Calcium-dependent Modulation of Poly(ADP-ribose) Polymerase-1 Alters Cellular Metabolism and DNA Repair

Melissa S. Bentle, Kathryn E. Reinicke, Erik A. Bey, Douglas R. Spitz, and David A. Boothman

From the Departments of Pharmacology and Biochemistry, Case Western Reserve University, Cleveland, Ohio 44106, the Department of Pharmacology, Laboratory of Molecular Stress Responses, and the Simmons Comprehensive Cancer Center, University of Texas Southwestern Medical Center, Dallas, Texas 75390, and the Department of Radiation Oncology, Free Radical and Radiation Biology Program, Holden Comprehensive Cancer Center, University of Iowa, Iowa City, Iowa 52242

After genotoxic stress poly(ADP-ribose) polymerase-1 (PARP-1) can be hyperactivated, causing (ADP-ribose)ation of nuclear proteins (including itself), resulting in NAD⁺ and ATP depletion and cell death. Mechanisms of PARP-1-mediated cell death and downstream proteolysis remain enigmatic. β-lapachone (β-lap) is the first chemotherapeutic agent to elicit a Ca²⁺-mediated cell death by PARP-1 hyperactivation at clinically relevant doses in cancer cells expressing elevated NAD(P)H:quinone oxidoreductase 1 (NQO1) levels. β-lap induces the generation of NQO1-dependent reactive oxygen species (ROS), DNA breaks, and triggers Ca²⁺-dependent γ-H2AX formation and PARP-1 hyperactivation. Subsequent NAD⁺ and ATP losses suppress DNA repair and cause cell death. Reduction of PARP-1 activity or Ca²⁺ chelation protects cells. Interestingly, Ca²⁺ chelation abrogates hydrogen peroxide (H₂O₂), but not N-Methyl-N′-nitro-N-nitrosoguanidine (MNNG)-induced PARP-1 hyperactivation and cell death. Thus, Ca²⁺ appears to be an important co-factor in PARP-1 hyperactivation after ROS-induced DNA damage, which alters cellular metabolism and DNA repair.

Alterations in the initiation and regulation of caspase-mediated apoptosis are associated with an array of pathological disease states, including chemotherapy resistance in cancer (1). Therefore, elucidating mechanisms that initiate non-caspase-mediated cell death are crucial for the development and use of novel anticancer agents.

A growing number of chemotherapeutic approaches focus on targeting specific DNA repair enzymes. In particular, inhibitors of poly(ADP-ribose) polymerase-1 (PARP-1) that sensitize cells to DNA-damaging agents are under extensive investigation (2). PARP-1 functions as a DNA damage sensor that responds to both single- and/or double-strand DNA breaks (SSBs, DSBs), facilitating DNA repair and cell survival. After binding to DNA breaks, PARP-1 converts β-NAD⁺ (NAD⁺) into polymers of branched or linear poly(ADP-ribose) units (PAR) and attaches them to various nuclear acceptor proteins, including XRCC1, histones, and PARP-1 for its autoregulation (3). However, in response to extensive DNA damage, PARP-1 can be hyperactivated, eliciting rapid cellular NAD⁺ and ATP pool depletion. PARP-1-mediated NAD⁺ and ATP losses have affects on mitochondrial function by decreasing the levels of pyruvate and NADH. Loss of mitochondrial membrane potential (MMP) ensues, causing caspase-independent cell death by as yet unknown mechanisms (3). PARP-1 hyperactivation was documented in the cellular response to trauma, such as ischemia-reperfusion, myocardial infarction, and reactive oxygen species (ROS)-induced injury (3). In each case, inhibition of PARP-1 was necessary for the long-term survival of damaged cells (4).

β-lapachone (β-lap) elicits a unique cell death process in various human breast, lung, and prostate cancers that have elevated levels of the two-electron oxidoreductase, NAD(P)H:quinone oxidoreductase 1 (NQO1) (EC 1.6.99.2) (5). β-lap induces an NQO1-dependent form of cell death wherein PARP-1 and p53 proteolytic cleavage fragments were noted (6), concomitant with μ-calpain activation (7). β-lap-induced lethality and proteolysis were abrogated by dicumarol (an NQO1 inhibitor), and were muted in cells deficient in NQO1.
enzymatic activity (5). Restoration of NQO1 caused increases in drug sensitivity (5). In contrast to staurosporine (STS), global caspase inhibitors had little effect on β-lap lethality (5). β-lap-mediated cell death exhibited classical features of apoptosis (e.g., DNA condensation, trypan blue exclusion, sub-G0, G1, and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive cells). β-lap cell death was not, however, dependent on typical apoptotic mediators, such as p53 or caspases (8). To date, the mechanisms responsible for the initiation of this unique cell death have not been delineated.

We report that β-lap induces an NQO1-dependent, PARP-1-mediated cell death pathway involving changes in cellular metabolism leading to cell death. NQO1-dependent reduction of β-lap results in a futile redox cycle between the parent β-lap molecule and its hydroquinone form (5), wherein ROS generation causes extensive DNA damage, H2AX phosphorylation (γ-H2AX) and PARP-1 hyperactivation. Decreases in NAD+ and ATP pools, in turn, inhibit DNA repair and accelerate cell death. In addition, chelation of intracellular Ca2+ by 1,2-bis-(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetra-acetoxymethyl ester (BAPTA-AM) abrogates β-lap-induced: (i) γ-H2AX formation, (ii) PARP-1 hyperactivation, (iii) atypical PARP-1 and p53 proteolysis, and (iv) cytotoxicity without affecting NQO1-dependent ROS production. A similar Ca2+-sensitive cell death is observed after hydrogen peroxide (H2O2) exposure. Interestingly, N-methyl-N′-nitro-N-nitrosoguanidine (MNNG)-induced PARP-1 hyperactivation is not sensitive to BAPTA-AM. These data support a critical role for Ca2+ as a regulator of cellular metabolism in response to ROS-induced DNA damage.

**EXPERIMENTAL PROCEDURES**

**Reagents**—β-lap was synthesized by Dr. William G. Bornmann (MD Anderson), dissolved in dimethyl sulfoxide (Me2SO) at 40 mM, and the concentration verified by spectrophotometric analyses (8). β-lap stocks were stored at −80 °C. Hoechst 33258, 3-aminobenzamide (3-AB), dicoumarol (DIC), H2O2, STS, and MNNG were obtained from Sigma. BAPTA-AM was dissolved in Me2SO and used at 5 μM unless otherwise stated. DPQ (3,4-dihydroxy-5-[1-piperidinyl]butoxy)-1(2H)-isoquinoline was dissolved in Me2SO and used at 20 μM. DPQ and BAPTA-AM were obtained from Calbiochem (La Jolla, CA). Z-VAD-fmk was obtained from Enzyme Systems Products (Dublin, CA). Different dilutions of 1:100–1:500 and purchased from Bethyl Laboratories (Montgomery, TX) and Upstate (Charlottesville, VA), respectively. An NQO1 antibody was generated and used directly for immunoblot analyses in medium containing 10% fetal bovine serum, 1× phosphate-buffered saline, and 0.2% Tween 20 (10).

**Cell Culture**—Human MCF-7 and MDA-MB-468-NQ+ breast cancer cells were maintained and used as described (5). Human MDA-MB-231 (231) breast cancer cells that contain a 609C→T polymorphism in NQO1 (9) and are deficient in enzyme activity, were obtained from the American Type Culture Collection (Manassas, VA). Cells were stably transfected with a CMV-driven NQO1 cDNA or the pcDNA3 vector alone as described (5). All cells were grown in high glucose-containing RPMI 1640 tissue culture medium containing 5% fetal bovine serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin at 37 °C in a 5% CO2, 95% air humidified atmosphere (6). 231- נQO1+ (NQ+) and −NQO1 (NQ−) cells were maintained in medium containing 400 μg/ml geneticin (8), but all experiments were performed without selection. All tissue culture components were purchased from Invitrogen (Carlsbad, CA) unless otherwise stated. All cells were routinely tested and found free of mycoplasma contamination.

**PARP-1 Knockdown**—A puromycin-selectable PS-HAG-MAGIC2 retroviral vector containing a short hairpin small interfering RNA against PARP-1 (PARP-1-shRNA) and a non-silencing sequence (ns-shRNA) control were used to infect both NQ+ and NQ− 231 cells. Cells were then selected and grown in 0.5 μg/ml puromycin and screened for PARP-1 protein expression and NQO1 enzymatic activity.

**Relative Survival Assays**—Relative survival assays were performed as previously described (5). MCF-7 cells were pretreated or not with BAPTA-AM (5 μM, 30 min) then treated with or without 2-h pulses of β-lap at the doses indicated, in the presence or absence of 40 μM dicoumarol. In some experiments, cells were exposed to 5 μM β-lap followed by delayed (t = 0–2 h) addition of 5 μM BAPTA-AM. After drug addition, media were removed and drug-free media added. Cells were then allowed to grow for an additional 6 days and relative survival, based on DNA content (Hoechst 33258 staining), was determined (5). Prior studies using β-lap showed that relative survival assays correlated directly with colony forming ability assays (5). Data were expressed as treated/control (T/C) from separate triplicate experiments (means, ± S.E.), and comparisons analyzed using a two-tailed Student’s t test for paired samples.

**Immunoblotting and Confocal Microscopy**—Western blots were prepared as previously described (8). α-PARP (sc-8007) and α-p53 (DO-1) antibodies were utilized at dilutions of 1:1000 (Santa Cruz Biotechnology). The α-PAR antibody was used at 1:2000 dilution (BD-Pharmingen, San Jose, CA). Antibodies to total levels of H2AX or γ-H2AX were used at dilutions of 1:100–1:500 and purchased from Bethyl Laboratories (Montgomery, TX) and Upstate (Charlottesville, VA), respectively. An NQO1 antibody was generated and used directly for immunoblot analyses in medium containing 10% fetal bovine serum, 1× phosphate-buffered saline, and 0.2% Tween 20 (10).

Confocal microscopy was performed as previously described (7). Cells were fixed in methanol/acetone (1:1) and incubated with α-PAR (10H; Alexis, San Diego, CA) or α-γ-H2AX (Trevigen, Gaithersburg, MD) for 2 h at room temperature. Nuclei were visualized by Hoechst 33258 staining at a 1:3000 dilution. Confocal images were collected at 488 nm excitation from a krypton/argon laser using a Zeiss LSM 510 confocal microscope (Thornwood, NY). Images were representative of experiments performed at least four times. The number of PAR-positive cells and γ-H2AX foci/cell were quantified by counting 60 or more cells from four independent experiments (means ± S.E.).

Formation of ROS was monitored by the conversion of non-fluorescent 6-carboxy-2’,7’-dichlorodihydrofluorescin diacetate, di(acetoxymethyl ester) to fluorescent 6-carboxy-2’,7’-dichlorofluorescein diacetate di(acetoxymethyl ester) (DCF) as previously described (11, 12). Briefly, MCF-7 cells were seeded

Ca2+-mediated PARP-1 Hyperactivation
at 2–3 × 10^5 cells in 35-mm glass bottom Petri dishes (MatTek Corp., Ashland, MA) and allowed to attach overnight. Cells were loaded with 5 μM DCF in media for 30 min at 37 °C. After loading, cells were washed twice with phosphate-buffered saline, and incubated for an additional 20 min at 37 °C to allow for dye de-esterification. Confocal images of DCF fluorescence were collected using 488-nm excitation from an argon/krypton laser, 560-nm dichroic mirror, and a 500–550 nm band pass filter. Three basal images were collected before drug addition (5–8 μM β-lap, +5 μM BAPTA-AM or 40 μM dicoumarol). Subsequent images were taken after the indicated treatments at 15-s intervals and similar results were found at 37 °C or rm. temp. BAPTA-AM was co-loaded with DCF where indicated. Mean pixel intensities were determined in regions of interest for at least 40 individual cells at each time point. Shown are representative traces of at least three independent experiments (means ± S.E.).

Single Cell Gel Electrophoresis (Alkaline Comet) Assays—DNA damage was assessed after different drug treatments by evaluating DNA “comet” tail area and migration distance (13). MCF-7 cells were pretreated with BAPTA-AM (5 μM, 30 min) or Me2SO (1:1000 dilution), and then exposed to H2O2 (500 μM, 1 h), β-lap (4 μM, various times), or vehicle alone, and harvested at various times. Cell suspensions (3 × 10^6/ml cold PBS) were mixed with 1% low melting temperature agarose (1:10 (v/v)) at 37 °C and immediately transferred onto a CometSlide™ (Trevisen). After solidifying (30 min at 4 °C), slides were submerged in prechilled lysis buffer (2.5 M NaCl, 100 mM EDTA pH 10, 10 mM Tris Base, 1% sodium lauryl sarcosinate, and 1% Triton X-100) for 45 min, incubated in alkaline unwinding solution (300 mM NaOH, and 1 mM EDTA) for 45 min at room temperature and washed twice (5 min) in neutral 1× TBE (89.2 mM Tris Base, 89 mM boric acid, and 2.5 mM EDTA disodium salt). Damaged and undamaged nuclear DNA was then separated by electrophoresis in 1× TBE for 10 min at 1 V/cm, fixed in 70% ethanol, and stained using SYBR-green (Trevisen). Comets were visualized using an Olympus fluorescence microscope (Melville, NY), and images captured using a digital camera. Images were analyzed using ImageJ software (14, 15) and comet tail length was calculated as the distance between the end of nuclei heads and the end of each tail. Tail moments were determined in regions of interest for at least 40 individual cells at each time point. Shown are representative traces of at least three independent experiments (means ± S.E.).

\[
\text{TA} = \frac{T_A \times T_A}{TA} = \frac{TA \times T_A}{100/(TA \times T_A)} + [H_A \times H_A] 
\]  

(Eq. 1)

where TA is the tail area, T_A is the tail area intensity, HA is the head area, and HAI is the head area intensity. Importantly, tail moment and tail area calculations yielded similar experimental results. Each datum point represented the average of 100 cells ± S.E., and data are representative of experiments performed three times.

Determination of NAD⁺ and ATP Levels—Intracellular NAD⁺ levels were measured as described (16) with modifications. Briefly, cells were seeded at 1 × 10^5 and allowed to attach overnight. Cells were pretreated for 2 h with 3-AB (25 mM), DPQ (20 μM), BAPTA-AM (5 μM), or Me2SO and then exposed to β-lap (2–20 μM) for the indicated times. Cell extracts were prepared in 0.5 M perchloric acid, neutralized (1 M KOH, 0.33 M KH2PO4/K2HPO4 (pH 7.5)), and centrifuged to remove KClO4 precipitates. Supernatants or NAD⁺ standards were incubated 4:1 (v/v) for 20 min at 37 °C with NAD⁺ reaction mixture as described (17). Measurements from extracts were taken at an absorbance of 570 nm and intracellular NAD⁺ levels were normalized to 1 × 10^6 cells. Data were expressed as %NAD⁺ ± S.E., for T/C samples from nine individual experiments.

ATP levels were analyzed using a luciferase-based bioluminescence assay as described (18). Data were graphed as means ± S.E. of experiments performed at least three times. Results were compared using the two-tailed Student’s t test for paired samples.

NQO1 Enzyme Activity Assays—NQO1 enzymatic assays were performed as described (19) using cytochrome c (practical grade, Sigma) in Tris-HCl buffer (50 mM, pH 7.5). NADH (200 μM) was the immediate electron donor, and menadione (10 μM) the electron acceptor. Changes in absorbance were monitored using a Beckman DU 640 spectrophotometer (Beckman Coulter, Fullerton, CA). Dicoumarol (10 μM) inhibitable NQO1 levels were calculated as nmol of cytochrome c reduced/min/μg protein based on initial rate of change in absorbance at 550 nm and an extinction coefficient for cytochrome c of 21.1 nmol/liter/cm (20). Results were expressed as means ± S.E. of three or more separate experiments.

Flow Cytometry and Apoptotic Measurements—Flow cytometric analyses of TUNEL-positive cells were performed as described using APO-DIRECT™ (BD Pharmingen) (5). Samples were analyzed in an EPICS Elite ESP flow cytometer using an air-cooled argon laser at 488 nm, 15 milliwatt (Beckman Coulter Electronics, Miami, FL) and Elite acquisition software. Experiments were performed a minimum of five times, and data expressed as means ± S.E. Statistical analyses were performed using a two-tailed Student’s t test for paired samples.

Glutathione Measurements—Disulfide glutathione and total glutathione (GSH + GSSG) levels were determined using a spectrophotometric recycling assay (21, 22). Following indicated treatments, pellets were thawed and whole cell homogenates prepared as described (21, 22). Data were expressed as the %GSSG normalized to protein content, as measured using the method of Lowry et al. (23). Shown are means ± S.E. for experiments performed at least three times.

RESULTS

Time and Ca²⁺ Dependence of β-lap-induced Cell Death—To elucidate the signaling events required for β-lap-induced cell death, log-phase human MCF-7 breast cancer cells, with high levels of endogenous NQO1 activity, were tested for their sensitivities to various concentrations of β-lap at various times. The purpose of these experiments was to determine the minimal time of β-lap exposure required to kill the entire cell population. Cells exposed to doses of ≤3 μM β-lap required ≥4 h to elicit cell death, whereas 2 h exposures of β-lap at ≥4 μM killed all MCF-7 cells (Fig. 1A).

Prior data from our laboratory demonstrated that Ca²⁺ was released within 2–5 min from endoplasmic reticulum (ER)
Ca\(^{2+}\) stores after β-lap treatment (24), suggesting that this was a required initiating factor in β-lap-induced cell death (24). To test this, MCF-7 cells were pretreated with the intracellular Ca\(^{2+}\) chelator BAPTA-AM (5 μM, 30 min), then exposed to 4 μM β-lap for various times (Fig. 1B). Under these conditions, we previously demonstrated that BAPTA-AM pretreatment was sufficient to block the rise in cytosolic Ca\(^{2+}\) caused by β-lap treatment (24). BAPTA-AM abrogated β-lap-induced cytotoxicity and nuclear condensation (Fig. 1B and supplemental Fig. S1A). To determine the kinetics of Ca\(^{2+}\)-dependent cell death, MCF-7 cells were treated with 5 μM β-lap, and 5 μM BAPTA-AM was added at various times thereafter, up to 2 h. A time-dependent decrease in survival was observed with delayed addition of BAPTA-AM (Fig. 1C), indicating that Ca\(^{2+}\) release was a necessary event, occurring before cells were committed to death. Abrogation of β-lap cytotoxicity by BAPTA-AM was equivalent to that noted with NQO1 inhibition by dicoumarol (Fig. 1B). Addition of BAPTA-AM also prevented β-lap-induced, atypical proteolysis (e.g. ~60 kDa PARP-1 and p53 cleavage fragments), in a manner as effective as dicoumarol (supplemental Fig. S1B, lanes 3 and 4). Interestingly, Z-VAD-fmk (50 μM), a pan-caspase inhibitor, did not block atypical PARP-1 and p53 proteolysis in β-lap-treated MCF-7 cells (lane 7). As expected, Z-VAD-fmk inhibited STS (1 μM)-induced caspase-mediated proteolysis (25). However, BAPTA-AM did not affect STS-induced apoptotic proteolysis (supplemental Fig. S1C). These data, in conjunction with our prior data showing β-lap-induced ER Ca\(^{2+}\) release (24), support a role for Ca\(^{2+}\) in the initiation of cell death induced by this drug.

PARP-1 Hyperactivation after β-lap Treatment Is NQO1-dependent and BAPTA-AM-sensitive—Because β-lap-induced cell death was accompanied by a ≥80% loss of ATP within 1 h (24), we suspected PARP-1 hyperactivation played a role in the mode of action for this drug. To investigate this, we generated 231 NQO1-proficient (231-NQ+) cells that are sensitive to β-lap (LD\(_{50}\); ~1.5 μM), after a 2-h pulse, and compared these cells to vector alone, 231 NQO1-deficient (231-NQ-) cells, that are resistant to the drug (LD\(_{50}\); 17.5 μM). Only β-lap-treated, 231-NQ+ cells exhibited an increase in PAR-modified proteins, mostly PARP-1, consistent with the role of PARP-1 as the predominant poly(ADP-ribose)ylated species. This response peaked ~30 min during β-lap exposure (Fig. 2A). In contrast, treatment of 231-NQ- cells with equal or significantly higher doses of β-lap did not induce PAR accumulation (data not shown). In contrast, both 231-NQ+ and 231-NQ- cells hyperactivated PARP-1 in response to H\(_2\)O\(_2\). The NQO1-dependence of PARP-1 hyperactivation after β-lap exposure was confirmed in a number of other cell lines (e.g. breast, prostate, and lung cancers) that have elevated NQO1 activity demonstrating that the responses to β-lap were not cell type-specific. In all cases, dicoumarol suppressed β-lap-induced PAR formation (supplemental Fig. S2, A–C).

We then examined a possible connection between the involvement of Ca\(^{2+}\) in lethality and PARP-1 hyperactivation. MCF-7 cells were pretreated with 5 μM BAPTA-AM, then exposed to 5 μM β-lap for the indicated times (Fig. 2B). The kinetics of PAR accumulation were faster in MCF-7 cells than in 231-NQ+ cells, (10 min versus 20 min Figs. 2, B and A,
FIGURE 2. β-lap induces NQO1- and Ca2+-dependent PARP-1 hyperactivation. A, immunoblots of PAR formation as a measure of PARP-1 hyperactivation, and steady-state PARP-1 protein levels from whole cell extracts of isogenic 231-NQ+ and 231-NQ− cells treated with 6 μM β-lap for 10–90 min. Relative PAR levels were calculated by densitometric analyses by NIH ImageJ using PARP loading controls wherein controls were set to 1.0. B, immunoblots of PAR formation and steady state α-tubulin expression in extracts from MCF-7 cells treated with β-lap or H2O2 (2 mM, control for PARP-1 hyperactivation). Other cells were pretreated with BAPTA-AM with or without β-lap (5 μM). Samples were harvested at the indicated times. Relative PAR levels were calculated by densitometric analyses by NIH ImageJ using α-tubulin loading controls wherein controls were set to 1.0. C, assessment of PARP-1 hyperactivation, measured by PAR formation, in MCF-7 cells treated with β-lap alone or in cells pretreated with BAPTA-AM for 30 min prior to β-lap exposure. PAR formation was visualized using confocal microscopy. D, quantified percentages of PAR-positive cells from confocal microscopy analyses of at least 60 cells from four independent experiments (means ± S.E.).
were monitored using a luciferase-based bioluminescence assay in MCF-7 cells treated with 3-AB or DPQ. The exposure and dissipation between 60–90 min (Fig. 2, C and D). Robust and extensive poly(ADP-ribose)lation of PARP-1, and efficient PAR hydrolysis by poly(ADP-ribose) glycohydrolase (PARG) (26). Interestingly, BAPTA-AM pretreatment abrogated PARP-1 hyperactivation induced by β-lap (Fig. 2B) as confirmed by confocal microscopy (Fig. 2C). Robust and extensive poly(ADP-ribosyl)ation occurred within 30 min (87% ± 17 PAR-positive cells) of drug exposure and dissipated between 60–90 min (Fig. 2, C and D). NQO1 levels in MCF-7 cells were exposed to varying concentrations of β-lap for 1 h, or β, 5 μM β-lap alone with or without pre- and co-treatment of PARP inhibitors, (20 μM DPQ or 25 μM 3-AB) or 5 μM BAPTA-AM for various times and harvested for NAD⁺ content. Student’s t test for paired samples, comparing experimental groups containing β-lap + 3-AB or DPQ versus β-lap alone are indicated (*, p ≤ 0.001; **, p ≤ 0.05, respectively). C, intracellular ATP levels were monitored using a luciferase-based bioluminescence assay in MCF-7 cells treated with β-lap, or in cells pre- and co-treated with 20 μM DPQ, or 25 μM 3-AB 2 h prior to β-lap addition. Differences were compared using two-tailed Student’s t test. Groups having *, p ≤ 0.05 values compared with β-lap alone are indicated.

D, apoptotic DNA fragmentation was assessed using TUNEL assays in β-lap-exposed, log-phase MCF-7 cells with or without pre- and co-treatment with DPQ or 3-AB. However, in the presence of BAPTA-AM PAR accumulation in β-lap-treated MCF-7 cells was prevented (Fig. 2, C and D). To determine the global nature of this response, other cancer cell lines such as NQO1+ PC-3 human prostate cancer cells were examined and similar responses noted (supplemental Fig. S2D). Collectively, these data suggested a role for Ca²⁺ in the modulation of PARP-1 hyperactivation after β-lap exposure. β-lap-induced PARP-1 Hyperactivation Alternates Cellular Energy Dynamics Causing Cell Death—PARP-1 hyperactivation can elicit depletion of cellular NAD⁺ levels and cause cell death in situations of extreme DNA damage or ischemia-reperfusion (27, 28). Treatment of MCF-7 cells with doses of β-lap ≥5 μM resulted in decreases (>80%) in NAD⁺ and ATP levels 1 h during treatment (Fig. 3, A–C). To determine if NAD⁺ and ATP losses were attributable to PARP-1 hyperactivation, MCF-7 cells were pretreated for 2 h with PARP inhibitors (i.e. 3-AB or DPQ), prior to 5 μM β-lap exposure. Similar to pretreatment with BAPTA-AM, NAD⁺ and ATP losses in β-lap-treated MCF-7 cells were abrogated by 3-AB or DPQ (Fig. 3, B and C). 3-AB was more effective at preventing nucleotide loss than DPQ presumably because it has two distinct modes of PARP-1 inhibition, preventing NAD⁺ binding to the catalytic site and competing with the PARP-1 DNA binding domain (29), whereas DPQ is a competitive inhibitor of NAD⁺ (30). Similar effects of DPQ on NAD⁺ and ATP losses after DNA damage have been reported (31). Neither 3-AB nor DPQ (used at >2-fold higher doses than in the above experiments) altered NQO1 activity in enzyme assays in vitro. Finally, 3-AB did not affect β-lap-induced ROS formation (data not shown).

To confirm that the energetic consequences of PARP-1 hyperactivation (e.g. NAD⁺ and ATP losses) were necessary and sufficient for β-lap-induced cell death, the effects of 3-AB or DPQ, on apoptosis, was measured by TUNEL assay. Pretreatment with either inhibitor resulted in a reduction in apoptosis (2% and 15% total apoptosis, respectively for 3-AB and DPQ) compared with 55% in β-lap-treated cells (Fig.
3D). Thus, PARP-1 inhibition by 3-AB or DPQ spared β-lap-induced apoptosis in NQO1+ MCF-7 cells, consistent with the effects of these inhibitors to prevent NAD+ and ATP losses. Cumulatively, these data strongly suggest that Ca2+-dependent PARP-1 hyperactivation caused NAD+ and ATP β-lap treatment causes DNA damage as assessed by alkaline or neutral filter elution, p53 induction, or covalent complex protein-DNA formation (8, 32, 33). However, many of these prior studies were performed in cells expressing little to no NQO1 (34). PARP-1 hyperactivation typically requires DNA damage,
BAPTA-AM exhibited less DNA damage compared with drofluorescein (DCF). Indeed, exposure of MCF-7 cells with 5 μM BAPTA-AM on drug exposure (Fig. 6A), rise in disulfide glutathione (GSSG) levels, that persisted during respectively) (Fig. 5C). Importantly, β-lap-induced γ-H2AX foci formation was partially abrogated by BAPTA-AM addition, with fewer γ-H2AX foci noted in 30–90 min (~25 foci/cell respectively) (Fig. 5C). These results were confirmed by immunoblot analyses (Fig. 5D).

β-lap Chelation Modulates DNA Repair after β-lap Treatment—We postulated that metabolism of β-lap by NQO1 would generate superoxide, peroxide, and other ROS (36). We directly monitored intracellular ROS formation, using the conversion of non-fluorescent 6-carboxy-2',7'-dichlorodihydrofluorescein to fluorescent 6-carboxy-2',7'-dichlorodihydrofluorescein (DCF). Indeed, exposure of MCF-7 cells with 5 or 8 μM β-lap treatment, caused an increase in fluorescence within 5 min compared with Me3SO-treated cells (Fig. 6A, left panel). Region-of-interest analyses showed an ~2000-fold increase in fluorescence with β-lap alone over control cells, which could be abrogated by inhibiting NQO1 activity with dicoumarol (Fig. 6A, right panel). Because BAPTA-AM has moderate affinity for divalent cations other than Ca(2+), we explored the possibility that BAPTA-AM may protect cells from DNA damage and subsequent cell death by interfering with Fenton chemistry. Cells pretreated with 5 μM BAPTA-AM and then exposed to 5 or 8 μM β-lap exhibited no significant difference in the rate or extent of ROS formation compared with β-lap alone-treated cells (Fig. 6A). These results were confirmed by examining the oxidative state of MDA-MB-468-NQ+ cells after treatment with 4 μM β-lap in the presence or absence of 5 μM BAPTA-AM. β-lap treatment caused an ~65% rise in disulfide glutathione (GSSG) levels, that persisted during drug exposure (Fig. 6B). Addition of BAPTA-AM did not alter the kinetics or levels of GSSG formation during β-lap exposure (Fig. 6B). These data suggest that the protective effects of BAPTA-AM on β-lap-treated NQO1+ cells were not caused by interference with β-lap-induced ROS formation. Similar results were found in 231-NQ- cells (data not shown).

To assess the effects of Ca(2+) on DNA damage and repair, β-lap-treated MCF-7 cells were analyzed by alkaline comet assays to monitor total DNA strand breaks with or without BAPTA-AM addition. β-lap-treated cells exhibited significant DNA strand breakage by 10 min, resembling the positive control (H2O2), and after 30 min, β-lap-induced DNA damage exceeded those levels (Fig. 6C and supplemental Fig. S3). Cells pretreated with BAPTA-AM exhibited less DNA damage compared with β-lap alone, and their repair of DNA damage correlated well with their ability (or lack thereof) to survive (Figs. 6C and 1B).

We then examined the kinetics of repair in MCF-7 cells following a 2-h β-lap exposure with or without BAPTA-AM pretreatment. After β-lap exposure, DNA damage persisted and gradually increased over time (Fig. 6C), indicative of inhibition of DNA repair and consistent with the drop in NAD+ and ATP levels (Fig. 3, B and C). Although cells treated with β-lap and BAPTA-AM at 2 h exhibited equivalent damage to 10 min of β-lap exposure alone (4.6 ± 0.2 versus 4.7 ± 0.4, p > 0.5 comet microns, respectively), BAPTA-AM pretreated cells were protected from PARP-1 hyperactivation (Fig. 2B), as well as decrements in NAD+ levels (Fig. 3B). BAPTA-AM pretreated cells showed a time-dependent recovery from DNA damage (Fig. 6C and supplemental Fig. S3). In contrast, β-lap-exposed cells showed extensive DNA damage with no signs of DNA repair. Collectively, these data suggest that NQO1-mediated metabolism of β-lap leads to the generation of ROS and subsequent DNA damage that hyperactivates PARP-1.

H2O2 Causes Ca(2+)-dependent PARP-1 Hyperactivation—To examine the universality of Ca(2+)-modulated PARP-1 function in response to other DNA damaging agents, we examined responses to H2O2 or MNNNG. Unlike β-lap, H2O2 treatment caused PARP-1 hyperactivation in both 231-NQ+ and 231-NQ- cells (Fig. 7A). However, expression of NQO1 required higher doses of H2O2 to cause PAR formation in 231 cells (Fig. 7A). These data suggest that NQO1 has a broader antioxidant role by protecting against ROS-induced damage as previously proposed (37–39). Consistent with β-lap, however, was the abrogation of H2O2-induced PAR formation by BAPTA-AM in 231 cells independent of NQO1 activity (Fig. 7B).

H2O2 treatment also caused a dose-dependent increase in apoptosis in both 231-NQ- and 231-NQ+ cells that was blocked by BAPTA-AM (Fig. 7C). However, 231-NQ+ cells were much less sensitive to H2O2 than 231-NQ- cells. ATP loss was seen within minutes of H2O2 exposure in 231-NQ-, but not in NQO1-positive 231-NQ+ cells (supplemental Fig. S4). In addition, PARP-1 hyperactivation and cell death in response to equivalent doses of H2O2 in 231-NQ- cells was much more robust than in 231-NQ+ cells (Fig. 7B and C). Interestingly, as noted with β-lap exposure, treatment of MCF-7 cells with ≥200 μM H2O2 for 2 h resulted in formation of a 60-kDa PARP-1 and 40-kDa p53 fragments. This atypical proteolysis was effectively inhibited by BAPTA-AM pretreatment (supplemental Fig. S5).

Finally, BAPTA-AM had no effect on PARP-1 hyperactivity or cytotoxicity caused by treatment with the monofunctional DNA-alkylating agent, MNNNG (Fig. 7E). Because MNNNG does not cause Ca(2+) release like β-lap or H2O2, these data suggest that Ca(2+) release is not a general phenomenon. Ca(2+) modulation of PARP-1 hyperactivation is unique to ROS-producing agents.

DISCUSSION

The regulatory mechanisms controlling PARP-1 function to either promote cell survival or cell death in response to DNA damage remain enigmatic. PARP-1 facilitates DNA repair and cell survival in response to a variety of DNA-damaging agents. However, it also mediates programmed necrosis (17), as well as caspase-inde-
FIGURE 5. β-lap-induced γ-H2AX foci formation is abrogated by BAPTA-AM pretreatment. A, immunoblot analyses of γ-H2AX, total H2AX, and α-tubulin protein levels in whole cell extracts from MCF-7 cells exposed to β-lap for various times, or IR (5 Gy) harvested after 15 min. B, visualization of γ-H2AX foci in MCF-7 cells at various times after treatment with 5 μM β-lap or 15 min post-IR (5 Gy) by confocal microscopy. C, BAPTA-AM pretreatment followed by β-lap exposure abrogates γ-H2AX foci formation in MCF-7 cells as visualized by confocal microscopy. The number of γ-H2AX foci per cell was determined from at least 60 cells for each treatment group from four independent confocal experiments (means ± S.E.). D, immunoblot of γ-H2AX, total H2AX, and α-tubulin protein levels in whole cell extracts from MCF-7 cells exposed to β-lap for various times with or without BAPTA-AM (5 μM) pretreatment, or IR (5 Gy) harvested after 15 min.
pendent apoptotic cell death following severe levels of DNA damage (40). The downstream pathways essential for the execution of cell death in response to PARP-1-mediated metabolic alterations remain poorly understood.

In elucidating the cell death pathway after exposure to β-lap, we uncovered a novel mechanism of PARP-1-mediated cell death. Our data suggest that this mechanism occurred selectively in response to ROS-generating agents. We demonstrated, for the first time, that Ca2+-mediated PARP-1 hyperactivation commits cells to death as a consequence of metabolic starvation without the involvement of caspases.

PARP-1 hyperactivation in response to β-lap treatment was not cell type-specific and has been observed in all cells that express elevated NQO1 levels (Fig. 2A, and supplemental Fig. S2, A–C). As a result, cells exposed to β-lap exhibited depletion of NAD+ and ATP, occurring 30–60 min during drug exposure. NAD+ and ATP losses were, in part, PARP-1-mediated since PARP inhibitors (e.g. 3-AB and DPQ) partially abrogated nucleotide loss (Fig. 3B and C). Chemical inhibition of PARP-1, or PARP-1 protein knock-down, not only prevented NAD+ and ATP losses, but also abrogated β-lap-induced apoptosis (Figs. 3D and 4, D–F). These data established PARP-1-mediated NAD+ and ATP losses as crucial upstream events in β-lap-mediated cell death.

PARP-1-mediated alterations in cellular metabolism caused by β-lap reported in NQO1-expressing cells in this study explain many of its purported effects in vitro and in vivo. These include, but are not limited to: (i) inhibition of NFκB activation via inhibition of IKK-α (41), (ii) lack of caspase activation (6) and p53 stabilization (8), and (iii) inhibition of Topoisomerase (Topo) I and Topo II-β (42). Furthermore, β-lap can initiate cell death independently from Bax and/or Bak activation as changes in mitochondrial outer membrane permeabilization (MOMP) can occur via PARP-1-mediated NAD+ loss.3 Thus, the results reported here appear to explain all prior phenomena reported in cells exposed to β-lap.

3 W. X., Zong, E. A. Bey, and D. A. Boothman, unpublished data.
Ca\textsuperscript{2+}-mediated PARP-1 Hyperactivation

A unique feature of PARP-1-mediated cell death stimulated by \(\beta\)-lap was that administration of BAPTA-AM abrogated PARP-1 hyperactivation (Fig. 2B, supplemental Fig. S2D), nucleotide pool loss (Fig. 3B), atypical proteolyses (assessed by \(\gamma\)-H2AX foci formation) (supplemental Fig. S1B), and cell death (Fig. 1, B and C). When BAPTA-AM was added \(>20\) min after \(\beta\)-lap treatment, cells were not protected from cell death (Fig. 1C), suggesting that events occurring within the first \(20\) min of drug exposure committed cells to death. BAPTA-AM (free acid form) did not alter NQO1 activity \textit{in vitro} (24). This appears to be confirmed by the inability of BAPTA-AM to prevent ROS generation, which arises from NQO1-mediated metabolism of \(\beta\)-lap. Instead, our data suggest that the ability of BAPTA-AM to prevent \(\beta\)-lap-induced lethality in NQO1+ cancer cells was caused by the specific prevention of PARP-1 hyperactivation. The observed differences in the amount of DNA damage and \(\gamma\)-H2AX foci formation between \(\beta\)-lap alone and that of \(\beta\)-lap co-administered with BAPTA-AM suggest that preventing PARP-1 hyperactivation and subsequent changes in cellular metabolism can allow for cell recovery, noted by more rapid and extensive DNA damage repair (Figs. S5C and 6C). Recent data suggest that protein phosphatase 2A (PP2A) dephosphorylates \(\gamma\)-H2AX and is required for DSB repair (43). It is possible that Ca\textsuperscript{2+} chelation not only prevents PARP-1 hyperactivation, but also augments \(\gamma\)-H2AX dephosphorylation through PP2A activity. ROS-induced ER Ca\textsuperscript{2+} release may poison PP2A. However, we favor the theory that NQO1-mediated \(\beta\)-lap-induced ER Ca\textsuperscript{2+} release has its predominant affect on PARP-1 hyperactivation, thereby inhibiting DNA repair and cell recovery.

The mechanism of cell death induced by \(\beta\)-lap could be recapitulated by treatment with high doses of ROS-generating agents, such as \(\text{H}_2\text{O}_2\) (Fig. 7, A–D). Notable similarities included: \(\text{H}_2\text{O}_2\)-mediated PARP-1 hyperactivation, \(\text{Ca}^{2+}\)-dependent proteolytic cleavage of PARP-1 and p53, and apoptotic DNA fragmentation. Furthermore, \(\text{H}_2\text{O}_2\)-induced lethality was abrogated by BAPTA-AM (Fig. 7, A–D and supplemental Fig. S5). Although \(\beta\)-lap and \(\text{H}_2\text{O}_2\) initiate a similar downstream death pathway, the compounds differed in their lethality in cells with respect to NQO1 expression. \(\beta\)-lap lethality was enhanced in cells that express NQO1, whereas \(\text{H}_2\text{O}_2\) caused greater cytotoxicity in NQO1-deficient cells (Fig. 7C). We noted striking similarities between \(\beta\)-lap- or \(\text{H}_2\text{O}_2\)-induced cell death and the caspase-independent cell death induced by ischemia-reperfusion. ROS produced during ischemia-reperfusion induces DNA strand breaks beyond a normal threshold that lead to PARP-1 hyperactivation, metabolic catastrophe, and an increase in intracellular \(\text{Ca}^{2+}\) levels leading to \(\mu\)-calpain activation (44). These data suggest that programmed PARP-1-mediated cell death is a global response to these types of cellular insults.

![FIGURE 7. \(\text{H}_2\text{O}_2\) causes \(\text{Ca}^{2+}\)-dependent PARP-1 hyperactivation and cell death. A, PARP-1 hyperactivation after \(\text{H}_2\text{O}_2\) exposure occurs regardless of NQO1 status. Immunoblot analyses of PAR, NQO1, and \(\alpha\)-tubulin protein levels in whole cell extracts from 231-NQ- and 231-NQ+ cells after exposure to varying doses of \(\text{H}_2\text{O}_2\) for 15 min. B, PARP-1 hyperactivation after \(\text{H}_2\text{O}_2\) treatment is \(\text{Ca}^{2+}\)-dependent. 231-NQ- (left) and 231-NQ+ cells (right) were pretreated with BAPTA-AM or vehicle alone for 30 min and then treated with varying doses of \(\text{H}_2\text{O}_2\) and harvested after 15 min. Immunoblots of PAR, and \(\alpha\)-tubulin protein levels from whole cell extracts were then analyzed. C, \(\text{Ca}^{2+}\) chelation protects 231-NQ- and 231-NQ+ cells from \(\text{H}_2\text{O}_2\)-induced apoptosis. TUNEL assays were performed in \(\text{H}_2\text{O}_2\)-exposed, log-phase 231-NQ- and 231-NQ+ cells, with or without pre-treatment with 5 \(\mu\text{M}\) BAPTA-AM. D, MNNG-induced PARP-1 hyperactivation is not blocked by \(\text{Ca}^{2+}\) chelation. Immunoblot analysis of PAR and \(\alpha\)-tubulin protein levels from whole cell extracts of MCF-7 cells treated with MNNG or in cells pretreated for 30 min with BAPTA-AM and then exposed to MNNG are shown. Cells were treated for the indicated times and immediately harvested.](image-url)
PARP-1 hyperactivation was also observed following high doses of MNNG, however, this response was not affected by BAPTA-AM (Fig. 7D). These data highlight two separate PARP-1 regulatory mechanisms. First, ROS-induced, PARP-1-mediated cell death appears to require Ca\(^{2+}\) as a cofactor, whereas alkylated PARP-1-induced cell death does not. We propose that Ca\(^{2+}\) release following ROS-induced stress directly influences PARP-1 and PARG function. Both Mg\(^{2+}\) and Ca\(^{2+}\) exert significant (≥3-fold increases) allosteric activation of PARP-1-auto(ADP-ribosyl)ation in vitro that is inhibited by EDTA addition (45). We, therefore, speculate that Ca\(^{2+}\) chelation modulates PAR synthesis by dampening PARP-1 auto(poly-ADP)-ribosylation. Furthermore, since increases in [Ca\(^{2+}\)] can inhibit PAR function by up to 50% in vitro, maintenance of homeostatic Ca\(^{2+}\) levels after drug treatment would, thereby, restore the normal turnover of PAR by PARG, lifting PARP-1 self-inhibition (46). Our data are consistent with the hypothesis that both PAR synthesis and degradation can be modulated by BAPTA-AM to spare the cell from metabolic catastrophe via Ca\(^{2+}\)-mediated NAD\(^{+}\) and ATP losses (Figs. 2B, and 3, B and C) (47). The remaining PARP-1 activity would be necessary for DNA break repair, ultimately providing a survival advantage to damaged cells (Figs. 1B, 5C, 6C, and 7C). We are currently exploring the mechanism by which Ca\(^{2+}\) modulates PARP-1 hyperactivation and subsequent DNA repair after H\(_2\)O\(_2\) or β-lap treatments versus MNNG.

There appears to be some disagreement as to the role of Ca\(^{2+}\) in PARP-1-dependent cell death. Ca\(^{2+}\) can hyperactivate PARP-1 in the absence of DNA breaks (48). In neuronal cells, glutamate caused Ca\(^{2+}\) -mediated ROS production through mitochondrial dysfunction, leading to DNA damage, PARP-1 hyperactivation, and cell death. Furthermore, Ca\(^{2+}\) chelators, such as BAPTA-AM, EGTA-AM, and Quin-2-AM, protected against other oxidative stress-induced apoptotic and necrotic cell death mechanisms (49, 50). In these studies, Ca\(^{2+}\) chelation did not directly inhibit PARP-1 activity, but rather prevented DNA damage by inhibiting ROS. Contrary to these observations, in our system BAPTA-AM did not alter the direct production of ROS or oxidative stress in H\(_2\)O\(_2\)- or β-lap-exposed cells (Fig. 6A). Therefore, there does not appear to be an interference with transition metal-mediated oxidant production by BAPTA-AM (e.g. Fenton reaction) as previously suggested after H\(_2\)O\(_2\) treatment (51, 52). In fact, our data demonstrate that β-lap caused equivalent ROS production in both β-lap alone and β-lap + BAPTA-AM treated cells. In contrast to β-lap alone-treated cells, cells pretreated with BAPTA-AM did not exhibit notable PARP-1 hyperactivation, associated NAD\(^{+}\) and ATP losses, and showed a decrease in DNA damage over time (supplemental Table S1). While these data are suggestive of ongoing DNA repair in the presence of Ca\(^{2+}\) chelators, we cannot discount that these observations could also be the result of a decrease in the initial amount of DNA damage created in NQO1+ cells in response to β-lap. Initial DNA damage and active DNA repair would be indistinguishable in these experiments. Thus, although we believe it is unlikely, the Ca\(^{2+}\)-dependence of PARP-1 hyperactivation could be an indirect consequence of a BAPTA-AM-mediated decrease (i.e. protection) in the initial amount of DNA lesions created in response to β-lap. Future studies will address this issue by utilizing DNA repair-compromised NQO1+ cells.

Collectively, our data suggest that PARP-1 is necessary for the initiation of cell death caused by β-lap. To date, however, the endonuclease responsible for the execution of cell death in response to β-lap treatment remains unknown. We therefore propose, that PARP-1-mediated NAD\(^{+}\) and ATP losses, in addition to PARG-liberated ADP-ribose, causes an influx of Ca\(^{2+}\) from extracellular and intracellular sources. Impairment of ATP-dependent membrane/organellar transporters (e.g. plasma membrane Ca\(^{2+}\) ATPases (PMCA) and sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\) ATPases (SERCA)) by ATP loss, and activation of plasma membrane cation channels (e.g. transient receptor potential-melastatin-like (TRMP)) by ADP-ribose, leads to high intracellular Ca\(^{2+}\) levels sufficient to activate the Ca\(^{2+}\)-dependent protease μ-calpain and commit the cell to death. Previous studies from our laboratory have demonstrated that β-lap causes the downstream activation of μ-calpain resulting in its translocation to the nucleus concomitant with nuclear proteolytic cleavage of p53 and PARP-1 (7). Studies from our laboratory indicate that β-lap treatment causes apoptosis-inducing factor (AIF) translocation from the mitochondria to the nucleus, leading to nuclear condensation following μ-calpain activation. To date, the mechanism responsible for PARP-1-mediated AIF release remains unclear. We speculate that AIF release under conditions of DNA damage may be mediated through a concerted effort of both μ-calpain and PARP-1. Disruption of the mitochondrial membrane potential through PARP-1-dependent NAD\(^{+}\) and ATP losses, in conjunction with μ-calpain-mediated cleavage of Bid or of AIF itself, may mediate its release from the mitochondria (54, 55).

In conclusion, our studies offer new insights into the signal transduction pathways necessary for PARP-1-mediated cell death, providing a connection between PARP-1 hyperactivation and cell death via fluctuations in Ca\(^{2+}\) homeostasis. Knowledge of this pathway may be used to understand, and effectively treat, a large number of human pathologies (e.g. ischemia-reperfusion during heart attacks and stroke, and diabetes), as well as to enhance current cancer chemotherapeutic agents through modulation of PARP-1 hyperactivation.

Acknowledgments—We thank Drs. John J. Pink (Case Western Reserve University), Craig Thompson (U. of Pennsylvania), Wei-Xing Zong (State University of New York), and Ying Dong (University of Texas Southwestern Medical Center) for helpful discussions.

REFERENCES

1. Green, D. R., and Evan, G. I. (2002) Cancer Cell 1, 19–30
2. Calabrese, C. R., Almassy, R., Barton, S., Batey, M. A., Calvert, A. H., Canan-Koch, S., Durakcz, B. W., Hostomsky, Z., Kumpf, R. A., Kyle, S., Li, J., Maegley, K., Newell, D. R., Notarianni, E., Stratford, I. J., Skalitzky, D., Thomas, H. D., Wang, L. Z., Webber, S. E., Williams, K. J., and Curtin, N. J. (2004) J. Natl. Cancer Inst. 96, 56–67
3. Kim, M. Y., Zhang, T., and Kraus, W. L. (2005) Genes Dev. 19, 1921–1967
4. Jagtap, P., and Szabo, C. (2005) Nat. Rev. Drug. Discov. 4, 421–440
5. E. A. Bey and D. A. Boothman, unpublished observations.
Ca\(^{2+}\)-mediated PARP-1 Hyperactivation

5. Pink, J. J., Planchnon, S. M., Tagliarino, C., Varnes, M. E., Siegel, D., and Boothman, D. A. (2000) J. Biol. Chem. 275, 5416–5424
6. Pink, J. J., Wuerzberger-Davis, S., Tagliarino, C., Planchnon, S. M., Yang, X., Froelich, C. I., and Boothman, D. A. (2000) Exp. Cell Res. 255, 144–155
7. Tagliarino, C., Pink, J. J., Reinicke, K. E., Simmers, S. M., Wuerzberger-Davis, S. M., and Boothman, D. A. (2003) Cancer Biol. Ther. 2, 141–152
8. Wuerzberger, S. M., Pink, J. J., Planchnon, S. M., Byers, K. L., Bornmann, W. G., and Boothman, D. A. (1998) Cancer Res. 58, 1876–1885
9. Ross, D., Ke(, J. K., Winski, S. L., Beall, H. D., Anwar, A., and Siegel, D. (2000) Chem. Biol. Interact. 129, 77–97
10. Siegel, D., Franklin, W. A., and Ross, D. (1998) Clin. Cancer Res. 4, 2065–2070
11. Nieminen, A. L., Byrne, A. M., Herman, B., and Lemasters, J. J. (1997) An. J. Physiol. 272, C1286–C1294
12. Dawson, T. L., Gores, G. J., Nieminen, A. L., Herman, B., and Lemasters, J. J. (1993) An. J. Physiol. 264, C961–C967
13. Olive, P. L., Banath, J. P., and Durand, R. E. (1990) Radiat. Res. 122, 86–94
14. Abramoff, M. D., Magelhaes, P. J., and Ram, S. J. (2004) Biophotons Int. 11, 36–42
15. Rasband, W. (1997–2005) Image J. Bethesda, MD
16. Jacobson, E. L., and Jacobson, M. K. (1997) Methods Enzymol. 280, 221–230
17. Zong, W. X., Ditsworth, D., Bauer, D. E., Wang, Z. Q., and Thompson, J. P. (1990) J. Biol. Chem. 265, 227–242
18. Chowdhury, D., Keogh, M. C., Ishii, H., Peterson, C. L., Buratowski, S., and Lin, J. I. (1998) Mol. Cell. 3, 287–295
19. Ueda, K., Kawaichi, M., and Hayaishi, O. (1981) J. Biol. Chem. 256, 1876–1885
20. Tagliarino, C., Pink, J. J., Dubay, G. R., Nieminen, A. L., and Boothman, D. A. (2001) J. Cell Biol. 154, 19150–19159
21. Jacobsen, M. D., Weil, M., and Raff, M. C. (1996) J. Cell Biol. 133, 1041–1051
22. D’Amours, D., Desnoyers, S., D’Silva, I., and Poirier, G. G. (1999) Biochem. J. 342, 249–268
23. Szabo, C., and Dawson, V. L. (1998) Trends Pharmacol. Sci. 19, 287–298
24. Spieth, A. A., Verma, A., Zhang, J., and Snyder, S. H. (1999) Trends Pharmacol. Sci. 20, 171–181
25. McClellan, J., Hamam, A., Bauer, P. I., Kun, E., Zacharias, D. E., and Glusker, J. P. (1987) Biochim. Biophys. Acta 909, 71–83
26. Southan, G. I., and Szabo, C. (2003) Curr. Med. Chem. 10, 321–340
27. Cipriani, G., Rapizzi, E., Vannacci, A., Rizzuto, R., Moroni, F., and Chiarugi, A. (2005) J. Biol. Chem. 280, 17227–17234
28. Boothman, D. A., and Pardee, A. B. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 4963–4967
29. Boothman, D. A., Wang, M., Schea, R. A., Burrows, H. L., Strickfaden, S., and Owens, I. K. (1992) Int. J. Radiat. Oncol. Biol. Phys. 24, 939–948
30. Boothman, D. A., Meyers, M., Fukunaga, N., and Lee, S. W. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7200–7204
31. Rogakou, E. P., Pilch, D. R., Orr, A. H., Ivanova, V. S., and Bonner, W. M. (1998) J. Biol. Chem. 273, 5858–5868
32. Reinicke, K. E., Bey, A. E., Bentle, M. S., Pink, J. J., Ingalls, S. T., Hoppel, C. L., Misico, R. I., Arzac, G. M., Burton, G., Bornmann, W. G., Sutton, D., Gao, J., and Boothman, D. A. (2005) Clin. Cancer Res. 11, 3055–3064
33. Leohard, E. L., and Jacobson, M. K. (1997) Radiat. Res. 122, 86–94
34. Lewis, A., Ough, M., Li, L., Hinkhouse, M. M., Ritchie, J. M., Spitz, D. R., and Cullen, J. J. (2004) Cancer Res. 64, 4550–4558
35. Cullen, J. J., Hinkhouse, M. M., Grady, M., Gauth, A. W., Liu, J., Zhang, Y. P., Weydert, C. I., Domann, F. E., and Oberley, L. W. (2003) Cancer Res. 63, 5513–5520
36. Yu, S. W., Wang, H., Poitras, M. F., Coombs, C., Bowers, W. I., Fedoff, H. J., Poirier, G. G., Dawson, T. M., and Dawson, V. L. (2002) Science 297, 259–263
37. Manna, S. K., Gad, Y. P., Mukhopadhyay, A., and Aggarwal, B. B. (1999) Biochem. Pharmacol. 57, 763–774
38. Pathak, A. B., Li, Y. Z., and Li, C. J. (2002) Curr. Cancer Drug Targets 2, 227–242
39. Chowdhury, D., Keogh, M. C., Ishii, H., Peterson, C. L., Buratowski, S., and Lieberman, J. (2005) Mol. Cell 20, 801–809
40. van Wijk, J. J., and Hageman, G. J. (2005) Free Radic. Biol. Med. 39, 81–90
41. Kun, E., Kirsten, E., Mendelevy, J., and Oradahl, C. P. (2004) Biochemistry 43, 210–216
42. Tanuma, S., Kawashima, K., and Endo, H. (1986) J. Biol. Chem. 261, 490–498
43. Ogata, N., Ueda, K., Kawaichi, M., and Hayaishi, O. (1981) J. Biol. Chem. 256, 4135–4137
44. Homburg, S., Visocek, L., Moran, N., Dantzer, F., Priel, E., Asculi, E., Schwartz, D., Rotter, V., Dekel, N., and Cohen-Armon, M. (2000) J. Cell Biol. 150, 293–307
45. Virag, L., Scott, G. G., Antal-Szalmas, P., O’Connor, M., Ohshima, H., and Szabo, C. (1999) Mol. Pharmacol. 56, 824–833
46. Barbouti, A., Doulias, P. T., Zhu, B. Z., Frei, B., and Galaris, D. (2001) Free Radic. Biol. Med. 31, 490–498
47. Jornot, L., Petersen, H., and Junod, A. F. (1998) Biochem. J. 335, 85–94
48. Britgan, B. E., Rasmussen, G. T., and Cox, C. D. (1998) Biochem. Pharmacol. 55, 287–295
49. Fonfria, E., Marshall, I. C., Benham, C. D., Boyfield, I., Brown, J. D., Hill, K., Hughes, J. P., Skaper, S. D., and McNulty, S. (2004) Br. J. Pharmacol. 143, 186–192
50. Polster, B. M., Basanez, G., Etxebarria, A., Hardwick, J. M., and Nicholls, D. G. (2005) J. Biol. Chem. 280, 6447–6454
51. Takano, J., Tomioi, M., Tsubuki, S., Higuchi, M., Iwata, N., Itohara, S., Maki, M., and Saido, T. C. (2005) J. Biol. Chem. 280, 16175–16184