Calcium Is a Key Signaling Molecule in β-Lapachone-mediated Cell Death*

Colleen Tagliarino‡§, John J. Pink‡, George R. Dubyak‡, Anna-Liisa Nieminen‡, and David A. Boothman*†

From the ‡Departments of Radiation Oncology and Pharmacology, §Department of Anatomy, and the ¶Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, Ohio 44106-4942

β-Lapachone (β-Lap) triggers apoptosis in a number of human breast and prostate cancer cell lines through a unique apoptotic pathway that is dependent upon NQO1, a two-electron reductase. Downstream signaling pathway(s) that initiate apoptosis following treatment with β-Lap have not been elucidated. Since calpain activation was suspected in β-Lap-mediated apoptosis, we examined alterations in Ca2+ homeostasis using NQO1-expressing MCF-7 cells. β-Lap-exposed MCF-7 cells exhibited an early increase in intracellular cytosolic Ca2+, from endoplasmic reticulum Ca2+ stores, comparable to thapsigargin exposures. 1,2-Bis(2-aminophenoxyn)ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl ester, an intracellular Ca2+ chelator, blocked early increases in Ca2+ levels and inhibited β-Lap-mediated mitochondrial membrane depolarization, intracellular ATP depletion, specific and unique substrate proteolysis, and apoptosis. The extracellular Ca2+ chelator, EGTA, inhibited later apoptotic end points (observed >8 h, e.g. substrate proteolysis and DNA fragmentation), suggesting that later execution events were triggered by Ca2+ influxes from the extracellular milieu. Collectively, these data suggest a critical, but not sole, role for Ca2+ in the NQO1-dependent cell death pathway initiated by β-Lap. Use of β-Lap to trigger an apparently novel, calpain-like-mediated apoptotic cell death could be useful for breast and prostate cancer therapy.

β-Lap† is a naturally occurring compound present in the bark of the South American Lapacho tree. It has antitumor activity against a variety of human cancers, including colon, prostate, promyelocytic leukemia, and breast (1–3). β-Lap was an effective agent (alone and in combination with taxol) against human ovarian and prostate xenografts in mice, with little host toxicity (4). We recently demonstrated that β-Lap kills human breast and prostate cancer cells by apoptosis, a cytotoxic response significantly enhanced by NAD(P)H:quinone oxidoreductase (NQO1, E.C. 1.6.99.2) enzymatic activity (5).2 β-Lap cytotoxicity was prevented by co-treatment with dicumarol (an NQO1 inhibitor) in NQO1-expressing breast and prostate cancer cells (5). NQO1 is a cytosolic enzyme elevated in breast cancers (6) that catalyzes a two-electron reduction of quinones (e.g. β-Lap, menadione), utilizing either NADH or NADPH as electron donors. Reduction of β-Lap by NQO1 presumably leads to a futile cycling of the compound, wherein the quinone and hydroquinone form a redox cycle with a net concomitant loss of reduced NAD(P)H (5).

Apoptosis is an evolutionarily conserved pathway of biochemical and molecular events that underlie cell death processes involving the stimulation of intracellular zymogens. The process is a genetically programmed form of cell death involved in development, normal turnover of cells, and in cytotoxic responses to cellular insults. Once apoptosis is initiated, biochemical and morphological changes occur in the cell. These changes include: DNA fragmentation, chromatin condensation, cytoplasmic membrane blebbing, cleavage of apoptotic substrates (e.g. PARP, lamin B), and loss of mitochondrial membrane potential with concomitant release of cytochrome c into the cytoplasm (7–9). Apoptosis is a highly regulated, active process that requires the participation of endogenous cellular enzymes that systematically dismantle the cell. The most well characterized proteases in apoptosis are caspases, aspartate-specific cysteine proteases, that work through a cascade that can be initiated by mitochondrial membrane depolarization leading to the release of cytochrome c and Apaf-1 into the cytoplasm (10), that then activates caspase 9 (11). Non-caspase-mediated pathways are less understood.

We previously showed that apoptosis following β-Lap administration was unique, in that an ~60-kDa PARP cleavage fragment, as well as distinct intracellular proteolytic cleavage of p53, were observed in NQO1-expressing breast or prostate cancer cells (5).2 These cleavage events were distinct from those observed when caspases were activated by topoisomerase I poisons, staurosporine, or administration of granzyme B (5, 12, 13). Furthermore, β-Lap-mediated cleavage events were blocked by administration of global cysteine protease inhibitors, as well as extracellular Ca2+ chelators (12). Based on these data, we concluded that β-Lap exposure of NQO1-expressing breast and prostate cancer cells caused the activation of a Ca2+-dependent protease with properties similar to calpain; in particular, the p53 cleavage pattern of β-Lap-exposed

* This work was supported by United States Army Medical Research and Materiel Command Breast Cancer Initiative Grant DAMD17-98-1-8260 (to D. A. B.), Predoctoral Fellowship DAMD17-00-1-0194 (to C. T.), and Postdoctoral Fellowship DAMD17-97-1-7221 (to J. J. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

‡ Partial fulfillment of the requirements for the Ph.D. degree, Case Western Reserve University, Dept. of Pharmacology.

§ To whom correspondence should be addressed: Dept. of Radiation Oncology and Pharmacology, Case Western Reserve University, 10900 Euclid Ave., Cleveland, OH 44106-4942. Tel.: 216-368-0840; Fax: 216-368-1142; E-mail: dab30@po.cwru.edu.

¶ The abbreviations used are: β-Lap, β-lapachone; MCP, MCF-7; W88; NQO1, NAD(P)H:quione oxidoreductase, DT-diaphorase (E.C. 1.6.99.2); PARP, poly(ADP-ribose) polymerase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; ER, endoplasmic reticulum; TG, thapsigargin; STS, staurosporine; BAPTA-AM, 1,2-bis-(2-aminophenoxyn)ethane-N,N',N'-tetraacetic acid-acetoxymethyl ester.

© 2001 by The American Society for Biochemistry and Molecular Biology, Inc. 
Printed in U.S.A.

This paper is available online at http://www.jbc.org

Downloaded from http://www.jbc.org/ by guest on July 25, 2018
cells was remarkably similar to the pattern observed after calpain activation (14, 15).

Ca\(^{2+}\) is recognized as an important regulator of apoptosis (16–21). The cytoplasmic Ca\(^{2+}\) concentration is maintained at \(\sim 100\) nM in resting cells by relatively impermeable cell membranes, active extrusion of Ca\(^{2+}\) from the cell by plasma membrane Ca\(^{2+}\)-ATPases, plasma membrane Na\(^+/Ca\(^{2+}\) exchangers, and active uptake of cytosolic Ca\(^{2+}\) into the endoplasmic reticulum (ER) by distinct Ca\(^{2+}\)-ATPases. In contrast, the concentration of Ca\(^{2+}\) in the extracellular milieu and in the ER is much higher (in the millimolar range). Evidence for involvement of Ca\(^{2+}\) influx into the cytosol as a triggering event for apoptosis has come from studies with specific Ca\(^{2+}\) channel blockers that abrogate apoptosis in regressing prostate following testosterone withdrawal (22). Other support for the involvement of Ca\(^{2+}\) in apoptosis comes from the observation that agents that directly mobilize Ca\(^{2+}\) (e.g., Ca\(^{2+}\) ionophores or the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase pump inhibitor, thapsigargin, TG) can trigger apoptosis in diverse cell types (23–27). Inhibition of the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase pump by TG causes a transient increase in cytoplasmic Ca\(^{2+}\) from ER Ca\(^{2+}\) stores, and a later influx of Ca\(^{2+}\) from the extracellular milieu, leading to the induction of apoptotic cell death (24, 27, 28). Consequently, emptying of intracellular Ca\(^{2+}\) stores may trigger apoptosis by disrupting the intracellular architecture and allowing key elements of the effector machinery (e.g., Apaf-1) to gain access to their substrates (e.g., caspase 9). Ca\(^{2+}\) has also been shown to be necessary for apoptotic endonuclease activation, eliciting DNA cleavage after many cellular insults (29–31). Buffering intracellular Ca\(^{2+}\) released from stored Ca\(^{2+}\) pools (e.g., ER) with BAPTA-AM, or removal of extracellular Ca\(^{2+}\) with EGTA, can protect cells against apoptosis (32, 33). Therefore, increases in intracellular Ca\(^{2+}\) levels appear to be important cell death signals in human cancer cells that might be exploited for anti-tumor therapy. Finally, Ca\(^{2+}\) may act as a signal for apoptosis by directly activating key proapoptotic enzymes (e.g., calpain); however, these proteolytic responses are poorly understood. The role of Ca\(^{2+}\) in cell death processes involving caspase activation has been examined in detail (28, 34–36). However, the role of Ca\(^{2+}\) in non-caspase-dependent cell death responses is relatively unexplored.

Recent studies have suggested that alterations in mitochondrial homeostasis play an essential role in apoptotic signal transduction induced by cytotoxic agents (37, 38). Various apoptotic stimuli have been shown to induce mitochondrial changes, resulting in release of apoptogenic factors, apoptosis-inducing factor (39), and mitochondrial cytochrome c (9) into the cytoplasm. These changes are observed during the early phases of apoptosis in human epithelial cells, and were linked to the initial cascade of events, sending the cell to an irreversible suicide pathway. During high, sustained levels of cytosolic Ca\(^{2+}\), mitochondrial Ca\(^{2+}\) uptake is driven by mitochondrial membrane potential to maintain Ca\(^{2+}\) homeostasis in the cytosol. In de-energized mitochondria, Ca\(^{2+}\) can be released by a reversal of this uptake pathway (40). These data, therefore, linked changes in Ca\(^{2+}\) homeostasis and mitochondrial membrane potential to the initiation of apoptosis. Li et al. (41) reported that β-Lap caused a decrease in mitochondrial membrane potential with release of cytochrome c into the cytoplasm in a number of human carcinoma cell lines, shortly after drug addition. Other alterations in metabolism (e.g., ATP depletion) have not been examined in β-Lap-treated cells.

We previously characterized the activation of a novel cysteine protease in various breast cancer cell lines with properties similar to the Ca\(^{2+}\)-dependent cysteine protease, calpain, after exposure to β-Lap (12). Using NQO1-expressing breast cancer cells, we show that β-Lap elicits a rise in intracellular Ca\(^{2+}\) levels shortly after drug administration that eventually leads to apoptosis. This paper suggests a critical, but not sufficient, role for Ca\(^{2+}\) in the cell death pathway initiated by NQO1-dependent bioactivation of β-Lap. Possible combinatorial effects (e.g., NADPH depletion as well as intracellular calcium alterations) that initiate β-Lap-mediated apoptosis in NQO1-expressing breast cancer cells will be discussed.

**Experimental Procedures**

**Reagents—β-Lapachone** (3,4-dihydro-2,2-dimethyl-2H-naphtho[1,2,3-b]pyran-5,6-dione) was synthesized by Dr. William G. Bornmann (Memorial Sloan Kettering, New York), dissolved in dimethyl sulfoxide at 10 mM, and the concentration verified by spectrophotometric analysis (2, 5). EGTA, Hoechst 33258, and thapsigargin were obtained from Sigma. BAPTA-AM (1,2-bis-(2-aminophenoxy)-ethane-N,N,N',N'-tetra-acetoxymethyl ester) and tetra-acetoxymethyl tetra-aminocarbocyanine iodide and Fluoro-4-AM were obtained from Molecular Probes, Inc. (Eugene, OR).

**Cell Culture—MCF-7/WS (MCF-7) human breast cancer cells were obtained from Dr. V. Craig Jordan, (Northwestern University, Chicago, IL). MDA-MB-468 cells were obtained from the American Type Culture Collection and transfected with Q1G1 cDNA (42) to obtain a constitutive expression vector as described previously (5). Tissue culture components were purchased from Life Technologies, Inc., unless otherwise stated. MCF-7 cells were grown in RPMI 1640 cell culture medium supplemented with 10% fetal bovine serum, in a 37 °C humidified incubator with 5% CO\(_2\), 95% air atmosphere as previously described (2, 5). For all experiments, log-phase breast cancer cells were exposed to 5 μM β-Lap for 4 h (unless otherwise indicated), after which fresh medium was added and cells were harvested at various times post-treatment.

**TUNEL Assay—**Cells were seeded at 1 × 10\(^6\) cells/cm Petri dish and allowed to grow for 24 h. Log-phase cells were then pretreated for 30 min with 10 μM BAPTA-AM, 3 mM EGTA, or 50 μM diconarol followed by a 4-h pulse of 5 μM β-Lap, as described above, or 24 h treatment of 10 μM ionomycin or 1 μM staurosporine. Medium was collected from experimental as well as control conditions 24 h later, and apoptosis was monitored using a PerkinElmer HTS 7000 Fluoro Bio Assay Plate Reader (Norwalk, CT) with 360 and 465 nm excitation and emission filters, respectively. Data were expressed as relative growth, T/C (treated/control), using experiments performed at least twice.

**Confocal Microscopy—**Cells were seeded at 2–3 × 10\(^5\) cells per 35-mm glass bottom Petri dishes (MatTek Corp., Ashland, MA) and allowed to attach overnight. Cells were rinsed twice in BSS, 130 mM NaCl, 5 mM KCl, 1.5 mM CaCl\(_2\), 1 mM MgCl\(_2\), 25 mM HEPES, pH 7.5, 5 mM glucose, and 2× L-glutamine (serum albumin) and loaded with the Ca\(^{2+}\)-sensitive fluorescent indicator, fluo-4-AM (5 μM), in BSS for ~20–30 min at 37 °C. Cells were rinsed twice in BSS and incubated for an additional 20 min at 37 °C to allow for hydrolysis of the AM-ester. Cells were imaged with a Zeiss 410 confocal microscope (Thornwood, NY) equipped with a ×63 N.A. 1.4 oil immersion planapochromatic objective at room temperature (the same results were observed at room temperature and 37 °C). Confocal images of fluo-4 fluorescence were collected using a 488-nm excitation light from an argon/krypton laser, a 560-nm dichroic mirror, and a 500–550 nm band-pass barrier filter. Three basal images were collected before drug addition (8 μM β-Lap, ± 50 μM diconarol or 200 mM TG). The mean pixel intensity was set to equal one for analyses of fold-increase in fluo-4 fluorescence intensity. Subsequently, images were collected after the indicated treatments at 90-s intervals. BAPTA-AM (20 μM) was co-

**Downloaded from http://www.jbc.org/ by guest on July 25, 2018**
load with fluo-4-AM where indicated. Mean pixels were determined in regions of interest for individual cells at each time point.

**Mitochondrial Membrane Potential Determinations**—MCF-7 cells were seeded at 2.5 × 10^4 cells per 6-well plate, and allowed to grow for 2 days. Log-phase cells were pretreated for 30 min with 10 μM BAPTA-AM, 3 mM EGTA, or 50 μM dicumarol followed by a 4-h pulse of 5 μM β-Lap, unless otherwise indicated. Cells were trypsinized and resuspended in phenol red-minus RPMI medium for analyses. Cells were maintained at 37 °C for the duration of the experiment, including during analyses. Prior to analyses, cells were loaded with 10 μg/ml JC-1 for 9–14 min and samples were analyzed using a Beckman Coulter EPICS Elite ESP (Miami, FL) flow cytometer. JC-1 monomer and aggregate emissions were excited at 488 nm and quantified using Elite acquisition software after signal collection through 525- and 590-nm band pass filters, respectively. Shifts in emission spectra were plotted on bivariant dot plots, on a cell-by-cell basis, to determine relative mitochondrial membrane potential of treated and control cells.

**ATP Measurements**—Cells were seeded at 2.5 × 10^4 cells per well in 6-well dishes and allowed to attach for 24 h. Fresh medium was added to the cells along with Ca^{2+} chelators or dicumarol 30 min prior to β-Lap exposure (4 h unless otherwise indicated). Floating cells were collected, pelleted, and lysed in 1.67 M perchloric acid. Attached cells were lysed directly in 1.67 M perchloric acid. Following a 20-min incubation at room temperature, attached cells were scraped and transferred to corresponding microcentrifuge tube, cooled on ice for several minutes, and spun to pellet protein precipitates. Deproteinized samples were neutralized with 3.5 M KOH and HEPES/KOH (25 mM HEPES, 15 mM KOH, pH 8), and incubated on ice for 15 min. Precipitates were removed by centrifugation and samples stored at −20 °C. Cell extracts were analyzed for ATP and ADP levels using a luciferase-based bioluminescent assay and rephosphorylation protocols, as described (42).

**Western Blot Analyses**—Whole cell extracts from control or β-Lap-exposed MCF-7 cells were prepared and analyzed by SDS-polyacrylamide gel electrophoresis/Western blot analyses as previously described (2, 5, 12). Loading equivalence and transfer efficiency were monitored by Western blot analyses of proteins that are known to be unaltered by experimental treatments (2), and using Ponceau S staining of the membrane, respectively. Probed membranes were then exposed to x-ray film for an appropriate time and developed. Prior to analyses, cells were loaded with fluo-4-AM where indicated. Mean pixels were determined by Hoescht 33258 fluorescence, and graphed as relative growth as the average of at least three independent experiments. The number in the upper right corner represents percent cells staining positive in the TUNEL assay. Results are graphically summarized in B as the average of at three independent experiments, mean ± S.E. Student’s t test for paired samples, experimental group compared with MCF-7 cells treated with β-Lap alone are indicated (*, p < 0.01). C, cells were exposed to a 4-h pulse of various concentrations of β-Lap either alone (closed), or after a 30-min pretreatment with 5 μM BAPTA-AM (open). Relative DNA per well was determined by Hoechst 33258 fluorescence, and graphed as relative growth (treated/control DNA); mean relative DNA per well, ± S.E. Shown are representative results of experiments performed at least twice. Student’s t test for paired samples, experimental group compared with MCF-7 cells treated with β-Lap alone are indicated (*, p < 0.05; and **, p < 0.005).

**Ca^{2+} Chelators Do Not Block Apoptosis Induced by Other Agents**—It was possible based on the data in Fig. 1 that calcium chelators may block β-Lap-mediated apoptosis by sequestering...
calcium required for the activation of apoptotic endonucleases. We, therefore, examined both intra- and extracellular Ca\(^{2+}\) chelators for their ability to prevent apoptosis in NQO1-transfected MDA-468 (MDA-468-NQ3) cells induced by \(\beta\)-Lap, ionomycin (which induces Ca\(^{2+}\)-mediated cell death (36)), and staurosporine (STS, which inhibits protein kinase C and works via a caspase-mediated cell death pathway (43, 44)). We used MDA-468-NQ3 cells to assay for caspase-mediated endonuclease activation and DNA fragmentation since they express the endonuclease-activating caspase 3, unlike MCF-7 cells (45). We previously demonstrated that MDA-468-NQ3 cells responded similarly to \(\beta\)-Lap as MCF-7 cells (Fig. 2 and Ref. 5). EGTA significantly protected MDA-468-NQ3 cells against ionomycin-induced apoptosis, but not against STS-induced apoptosis (Fig. 2). MDA-468-NQ3 cells treated for 24 h with 10 \(\mu\)M ionomycin exhibited 49% apoptotic cells, whereas, MDA-468-NQ3 cells pretreated for 30 min with 3 mM EGTA followed by a 24-h exposure to ionomycin exhibited only 4% apoptotic cells. Cells treated for 24 h with 1 \(\mu\)M STS in the absence or presence of 3 mM EGTA exhibited 56% and 46% apoptosis, respectively. BAPTA-AM (10 \(\mu\)M) did not significantly block apoptosis induced by ionomycin. BAPTA-AM pretreatment of STS-exposed MDA-468-NQ3 cells did not significantly decrease apoptosis (\(p < 0.4\)) compared with cells exposed to STS alone; the modest effect of BAPTA-AM on STS-induced apoptosis may reflect the Ca\(^{2+}\) dependence of the apoptotic endonucleases involved in this response. Neither BAPTA-AM nor EGTA alone elicited apoptotic responses at the doses used in the aforementioned experiments (Figs. 1B and 2). Furthermore, preliminary data suggest that DFF45 (ICAD) was cleaved in NQO1-expressing MCF-7 or MDA-468-NQ3 cells at 8 h after \(\beta\)-Lap treatment, in a temporal manner corresponding to the induction of apoptosis (data not shown). Cleavage of DFF45, an endogenous inhibitor of the magnesium-dependent and Ca\(^{2+}\)-independent apoptotic endonuclease, DFF40 (CAD), suggests that DFF40 is activated following treatment with \(\beta\)-Lap. Taken together with results in Fig. 1, these data strongly suggest that a rise in intracellular Ca\(^{2+}\) levels is part of a critical signaling pathway for the induction of apoptosis in NQO1-expressing human breast cancer cells following \(\beta\)-Lap exposure.

**Exposure of NQO1-expressing MCF-7 Cells to \(\beta\)-Lap Results in Increased Intracellular Ca\(^{2+}\)**—We next directly examined whether intracellular Ca\(^{2+}\) levels were increased in log-phase MCF-7 cells after \(\beta\)-Lap treatment using the cell-permeant intracellular Ca\(^{2+}\) indicator dye, fluo-4. Cells were loaded with 5 \(\mu\)M fluo-4-AM, and where indicated, 20 \(\mu\)M BAPTA-AM, incubated for -25 min to allow for the dye to permeate cells, rinsed, and then incubated for an additional -20 min for hydrolysis of the AM-ester. Following drug addition, images were collected every 90 s for -60 min using confocal microscopy. Three basal images were recorded before drug addition and average pixels per cell were determined (indicative of fluo-4 fluorescence and, therefore, basal intracellular Ca\(^{2+}\) levels) and used for analyses over time. The fluorescence of basal images were averaged and set to equal one; fold increases were determined from changes in fluo-4 fluorescence over control. After exposure to 8 \(\mu\)M \(\beta\)-Lap, MCF-7 cells exhibited an ~2-fold increase in fluo-4 fluorescence from 4 to 9 min, after which time Ca\(^{2+}\) levels returned to basal levels in a majority of cells examined (43 of 50, 86\%) (Fig. 3A). The rise in intracellular Ca\(^{2+}\) levels in MCF-7 cells following \(\beta\)-Lap exposure was prevented by preloading cells with BAPTA-AM (20 \(\mu\)M) (Fig. 3B). Interestingly, not all \(\beta\)-Lap-exposed MCF-7 cells were affected by pretreatment with BAPTA-AM; 3 of 26 cells (12\%) exhibited a rise in intracellular Ca\(^{2+}\) levels after exposure to \(\beta\)-Lap despite the presence of this Ca\(^{2+}\) chelator. However, BAPTA-AM pretreated MCF-7 cells that did exhibit a rise in intracellular Ca\(^{2+}\) levels following \(\beta\)-Lap treatment exhibited a similar, but delayed Ca\(^{2+}\) increase (10–20 min), as compared with \(\beta\)-Lap-exposed MCF-7 cells in the absence of BAPTA-AM (4–9 min). This may be due to a saturation of the chelator or heterogeneity of the tumor cell population. These results are consistent with previous reports that the buffering capacity of BAPTA-AM may be overwhelmed with time (34, 46). Higher doses of BAPTA-AM were not used due to toxicity caused by the drug alone (data not shown).

Since the ER is a major store of Ca\(^{2+}\) in the cell, we tested if the initial rise in intracellular Ca\(^{2+}\) levels after exposure of MCF-7 cells to \(\beta\)-Lap was due to release of Ca\(^{2+}\) from this organelle. If \(\beta\)-Lap exposure led to release of Ca\(^{2+}\) stored in the ER, then TG (a sarcoplasmic reticulum Ca\(^{2+}\)-ATPase pump inhibitor) administration should not cause additional Ca\(^{2+}\) release. Similarly, if the sequence of drug administration were reversed, additional Ca\(^{2+}\) release would also not be observed. When \(\beta\)-Lap was added after TG-induced depletion of ER Ca\(^{2+}\) stores, no measurable rise in intracellular Ca\(^{2+}\) levels occurred in 25 of 27 (93\%) cells analyzed (Fig. 3C). Similarly, when TG was added to cells after \(\beta\)-Lap, only 1 of 18 (6\%) cells that initially responded to \(\beta\)-Lap exhibited a rise in intracellular Ca\(^{2+}\) levels following subsequent TG administration (Fig. 3D). At the end of the experiment, all cells analyzed remained responsive to ionomycin. Thus, cells exposed to \(\beta\)-Lap and/or TG were still capable of altering Ca\(^{2+}\) levels of Ca\(^{2+}\) pool of Ca\(^{2+}\). All cells analyzed started with comparable basal Ca\(^{2+}\) and appeared to load equal amounts of the indicator dye, fluo-4. Cells were loaded with 8 S.E. Untreated or MDA-468-NQ3 cells pretreated for 30 min with either 3 mM EGTA or 30 \(\mu\)M BAPTA-AM for 30 min prior to drug addition; either a 4-h pulse of 8 \(\mu\)M \(\beta\)-Lap, or 24 h continuous treatment of 10 \(\mu\)M ionomycin or 1 \(\mu\)M STS. Cells were then analyzed using the TUNEL assay for DNA fragmentation. Shown are mean ± S.E. of at least two independent experiments. Student’s t test for paired samples, experimental group compared with cells treated with drug alone are indicated (*, \(p < 0.05\)).
Loss of Mitochondrial Membrane Potential After β-Lap Is Attenuated by Intracellular, but Not Extracellular, Ca\(^{2+}\) Chelation—Mitochondrial membrane potential was previously shown to drop from a hyperpolarized state to a depolarized state after treatment of various human cancer cells with β-Lap (41). A drop in mitochondrial membrane potential in β-Lap-treated cells was accompanied by a concomitant release of cytochrome c into the cytosol (41). To explore whether early changes in intracellular Ca\(^{2+}\) levels were upstream of mitochondrial changes in NQO1-expressing breast cancer cells, log phase MCF-7 cells were pretreated for 30 min with either 10 μM BAPTA-AM or 3 mM EGTA and then exposed to 5 μM β-Lap for 4 h. Prior to analyses, cells were loaded with JC-1, a cationic dye commonly used to monitor alterations in mitochondrial membrane potential (47, 48). Mitochondrial depolarization measurements using JC-1 were indicated by a decrease in the red/green fluorescence intensity ratio (a movement of events from upper left to lower right, Fig. 4), as seen following a 10-min treatment with the potassium ionophore, valinomycin (100 nM), which causes a collapse of mitochondrial membrane potential by uncoupling mitochondrial respiration (Fig. 4e) (49); cells in the upper left-hand quadrant exhibited high mitochondrial membrane potential, whereas, cells in the lower right-hand quadrant have low mitochondrial membrane potential and are depolarized. Cells in the upper right-hand quadrant exhibited intermediate membrane potential. Mitochondrial membrane potential decreased in MCF-7 cells in a time- and dose-dependent manner following exposure to β-Lap (Figs. 4, a–d, and data not shown). By 4 h, the majority of β-Lap-treated MCF-7 cells exhibited low mitochondrial membrane potential (53%), while the majority of control cells maintained high mitochondrial membrane potential (51%) (Fig. 4, b, a and g, f, respectively). This drop in mitochondrial membrane potential observed 4 h after treatment with β-Lap (low, 53%) was abrogated by pretreatment with BAPTA-AM (low, 23%), but not by EGTA (low, 48%) (Fig. 4, g, i, respectively). Pretreatment with 10 μM BAPTA-AM prevented the decrease in mitochondrial membrane potential (low, 23%); however, BAPTA-AM did not maintain β-Lap-exposed cells in a high-potential state (high, 28%) as observed in control untreated cells (high, 51%). Approximately half of the BAPTA-AM-exposed cells were in an intermediate membrane potential state (45%) (Fig. 4h). We noted, however, that BAPTA-AM or EGTA exposures alone caused depolarization of the mitochondria, with a majority of the cells residing in the same intermediate energized state as observed following BAPTA-AM and β-Lap (Fig. 4, j–k). Therefore, BAPTA-AM prevented mitochondrial depolarization induced by β-Lap to the same extent as in cells treated with BAPTA-AM alone. Pretreatment with 3 mM EGTA did not affect the loss of mitochondrial membrane potential caused by β-Lap (low 48%), implying that an early rise in intracellular Ca\(^{2+}\) levels from intracellular stores was sufficient to cause a drop in mitochondrial membrane potential, and that extracellular calcium was not needed for these effects in β-Lap-treated cells (Fig. 4, h-i).

Loss of ATP After β-Lap Is Attenuated by Intracellular Ca\(^{2+}\) Chelation—The bioactivation of β-Lap by NQO1 is thought to lead to a futile cycling between quinone and hydroquinone forms of the compound, presumably due to the instability of the
hydroquinone form of β-Lap (5). This futile cycling led to depletion of NADH and NADPH, electron donors for NQO1 in intracellular assays (5). Exhaustion of reduced enzyme co-factors may be a critical event for the activation of the apoptotic pathway in NQO1-expressing cells following β-Lap exposure. We, therefore, measured intracellular ATP and ADP in log-phase MCF-7 cells after various doses and times of β-Lap (using a luciferase-based bioluminescent assay (42)). Intracellular ATP levels were reduced in MCF-7 cells after treatment with β-Lap in a dose- and time-dependent manner (Fig. 5A). At all doses of β-Lap above the LD_{50} of the drug (~2.5 μM) in MCF-7 cells (2), intracellular ATP levels were reduced by >85% at 4 h, the time at which drug was removed (Fig. 5A, left); the loss of ATP correlated well with β-Lap-induced cell death in MCF-7 cells (Fig. 1C). ADP levels remained relatively unchanged after various doses of β-Lap, however, the [ATP]/[ADP][P_1] ratio decreased dramatically. Intracellular ATP levels began to drop to 70% of control levels 2 h after 5 μM β-Lap exposure, the time at which β-Lap began to elicit mitochondrial membrane depolarization (Figs. 5A, A, right, and 4, c). ATP levels continued to drop to 8% of control levels by 4 h after drug exposure (Fig. 5A, right). In contrast, ADP levels remained relatively unchanged during the course of the experiment, with an increase at 30 min (172% control levels) that returned to control levels by 1 h during the course of the experiment, with an increase at 30 min right). In contrast, ADP levels remained relatively unchanged.

Chelators Prevent β-Lap-induced Proteolysis—We previously showed that apoptosis in various breast cancer cell lines induced by β-Lap was unique, causing a pattern of PARP and p53 intracellular cleavage events distinct from those induced by caspase activating agents (12). After β-Lap treatment, we observed an ~60-kDa PARP cleavage fragment and specific cleavage of p53 in NQO1-expressing breast cancer cells. Furthermore, we showed that this proteolysis in β-Lap-treated cells was the result of activation of a Ca^{2+}-dependent protease with properties similar to μ-calpain (12). PARP and p53 proteolysis in β-Lap-exposed, NQO1-expressing cells was prevented by pretreatment with the extracellular Ca^{2+} chelators, EGTA and EDTA, in a dose-dependent manner (at 8 and 24 h) (Ref. 12, and data not shown). Additionally, PARP, p53, and lamin B proteolysis induced at 24 h in MCF-7 cells following β-Lap treatment were abrogated by pretreatment with 10 or 30 μM BAPTA-AM (Fig. 6). These data strongly suggest that a Ca^{2+}-dependent path and potentially a Ca^{2+}-dependent protease are operative in β-Lap-mediated apoptosis.

A simple explanation for the aforementioned results could be that BAPTA blocks bioactivation of β-Lap by NQO1 in a manner similar to that of dicumarol (5). However, BAPTA (free acid) did not affect the enzymatic activities of NQO1 using standard enzymatic assays (data not shown) (5). The free acid (active) form of BAPTA, instead of its −AM ester form, was used in these assays since intracellular accumulation of the Ca^{2+} chelator was not necessary and was physiologically relevant in the in vitro enzyme assay. Using β-Lap as a substrate, NQO1 enzymatic activity in the presence of 10 mM BAPTA (a dose of the free acid form of BAPTA that was >1000-fold higher than that used in the experiments of Figs. 1–6) was reduced by <20%. Thus, BAPTA-AM did not affect the activity of NQO1, a two-electron reductase required for β-Lap cytotoxicity (5). We conclude that BAPTA-AM prevents β-Lap-induced apoptosis by blocking Ca^{2+}-mediated signaling events via chelating intracellular Ca^{2+}.

β-Lap Bioactivation by NQO1 Is Critical for Ca^{2+}-mediated Signaling—We previously reported that cells expressing NQO1 are more sensitive to the cytotoxic effects of β-Lap (5). NQO1 is inhibited by dicumarol, which competes with NADH or NADPH for binding to the oxidized form of the enzyme. Dicumarol thereby prevents reduction of quinones (50, 51). We demonstrated that dicumarol attenuates β-Lap-mediated proteolysis of apoptotic substrates (e.g. PARP and p53), apoptosis, and survival in NQO1-expressing cells (5). As expected, increases

FIG. 4. β-Lap-induced loss of mitochondrial membrane potential is mediated by alterations in Ca^{2+} homeostasis. Mitochondrial membrane potential was measured in control or drug-treated MCF-7 cells with the JC-1 dye. A, cells were treated with 5 μM β-Lap and assayed for changes in mitochondrial membrane potential at 1, 2, and 4 h post-treatment. Exposure of MCF-7 cells to 100 nM valinomycin for 15 min served as a positive control as described (49). Cells in the upper left-hand quadrant exhibit high mitochondrial membrane potential, while cells in the lower right-hand quadrant exhibit low mitochondrial membrane potential. B, cells were treated for 30 min with either 10 μM BAPTA-AM or 3 mM EGTA prior to a 4-h treatment with 5 μM β-Lap. At 4 h, cells were harvested for analyses of changes in mitochondrial membrane potential using JC-1 as described above. Shown are representative experiments performed at least three times, and numbers in each quadrant represent the average of cells in that quadrant of at least three independent experiments. S.E. for any single number was not more than 11%.
Ca\textsuperscript{2+} Signaling in \textbeta-Lap-mediated Apoptosis

in intracellular Ca\textsuperscript{2+} levels in NQO1-expressing human cancer cells elicited by \textbeta-Lap were abrogated by co-treatment with 5 \mu M dicumarol in 26 of 27 cells (96\%) examined (Fig. 7A, lower panel). The ability of dicumarol to inhibit increases in intracellular Ca\textsuperscript{2+} levels was greater than that observed with BAPTA-AM, where intracellular Ca\textsuperscript{2+} level increases were prevented in only 89\% of cells examined (Fig. 3B). Thus, NQO1 was critical for the rise in intracellular Ca\textsuperscript{2+} levels observed in MCF-7 cells after \textbeta-Lap exposure.

Mitochondrial membrane depolarization induced by \textbeta-Lap was also abrogated by pretreatment with dicumarol (Fig. 7B). By 4 h, the majority of \textbeta-Lap-treated cells exhibited low mitochondrial membrane potential (58\%), while very few control cells were depolarized (9\%) (Fig. 7B). Pretreatment with dicumarol attenuated this response to \textbeta-Lap, with only 34\% being depolarized. The inability of dicumarol to prevent mitochondrial depolarization in 34\% of \textbeta-Lap-treated cells was probably due to the high background of control cells (20\%) that were depolarized after exposure to dicumarol alone. Comparison with intracellular Ca\textsuperscript{2+} buffering, BAPTA-AM elicited only a minor depolarization of the mitochondria on its own (low, 14\%) and thus was able to elicit a greater protective effect (Fig. 4B); only 23\% of cells exposed to BAPTA-AM and \textbeta-Lap exhibited low mitochondrial membrane potential as compared with \textbeta-Lap exposed cells in the presence of dicumarol (34\%).

The dramatic loss of intracellular ATP in MCF-7 cells following \textbeta-Lap exposure was inhibited by a 30-min pretreatment with 50 \mu M dicumarol (Fig. 7C). \textbeta-Lap-treated MCF-7 cells pretreated with dicumarol exhibited only 34\% loss of intracellular ATP, compared with 92\% loss after \textbeta-Lap treatment alone (Fig. 7C). ATP levels were not altered by any of the treatments used, however, the [ATP]/[ADP]/[P\textsubscript{i}] ratio decreased dramatically in \textbeta-Lap-treated cells, and was only partially decreased with dicumarol pretreatment alone, as compared with control untreated cells.

Dicumarol also abrogated DNA fragmentation induced by \textbeta-Lap in MCF-7 cells. MCF-7 cells exhibited 94\% apoptosis following \textbeta-Lap exposure that was prevented by a 30-min pretreatment with 50 \mu M dicumarol; only 6\% of the cells staining positive in a TUNEL assay at 24 h post-treatment (Fig. 7D). These data are consistent with prior results (5), and correlate well with the survival protection afforded by dicumarol to \textbeta-Lap-treated cells. Dicumarol did not induce DNA fragmentation on its own. These data are consistent with the protection from apoptosis observed with either intra- and extracellular Ca\textsuperscript{2+} chelators. BAPTA-AM or EGTA protected \textbeta-Lap exposed MCF-7 cells from apoptosis (Fig. 1, A and B). Collectively, these data implicate the bioactivation of \textbeta-Lap by NQO1 as a critical step in the rise of intracellular Ca\textsuperscript{2+} levels following \textbeta-Lap exposure, and thus \textbeta-Lap-mediated downstream apoptotic events.

**DISCUSSION**

When homeostatic mechanisms for regulating cellular Ca\textsuperscript{2+} are compromised, cells may die, either by necrosis or apoptosis (20, 21, 36). We demonstrated that bioactivation of \textbeta-Lap by NQO1 induced cell death in a manner that was dependent upon Ca\textsuperscript{2+} signaling (Figs. 1–6). \textbeta-Lap can be reduced by NQO1 and

**Fig. 6. Intracellular Ca\textsuperscript{2+} chelators prevent apoptotic proteolysis after \textbeta-Lap treatment.** Apoptotic proteolysis was measured in MCF-7 cells exposed to a 4-h pulse of 5 \mu M \textbeta-Lap, with or without a 30-min pretreatment of the indicated dose of BAPTA-AM. Whole cell extracts were prepared 24 h after drug addition, and analyzed using standard Western blotting techniques with antibodies to PARP, p53, and lamin B. Shown is a representative Western blot of whole cell extracts from experiments performed at least three times.
**Fig. 7.** NQO1-dependent activation of β-Lap is critical for Ca\(^{2+}\) signaling. A, intracellular Ca\(^{2+}\) was measured on live cells using the Ca\(^{2+}\) indicator dye, fluo-4-AM, and confocal microscopy as described in the legend to Fig. 3. Three basal images were recorded before drug treatments. β-Lap (8 μM) was then added to MCF-7 cells, either alone (upper panel) or in combination with 50 μM dicumarol (lower panel). Images were collected every 90 s for 50–60 min. Shown are representative graphs displaying changes in fluo-4 fluorescence for the duration of the experiment. Each line represents the fold change in fluo-4 fluorescence emission (as compared with basal levels) of an individual cell from one experiment, and the graph is representative of experiments performed at least three times. B, mitochondrial membrane potential was measured using the JC-1 dye as described in the legend to Fig. 4. MCF-7 cells were treated with 50 μM dicumarol 30 min prior to β-Lap exposure. Four hours later, cells were harvested for analyses of mitochondrial membrane potential. Shown are mean ± S.E. of the percentage of cells with low mitochondrial membrane potential of at least two independent experiments. C, ATP and ADP levels were assayed as described in the legend to Fig. 5. Cells were pretreated with dicumarol for 30 min prior to drug addition, 5 μM β-Lap was added for 4 h, and cells were harvested immediately thereafter for analyses. Results represent the mean of at least three independent experiments ± S.E. Student’s t test for paired samples, experimental groups compared with drug alone are indicated (* p < 0.05). D, apoptosis, using the TUNEL assay, was assessed as per Fig. 1. MCF-7 cells were treated with 50 μM dicumarol 30 min prior to a 4-h exposure of 5 μM β-Lap. Cells were then harvested for TUNEL analyses at 24 h post-treatment. Shown are mean ± S.E. of at least three independent experiments. Student’s t test for paired samples, experimental groups compared with β-Lap exposure alone are indicated (*, p < 0.005). DC, 50 μM dicumarol.

**Fig. 8.** Proposed model for β-lapachone-mediated apoptosis in NQO1-expressing cells. In cells that express NQO1, β-Lap is reduced from the quinone (β-Lap-Q) to the hydroquinone (β-Lap-HQ) form in a futile cycle that results in dramatic losses of NAD(P)H (5). During the metabolism of β-Lap by NQO1, Ca\(^{2+}\) is subsequently released from the ER causing a rise in cytosolic Ca\(^{2+}\) levels by an as yet unknown mechanism. To maintain low cytoplasmic Ca\(^{2+}\) levels, we theorize that mitochondria sequester Ca\(^{2+}\) and numerous cellular ATPases probably function to pump Ca\(^{2+}\) out of the cytosol. This leads to mitochondrial depolarization and ATP hydrolysis, respectively (Figs. 4 and 5). Sustained depolarization of the mitochondrial membrane leads to further loss of ATP and prevents ATP synthesis by inhibiting respiration. The loss of ATP disrupts ionic homeostasis within the cell and thereby allows extracellular Ca\(^{2+}\) to enter the cell down its concentration gradient (see “Discussion”). The secondary rise in cytosolic Ca\(^{2+}\) levels leads to protease (presumably activation of calpain or a calpain-like protease) and, thus, endonuclease (DFF40) activation, ultimately resulting in apoptosis.

The rise in intracellular Ca\(^{2+}\) appears to be dependent upon the bioactivation of β-Lap by NQO1, suggesting a critical and necessary signaling role for Ca\(^{2+}\) in the downstream apoptotic pathway induced by this drug. Dicumarol completely abrogated intracellular Ca\(^{2+}\) changes (Fig. 7), as well as apoptosis and survival, following β-Lap exposure of NQO1-expressing cells (5). When increases in intracellular Ca\(^{2+}\) levels in response to β-Lap, activate a novel caspase-independent apoptotic pathway, as described in this paper and previously (2, 5, 12).
suggest that DNA fragmentation, mitochondrial membrane depolarization, ATP loss, and apoptotic proteolysis were a consequence of the increase in intracellular Ca\(^{2+}\) levels (Figs. 1–6 and 8). Interestingly, the cell death pathway induced by \(\beta\)-Lap was quite distinct from that observed after exposure to TG, an agent known to specifically cause release of Ca\(^{2+}\) from ER stores and mediate caspase-dependent apoptosis (24, 28, 33, 52). Thus, Ca\(^{2+}\) release was necessary for \(\beta\)-Lap-induced cytotoxicity, but apparently not sufficient for the unique apoptotic responses induced by \(\beta\)-Lap.

\(\beta\)-Lap and TG-induced Similar Ca\(^{2+}\) Responses, but Different Patterns of Apoptosis—\(\beta\)-Lap elicited an early rise in intracellular Ca\(^{2+}\) levels from the same ER store as released by TG, however, subsequent cell death processes were remarkably different between the two compounds. TG is known to cause transient increases in intracellular Ca\(^{2+}\) levels, however, these were insufficient to induce apoptosis. Much like \(\beta\)-Lap, Ca\(^{2+}\) was needed from the extracellular milieu, along with a sustained increase in intracellular Ca\(^{2+}\) levels, for TG-induced apoptosis (23) in MCF-7 cells (27). Depolarization of the mitochondrial membrane potential and loss of intracellular ATP in cells exposed to \(\beta\)-Lap, may have prevented plasma membrane Ca\(^{2+}\) pumps and ER Ca\(^{2+}\) pumps from functioning and maintaining Ca\(^{2+}\) homeostasis. This, in turn, may have facilitated Ca\(^{2+}\) leakage down its concentration gradient into the cytosol, providing a secondary and sustained elevation of Ca\(^{2+}\) that initiated a protease cascade(s) and ultimately caused apoptosis after exposure to \(\beta\)-Lap. This is consistent with what we observed in NQO1-expressing cells after \(\beta\)-Lap treatment and co-administration of Ca\(^{2+}\) chelators. Buffering intracellular Ca\(^{2+}\) with BAPTA-AM partially abrogated all of the downstream events induced in MCF-7 cells by \(\beta\)-Lap (and thus prevented secondary Ca\(^{2+}\) entry by buffering the initial rise in cytosolic Ca\(^{2+}\)). In contrast, extracellular chelation by EGTA only prevented those events initiated by secondary Ca\(^{2+}\) entry (e.g. protease activation and DNA fragmentation). Thus, a secondary rise in intracellular Ca\(^{2+}\) levels after exposure to \(\beta\)-Lap seems probable, and necessary, for protease activation and DNA fragmentation as was observed for TG-induced caspase-mediated apoptosis (23, 27). However, a secondary influx of Ca\(^{2+}\) does not appear to be necessary for reduction in mitochondrial membrane potential or loss of intracellular ATP after \(\beta\)-Lap exposure, since EGTA did not prevent these responses.

Although MCF-7 cells treated with \(\beta\)-Lap had similar calcium responses, as do TG-exposed cells, \(\beta\)-Lap-exposed cells exhibited a very different pattern of apoptosis than TG-treated cells. \(\beta\)-Lap-exposed cells exhibit loss of intracellular ATP and a decrease in the [ATP]/[ADP]/[Pi] ratio. In contrast, TG-exposed cells did not exhibit loss of ATP (Fig. 5, and as reported by Ref. 53). Our data suggest that in contrast to TG where ATP-dependent caspase activation results in cell death (28, 33, 34, 54), an ATP-independent protease is activated after exposure to \(\beta\)-Lap. Ca\(^{2+}\) may regulate apoptosis by activating Ca\(^{2+}\)-dependent protein kinases and/or phosphatases leading to alterations in gene transcription. However, with the rapid loss of intracellular ATP after exposure to \(\beta\)-Lap (2–4 h, Fig. 5), \(\beta\)-Lap-mediated cell death unlikely involves stimulated kinases or phosphatases or new protein synthesis. Instead, indirect kinase inhibition, due to ATP depletion, along with continued phosphatase activity is likely. Consistent with this notion, we found dramatic de-phosphorylation of pRb in cells exposed to \(\beta\)-Lap at 3 h (2), a time consistent with loss of ATP following exposure to this drug. Furthermore, loss of ATP at 2 h may also be responsible for inhibition of NF-\(\kappa\)B activation induced by tumor necrosis factor-\(\alpha\) in \(\beta\)-Lap pre-exposed cells (55), since significant loss of ATP would prevent proteosome-mediated I\(\kappa\)B degradation. Thus, Ca\(^{2+}\)-dependent loss of ATP in NQO1-expressing cells following \(\beta\)-Lap treatment may explain the reported pleiotropic effects of this agent.

\(\beta\)-Lap-exposed cells also exhibited a very different pattern of substrate proteolysis compared with that observed after TG (2, 12, 28). We previously showed that \(\beta\)-Lap elicited a unique cleavage of PARP (−60-kDa fragment), compared with the classical caspase-3-mediated fragmentation of the protein (−89 kDa) observed after TG exposure (data not shown and Ref. 28). In a variety of NQO1-expressing cells exposed to \(\beta\)-Lap, atypical PARP cleavage was inhibited by the global cysteine protease inhibitors, iodoacetamide and N-ethylmaleimide, as well as the extracellular Ca\(^{2+}\) chelators, EGTA and EDTA (12). In addition, \(\beta\)-Lap-mediated apoptotic responses were insensitive to inhibitors of caspases, granzyme B, cathepsins B and L, trypsin, and chymotrypsin-like proteases (12). In contrast, classic caspase inhibitors blocked TG-induced caspase activation and apoptosis (28). Caspase activation, as measured by pro-caspase cleavage via Western blot analyses, does not occur following \(\beta\)-Lap exposures. Thus, protease activation after \(\beta\)-Lap treatment appears to be Ca\(^{2+}\)-dependent, or alternatively, is activated by another protease or event that is Ca\(^{2+}\)-dependent (Figs. 1–6 and Ref. 12).

**Loss of Reducing Equivalents Is Also Necessary for \(\beta\)-Lap-mediated Apoptosis, Similar to Menadione-mediated Apoptosis.—Menadione is a quinone that can be detoxified by NQO1 two-electron reduction. However, menadione can also be reduced through two, one-electron reductions via other cellular reductases (56), thus eliciting menadione's toxic effects. Menadione toxicity, elicited via two, one-electron reductions, exhibited many similarities to \(\beta\)-Lap-mediated, NQO1-dependent, toxicity (5). These included: (a) elevations in cytosolic Ca\(^{2+}\) (57, 58); (b) NAD(P)H depletion (5, 59, 60); (c) ATP depletion (−0.1% control) (61–63); and (d) mitochondrial potential depolarization (64). We previously demonstrated that menadione caused similar substrate proteolysis (53 and atypical PARP cleavage) in NQO1-deficient cells, or at high doses in cells that express NQO1 where detoxification processes were over-ridden (5). The semiquinone form of menadione can undergo spontaneous oxidation to the parent quinone (59, 63, 65, 66); a pattern similar to the futile cycle observed after \(\beta\)-Lap bioactivation by NQO1 (5). Loss of reducing equivalents, such as NADH, due to the futile cycle of menadione may cause inactivation of the electron transport chain with the concomitant loss of mitochondrial membrane potential, and thus, loss of ATP (67, 68). These responses were also observed in MCF-7 cells exposed to \(\beta\)-Lap (Figs. 4 and 5). Extensive mitochondrial Ca\(^{2+}\) accumulation can also mediate mitochondrial depolarization (69, 70). Thus, Ca\(^{2+}\) sequestration may elicit mitochondrial membrane depolarization and consequent ATP depletion in cells exposed to \(\beta\)-Lap. These data further suggest that Ca\(^{2+}\) is necessary for \(\beta\)-Lap-mediated cell death, but other factors are apparently needed for the initiation of the novel execution apoptotic pathway observed in cells treated with this compound.

The rise in intracellular Ca\(^{2+}\) appears to be dependent on the bioactivation of \(\beta\)-Lap by NQO1, suggesting a critical and necessary signaling role for Ca\(^{2+}\) in the downstream apoptotic pathway induced by this drug. These data suggest that DNA fragmentation, mitochondrial membrane depolarization, ATP loss, and apoptotic proteolysis were a consequence of the increase in intracellular Ca\(^{2+}\) levels. Work in our laboratory is focused on elucidating the signaling response(s) that elicits ER Ca\(^{2+}\) release following \(\beta\)-Lap bioactivation by NQO1. The cell

---

3 C. Tagliarino, J. J. Pink, and D. A. Boothman, unpublished results.
death pathway induced by β-Lap is quite distinct from that observed after exposure to TG, and β-Lap-mediated apoptosis exhibited many similarities to menadione-mediated apoptosis. These observations further suggest that early release of Ca\(^{2+}\) from ER stores, as well as influx of Ca\(^{2+}\) from the extracellular milieu are necessary, but not sufficient for the novel apoptotic execution pathway induced by β-Lap. Thus, changes in Ca\(^{2+}\) homeostasis in conjunction with the presumed loss of reducing equivalents are both necessary and sufficient for β-Lap-mediated apoptosis. We propose that development of β-Lap for treatment of human cancers that have elevated NQO1 levels (e.g. breast and lung) is warranted (6). Since most clinical agents used to date kill cells by caspase-dependent and p53-dependent pathways, and many cancers evade death by altering these pathways, development of agents that kill by specific targets (NQO1-mediated) and in p53- and caspase-independent manners are needed.

Acknowledgments—We thank Sara Simmers and Rich Tarin for all their technical help, as well as R. Michael Sramkoski, MT-ASC(P). We are grateful to Dr. William G. Bornmann for synthesizing β-lapachone, and Edmunds Z. Reineks and Philip A. Verhoef for critical review of this manuscript. We are also indebted to Sarah Hildebrand for her enduring support of our research.

REFERENCES

1. Planchon, S. M., Wuerzberger, S., Frydman, B., Witiak, D. T., Hutson, P., Church, D. R., Wilding, G., and Boothman, D. A. (1995) Cancer Res. 55, 3706–3712.

2. Wuerzberger, S., Pink, J. J., Planchon, S. M., Byers, K. L., Bornmann, W. G., and Boothman, D. A. (1998) Cancer Res. 58, 1876–1885.

3. Li, C. J., Wang, C., and Pardue, A. B. (1995) Cancer Res. 55, 3712–3715.

4. Li, C. J., Li, Y. Z., Pinto, A. V., and Pardue, A. B. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 13369–13374.

5. Pink, J. J., Planchon, S. M., Taglierino, C., Yarnes, M. E., Siegel, D., and Boothman, D. A. (2000) J. Biol. Chem. 275, 5416–5424.

6. Marin, A., Lopez de Cerain, A., Hamilton, E., Lewis, A. D., Martinez-Penuela, E. S., and Wang, X. (1997) Cell 89, 479–489.

7. Patel, T., Gores, G. J., and Kaufmann, S. H. (1996) FASEB J. 10, 232–239.

8. Beigt, R. D., and Dubayk, G. R. (2000) J. Immunol. 165, 7189–7198.

9. Tang, D., Lihti, J. M., and Kidd, V. J. (2000) J. Biol. Chem. 275, 9303–9307.

10. Kim, J. E., Oh, J. H., Choi, W. S., Chang, I. G., Sihn, S., Krajewski, S., Reed, J. C., and O’Malley, K. L. (1998) J. Neurochem. 72, 2456–2463.

11. Kurokawa, H., Nishio, K., Fukushima, H., Tomonari, A., Suzuki, T., and Sajii, N. (1999) Oncol. Rep. 6, 33–37.

12. Yigit, Y., and Tsien, R. Y. (1999) J. Gen. Physiol. 109, 703–715.

13. Cossarizza, A., Baracani-Contri, M., Kalashnikova, G., and Franceschi, C. (1999) Biochem. Biophys. Res. Commun. 197, 49–55.

14. Sasaki, S., Ardizzone, A., Franceschi, C., and Cossarizza, A. (1997) FEBS Lett. 411, 77–82.

15. Inai, Y., Yahuki, M., Kanno, T., Akiyama, J., Yasuda, T., and Utsumi, K. (1997) Cell Struct. Funct. 22, 555–562.

16. Holmander, P. M., and Ermst, L. (1975) Arch. Biochem. Biophys. 160, 560–567.

17. Hosoda, S., Nakamura, W., and Hayashi, K. (1974) J. Biol. Chem. 249, 6416–6423.

18. Distelhorst, C. W., and McCormick, T. S. (1996) Cell Calcium 19, 473–483.

19. Waring, P., and Beaver, J. (1996) Exp. Cell Res. 227, 264–276.

20. Qi, X. M., He, H., Zhong, H., and Distelhorst, C. W. (1997) Oncogene 15, 1207–1212.

21. Mann, S. K., Gad, Y. P., Mukhopadhyay, A., and Aggarwal, B. B. (1999) Biochem. Pharmacol. 57, 763–774.

22. Iyano, T., and Yamazaki, I. (1970) Biochim. Biophys. Acta 176, 282–294.

23. Jewell, S. A., Bellomo, G., Thor, H., Orrenius, S., and Smith, M. (1982) Science 217, 1257–1259.

24. Nicotera, P., McConkey, D., Svensson, S. A., Bellomo, G., and Orrenius, S. (1988) Toxicology 52, 55–63.

25. Di Monte, D., Bellomo, G., Thor, H., Nicotera, P., and Orrenius, S. (1984) Arch. Biochem. Biophys. 235, 343–350.

26. Smith, P. F., Alberts, D. W., and Rush, G. F. (1987) Toxicol. Appl. Pharmacol. 91, 345–349.

27. Akman, S. A., Durachow, J. H., Dietrich, M. F., Chlebovskii, R. T., and Block, J. S. (1987) J. Biol. Chem. Exp. Ther. 240, 486–491.

28. Hemscheidt, H. M., Svensson, S. A., Balld, C., and Orrenius, S. (1985) Eur. J. Immunol. 149, 201–206.

29. Reedegel, F. A., Moison, B. M., Koster, A. S., and Noordhoek, J. (1989) Arch. Biochem. Biophys. 273, 215–222.

30. Saxena, K., Henry, T. R., Solen, L. E., and Wallace, K. B. (1995) Arch. Biochem. Biophys. 317, 79–84.

31. Mirabelli, F., Salis, A., Marinoni, V., Finardi, G., Bellomo, G., Thor, H., and Orrenius, S. (1988) Arch. Biochem. Biophys. 264, 261–269.

32. Frei, B., Winterhalter, K. H., and Richter, C. (1986) Biochemistry 25, 438–443.

33. Reedegel, F. A., Moison, R. M., Barentsen, H. M., Koster, A. S., and Noordhoek, J. (1990) Arch. Biochem. Biophys. 280, 130–136.

34. Bellomo, G., Jewell, S. A., and Orrenius, S. (1982) J. Biol. Chem. 257, 11558–11562.

35. Ackerman, K. E. (1978) Biochim. Biophys. Acta 502, 359–366.

36. Budd, S. L., Tenneti, L., Lishnak, T., and Lipton, S. A. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 6161–6166.
