophilicity appear to cause greater enhancement of hyperthermia-induced apoptosis.

When the cells were treated with hyperthermia and lidocaine in the range of toxic concentrations a decrease in DNA fragmentation, early apoptosis, and an increase in secondary necrosis were observed. It is well known that a fall in ATP eventually causes various cellular abnormalities, which may lead to cell death (28), and changes the type of cell death from apoptosis to necrosis (29). Therefore, it is possible that the decrease in intracellular ATP concentration is related to this change of cell death from apoptosis to necrosis. In this study the promotion of a decrease in the intracellular ATP concentration and a subsequent drop in DNA fragmentation by hyperthermia combined with lidocaine was observed.

The increase in intracellular Ca²⁺ concentration after treatment of hyperthermia combined with lidocaine and the suppression of DNA fragmentation by a Ca²⁺ chelator (BAPTA-AM) suggest that a Ca²⁺-dependent pathway is involved in the enhancement of hyperthermia-induced apoptosis by lidocaine. The small increase in [Ca²⁺]i after hyperthermia appears to be caused by an initial release of Ca²⁺ (derived from the intracellular storage sites) because expression of IP₃R1 was up-regulated immediately after hyperthermia. Subsequently, the decrease in intracellular ATP concentration facilitated by
lidocaine led to a rapid decline in the Ca\(^{2+}\)-ATPase activity, and the accumulation of intracellular Ca\(^{2+}\) due to an abatement of Ca\(^{2+}\) exclusion (33) appears to cause the activation of Ca\(^{2+}\)-dependent endonucleases and proteases in apoptosis.

Involvement of the mitochondrial caspase-dependent apoptosis pathway was also indicated because of the lowering of the mitochondrial membrane potential, the increase in O\(_2^-\) production, and the activation of intracellular caspase-3. There have been some reports that LAs alone with high lipophilicity could reach mitochondria (36) and that LAs not only induce uncoupling of mitochondria by an electrophoteric mechanism but also inhibit adenine nucleotide transport in mitochondria (37). Another study has reported that LAs at high concentrations induced apoptosis and appear to be related to activation of the mitochondria-caspase pathway, but they may not interact directly with mitochondria in HL-60 cells to induce apoptosis (38). Because LAs at toxic concentrations induced apoptosis dependent on the mitochondria-caspase pathway whether they interacted with the mitochondria directly or indirectly, it is possible that hyperthermia further enhanced activation of the mitochondrial caspase-dependent pathway in apoptosis induced by LAs even at lower nontoxic concentrations.

To understand better the significance of the increase in [Ca\(^{2+}\)], in cells treated with hyperthermia and lidocaine, various parameters of the mitochondrial caspase-dependent apoptotic pathway were examined in the presence of a cytosolic free Ca\(^{2+}\) chelator, BAPTA-AM, at a concentration of 50 \(\mu\)M (because 50 \(\mu\)M BAPTA-AM has shown significant inhibition of hyperthermia-induced DNA fragmentation in U937 cells) (21). When cells were treated and incubated for 6 h in the presence of BAPTA-AM, enhancement of DNA fragmentation by lidocaine combined with hyperthermia was decreased by 60.9 \(\pm\) 5.1%. In addition decreases in MMP, superoxide formation, and activation of intracelular caspase-3 by hyperthermia combined with lidocaine were suppressed by approximately 40–50\% (Fig. 10). These results indicate that the rise in intracellular free Ca\(^{2+}\) takes part not only in the Ca\(^{2+}\)-dependent pathway but also in the mitochondrial caspase-dependent pathway. In addition because a series of parameters on the mitochondrial caspase-dependent pathway in cells treated with hyperthermia and lidocaine was suppressed by BAPTA-AM, direct involvement of intracellular free Ca\(^{2+}\) upon the mitochondria is suggested. These results support the view that elevations in cytosolic Ca\(^{2+}\) act directly on mitochondria to induce the rupture of the outer membrane and the release of caspase activation proteins (reviewed in Ref. 39).

In contrast, the reduced intracellular ATP concentration (another parameter that relates to mitochondrial function and cell death) in the cells treated with hyperthermia and lidocaine was further decreased by 75.7 \(\pm\) 5.2\% in the presence of BAPTA-AM. However, when cells were treated with BAPTA-AM alone the ATP concentration was decreased very markedly by 93.2 \(\pm\) 5.1\%. For this reason, the relationship between the rise in intracellular free Ca\(^{2+}\) and the decrease in intracellular ATP concentration in the cells treated with hyperthermia combined with lidocaine has not been resolved. Therefore, the role of intracellular ATP in the mitochondrial caspase-dependent pathway cannot be fully established because BAPTA-AM did not prevent the decrease of ATP concentration.

The molecular mechanism of the enhancement of apoptosis by LAs at hyperthermic temperature has not been established. However, we obtained evidence for the cardinal roles of the initial release of Ca\(^{2+}\) from intracellular store sites caused by hyperthermia and the subsequent increase of [Ca\(^{2+}\)], and for the additional activation of the mitochondrial caspase-depend-
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J. Biol. Chem. 2002, 277:18986-18993.
doi: 10.1074/jbc.M108084200 originally published online February 22, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M108084200

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