Mechanisms of Amiodarone and Desethylamiodarone Cytotoxicity in Nontransformed Human Peripheral Lung Epithelial Cells

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ABSTRACT
Amiodarone (AM) is a potent antidysrhythmic agent that can cause potentially life-threatening pulmonary fibrosis, and N-desethylamiodarone (DEA), an AM metabolite, may contribute to AM toxicity. Apoptotic cell death in nontransformed human peripheral lung epithelial 1A (HPL1A) cells was assessed by annexin V-fluorescein isothiocyanate (ann-V) staining and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL), and necrotic cell death was assessed by propidium iodide (PI) staining. The percentage of cells that were PI-positive increased more than six times with 20 μM AM and approximately doubled with 3.5 μM DEA, relative to control. The percentage of cells that were ann-V-positive decreased by more than 80% after 24-h exposure to 10 μM AM but more than doubled after 24-h incubation with 3.5 μM DEA. Incubation for 24 h with 5.0 μM DEA increased the percentage of cells that were TUNEL-positive more than six times. Incubation with AM (2.5 μM) or DEA (1–2 μM) for 24 h did not significantly alter angiotensinogen mRNA levels. Furthermore, angiotensin II (100 pM–1 μM) alone or in combination with AM or DEA did not alter cytotoxicity, and pretreatment with the angiotensin-converting enzyme inhibitor and antioxidant captopril (3–6 μM) did not protect against AM or DEA cytotoxicity. In conclusion, AM activates primarily necrotic pathways, whereas DEA activates both necrotic and apoptotic pathways, and the renin-angiotensin system does not seem to be involved in AM or DEA cytotoxicity in HPL1A cells.

Introduction
Amiodarone (AM), an iodinated benzofuran, is considered to be the most efficacious antidysrhythmic drug currently available (Lafuente-Lafuente et al., 2009). However, long-term treatment with AM is associated with several adverse effects, the one of greatest concern being AM-induced pulmonary toxicity (AIPT), because it can progress to potentially life-threatening pulmonary fibrosis. Recent studies have reported that the incidence of AIPT occurs in 5 to 13% of patients treated with AM in a dose- and duration-dependent manner (Oyama et al., 2005). The prognosis of a patient with AIPT is poor, with a 10 to 23% mortality rate (Vrobel et al., 1989; Oyama et al., 2005).

AM and its major pharmacological active metabolite, desethylamiodarone (DEA), have large apparent volumes of distribution and slow clearances from adipose tissue, liver, lungs, and lymph nodes and therefore accumulate to high concentrations in these tissues (Freedman and Somberg, 1991). After long-term therapy, AM can accumulate in lung to >1 mmol/kg wet tissue (Brien et al., 1987). In addition, DEA has greater cytotoxic potency than AM and can accumulate in lung to up to four times greater levels than AM (Broekhuysen et al., 1969; Wilson and Lippmann, 1990; Reasor and Kacew, 1996). Hence, there is compelling evidence that DEA plays a role in AIPT.

The underlying etiology of AIPT is unknown; however, both indirect inflammatory processes and direct toxic effects have been proposed previously (Reasor and Kacew, 1996). AM and DEA are directly toxic to bovine arterial endothelial
Many mechanisms have been hypothesized to cause the di-
cells, alveolar macrophages, interstitial lung fibroblasts (Martin and Howard, 1985), human pulmonary arterial end-
epithelial cells (Powis et al., 1990), bronchial epithelial cells (Colgan et al., 1984), and hepatocytes (Gross et al., 1989).
Many mechanisms have been hypothesized to cause the di-
rect toxicity of AM and DEA, including increased intracellular influx of Ca²⁺ (Powis et al., 1990), mitochondrial disruption (Bolt et al., 2001), free radical and reactive oxygen species formation (Pollak, 1999), and up-regulation of the renin-angiotensin system (RAS) (Uhal et al., 2007).
Studies have indicated that rat lung epithelial cells and human lung adenocarcinoma cells have an intrinsic RAS,
with the ability to generate angiotensin II (Ang II) de novo (Li et al., 2003). Ang II signaling, mediated via the angioten-
sin receptors AGTR1 and AGTR2, plays a role in tissue remodeling in fibrosis (Konigshoff et al., 2007). However, the downstream effects of AGTR1 and AGTR2 activation are quite different. The classic physiologic effects of Ang II, including vasoconstriction, aldosterone and vasopressin release, sodium and water retention, and cell proliferation, are mediated by AGTR1, whereas the established role of AGTR2 includes modulation of biological processes involved in develop-
ment, cell differentiation, tissue repair, and apoptosis (Kaschina and Unger, 2003). Ang II can induce concentra-
tion-dependent apoptosis in human lung epithelial cells and in primary type II pneumocytes isolated from adult
Wistar rats, an effect that can be abrogated by the nonselective AGTR antagonist saralasin (Wang et al., 1999b). In addition, treatment with the angiotensin-converting enzyme inhibitor captopril can attenuate apoptosis in human lung adenocarcinoma cells and primary rat alveolar epithelial cells treated with AM or DEA (Bargout et al., 2000) and can inhibit alveolar wall collagen formation in lungs of AM-
treated rats (Uhal et al., 2003). Furthermore, a retrospective review of patients taking AM suggests that patients who developed AIPIT were administered a lower dose of RAS inhi-
bitor than those who did not develop AIPIT (Nikaido et al., 2008). Thus, the RAS may play a contributing role in the initiation and/or progression of AIPIT.
Surfactant-secreting type II alveolar epithelial cells pro-
vide antioxidant defense, local immunomodulation, and a stem cell reserve for alveolar epithelial repair and are critical for normal re-epithelialization and healing without fibrosis of the alveolar surface (Thannickal et al., 2004). Histological analysis of lung tissue from patients treated with AM dem-
onstrates alveolar interstitial damage, including hyperplasia of type II pneumocytes (Brien et al., 1987). In addition, AM is toxic to epithelial cells in vitro (Bargout et al., 2000), thereby implicating epithelial injury in the initiation of AIPIT. To better understand the etiology of AIPIT, the present study investigated the cytotoxic pathways activated by AM or DEA individually and whether an intrinsic RAS is linked to AM- or DEA-induced cell death. HPL1A cells, which were estab-
lished by immortalization from a normal adult lung speci-
men, were employed because they retain morphological and biochemical features characteristic of normal adult human peripheral lung epithelial cells (Masuda et al., 1997).

Materials and Methods
Reagents. Chemicals and reagents were obtained as follows: AM HCl (98% purity), bovine insulin, fetal bovine serum, hydrocortisone, HEPES, Ang II, captopril, propidium iodide (PI), and trypan blue from Sigma-Aldrich (Oakville, ON, Canada); annexin V-fluorescein isothiocyanate (ann-V) from BD Biosciences (Mississauga, ON, Canada); glacial acetic acid, KH₂PO₄, NaCl, NaHCO₃, NaOH, and Na₂HPO₄·7H₂O from Thermo Fisher Scientific (Nepean, ON, Canada); and antibiotic-antimycotic, l-glutamine, Ham’s F-12 nutrient mixture, human transferrin, and trypsin-EDTA from Invitrogen Canada Inc. (Burlington, ON, Canada). DEA HCl (99.9% purity) was synthesized by Dr. Manlio Alessi (Department of Chemistry at Queen’s University, Kingston, ON). All other reagents were of ana-
lytical grade and were purchased from standard commercial suppli-
ers. Stock solutions of 4.0 mM AM, 1.0 mM DEA, 4.8 mM Ang II, and 2.0 mM captopril were prepared fresh in reverse osmosis purified distilled water at 65°C (AM and DEA) or room temperature (Ang II and captopril).

Cell Culture. HPL1A cells (Masuda et al., 1997) were cultured in Ham’s F-12 nutrient mixture medium, pH 7.2, supplemented with 1.18 g/liter sodium bicarbonate, 1% fetal bovine serum, 15 mM HEPES buffer, 1× antibiotic-antimycotic, 0.1 mM hydrocortisone, 0.13 ng/ml triiodothyronine, 5.0 μg/ml human transferrin, and 5.0 μg/ml bovine insulin (HPL1A medium). Cells were grown in T-75 flasks (Corning Inc., Corning, NY). Culture medium was replaced every 3 to 4 days, and cells were subcultured approximately every 7

days between passages 8 and 12. Cells were incubated at 37°C under 95% air, 5% CO₂. After reaching 80 to 90% confluence, cells were removed from tissue culture flasks by washing twice with 10 ml of phosphate-buffered saline (PBS) (0.2 g/liter KH₂PO₄, 0.8 g/liter NaCl, 2.16 g/liter Na₂HPO₄·7H₂O, pH 7.3), and then treated with 0.1% trypsin and 1.06 mM EDTA in PBS. The cells were then incubated at 37°C for 5 to 10 min and resuspended in HPL1A medium. Cells were seeded at a density of 2.1 × 10⁵ cells/well in 12-well plates or 2.8 × 10⁶ cells/100-mm dish and allowed to accli-
matize for approximately 24 h before drug treatment.

Quantification of AM and DEA in HPL1A Cells. To determine whether HPL1A cells are able to convert AM to DEA, the amount of AM and DEA within HPL1A cells after 24 h of treatment was determined by high-performance liquid chromatography (HPLC). In brief, cells in 100-mm dishes were treated with AM for 24 h. Cells were then harvested and centrifuged at 700g for 10 min at 4°C. The supernatant was removed, and the resulting cell pellet was resuspen-
ded in 1.0 ml of 4°C PBS and then centrifuged at 700g for 5 min at 4°C. The supernatant was removed, and the resulting cell pellet was immedi-
ately frozen in liquid nitrogen and stored at −80°C until analysis.

The amount of AM and DEA in cell pellets was measured as described by Bolt et al. (1998). In brief, each cell pellet was thawed, and 100 μl of mobile phase [acetonitrile/5% aqueous acetic acid, 8:2 (v/v) adjusted to pH 5.9 with ammonium hydroxide] was added. Cell pellets were mixed in the mobile phase for 1 min, and the mixture was centrifuged at 16,000g for 3 min at room temperature. The supernatants were analyzed quantitatively for AM and DEA by reverse-phase HPLC with UV-visible spectrophotometric detection at 254 nm, with a with-in day precision of 7.00% (Brien et al., 1983, 1987). The percentage conversion of AM to DEA for each treatment condition was calculated. The mean value from three independent experiments was used to calculate the overall percent conversion of AM to DEA for each treatment condition. The lower limit of quanti-
fiable detection for DEA was 0.20 μg/ml (0.31 μM). The concentra-
tions of AM and DEA injected onto the HPLC column from biological samples were within the range of standards employed (11.3–180 μg/ml for AM and 0.25–4.00 μg/ml for DEA).

Annexin-V-Fluorescein Isothiocyanate and Propidium Iodi-
dine Dual Staining. HPL1A cells were stained with ann-V to indicate apoptosis and PI to indicate necrosis. For AM and DEA cytotoxicity experiments, HPL1A cells in 12-well plates were treated with AM or DEA for 6, 12, or 24 h. For Ang II cytotoxicity experiments, HPL1A cells in 12-well plates were treated with AM or DEA in combination with Ang II for 24 h. Ang II was administered every 12 h in the 24-h treatment period. For captopril protection experiments,
HPLIA cells in 12-well plates were pretreated for 2 h with captopril to allow captopril to enter the cells and interact with angiotensin-converting enzyme before the addition of AM or DEA. After captopril pretreatment, AM or DEA was added for an additional 24 h. After treatment, culture medium was removed, and cells were washed once with PBS. Cells were detached by incubation with trypsin-EDTA for 5 min at 37°C. The original culture medium, PBS wash, and cell suspension were combined to retain all dead and living cells for analysis. The resulting cell suspension was centrifuged for 4 min at 215g at room temperature. The supernatant was removed, and the cell pellet was resuspended in 0.5 ml of a buffer consisting of 10 mM HEPES, 140 mM NaCl, and 5 mM CaCl2. The cell suspension was then treated with 5 µl of stock annex-V solution (BD Biosciences) and 5 µl of 0.5 mg/ml PI and placed on ice in the dark for 20 min. Samples were then centrifuged for 4 min at 215g, and the cell pellets were resuspended in 0.5 ml of PBS. Analysis was completed via flow cytometry (EPICS ALTRA; Beckman Coulter, Mississauga, ON, Canada). In addition, total cell death percentages were fitted to a sigmoidal dose-response curve (variable slope), and the concentrations of drug producing 50% cell viability loss (LC50) were interpolated using Prism (ver. 5.0; GraphPad Software, San Diego, CA). Analysis was performed on the mean values of triplicates (values from three independent experiments).

**Modified Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling Assay.** A modified fluorometric terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) kit was used to detect fragmented DNA, a characteristic of apoptotic cells, according to the manufacturer’s protocol (Promega, Madison, WI). Cells in 12-well plates were treated for 24 h with AM or DEA. After treatment, the culture medium was removed, and cells were washed once with PBS. Cells were detached by incubation with trypsin-EDTA for 5 min at 37°C and centrifuged at 215g at 4°C for 5 min, and the cell pellet was resuspended in 0.5 ml of 4°C PBS. Cells were fixed using 3.5 ml of 1% paraformaldehyde for 20 min on ice. After fixation, cells were centrifuged and resuspended in 0.5 ml of PBS. Seventy percent of ice-cold ethanol (3.5 ml) was then added to the cell suspensions, and samples were stored at −20°C overnight.

In the next day, cells were washed with PBS, resuspended in 80 µl of equilibration buffer (200 mM potassium cacodylate, 25 mM Tris-HCl, 0.2 mM dithiothreitol, 0.25 mg/ml bovine serum albumin, and 2.5 mM cobalt chloride) and left to incubate for 5 min at room temperature. Cells were then resuspended in 50 µl of reverse terminal deoxynucleotidyl transferase incubation buffer and left to incubate in a 37°C water bath for 60 min. The reaction was terminated by adding 1.0 ml of 20 mM EDTA, followed by centrifugation at 175g for 6 min. Cells were resuspended in 1.0 ml of 0.1% Triton X-100 solution in PBS containing 5 mg/ml bovine serum albumin, followed by centrifugation at 175g for 5 min. Cells were resuspended in 0.5 ml of propidium iodide containing 250 µg of DNase-free RNase A and left to incubate at room temperature in the dark for 30 min. Cell samples were analyzed by flow cytometry, with fluorescein-12-dUTP measured at 520 ± 20 nm and PI measured at >620 nm. Analysis was performed on the mean values of triplicates (values from three individual wells) from three independent experiments.

**Trypan Blue Exclusion.** HPLIA cells in 12-well plates were treated with Ang II for 24 h. After treatment, cells were detached by incubation with trypsin-EDTA for 5 min at 37°C. Cells were then stained with 0.5% trypan blue dye. Cell viability was assessed by trypan blue exclusion, using a hemocytometer and light microscope (Reichert Scientific Instruments, Buffalo, NY). Analysis was performed on the mean values of triplicates (values from three individual wells) from three independent experiments.

**Angiotensinogen mRNA Levels.** Total RNA was isolated from HPLIA cells using the RNeasy Mini Kit (Qiagen, Valencia, CA), with an additional on-column DNase treatment step in accordance with the manufacturer’s instructions. The quality of the RNA samples was determined by electrophoretic analysis of 1 µg of RNA on a denaturing gel. Ethidium bromide staining of the gel revealed distinct 28S and 18S rRNA bands, with an intensity ratio of 28S/18S of at least 2. The UV absorbance ratio (260/280 nm) ranged from 1.9 to 2.1 for all RNA samples. cDNA was synthesized from 1 µg of total RNA in a reaction volume of 20 µl using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) in accordance with manufacturer’s instructions. For quantitative real-time PCR, 2.0-µl aliquots of cDNA were amplified using an angiotensinogen TaqMan primer and probe set (Applied Biosystem Assay ID Hs00174854_m1) according to the manufacturer’s recommendations. Amplification, detection, and analysis were performed using Smart Cycler II instrumentation and software (Cepheid, Sunnyvale, CA). For mRNA quantitation, the standard curve method of relative quantitation was used. PCR product specificity was verified by agarose gel electrophoresis with ethidium bromide staining.

**Data Analysis.** Data are expressed as the mean ± S.E.M. for each experimental group. Experiments were conducted in the same culture of cells sequentially. To assess viability of treated and untreated cells at multiple concentrations, repeated-measures one-way analysis of variance (ANOVA) was performed, followed by Student Newman-Keuls post hoc test. In all cases, analysis was performed using Prism software, and statistical significance was defined as p < 0.05.

**Results**

**Conversion of Amiodarone to Desethylamiodarone in HPLIA Cells.** Treatment of HPLIA cells with 5 to 20 µM AM for 24 h resulted in minimal production of DEA; with 5 µM AM, 1.86 ± 0.42% AM was converted to DEA, whereas 0.99 ± 0.36 and 0.92 ± 0.35% AM were converted to DEA after treatment of 10 and 20 µM, respectively. Treatment of HPLIA cells with 20 µM AM for 0 h (treated medium was added to cultures and cultures were immediately harvested for HPLC analysis) resulted in no detectable DEA (i.e., <0.31 µM), confirming that the conversion of AM to DEA occurred during 24-h incubation and was not artifactual. The recovery of AM from the cell pellet after 24-h incubation was 98±5% for the concentrations of AM tested.

**AM- and DEA-Induced Necrosis and Apoptosis.** Treatment of HPLIA cells with 1 to 20 µM AM for 6 or 12 h increased the percentage of PI-positive cells (necrosis) and decreased the percentage of ann-V-positive cells (apoptosis) (Fig. 1, A and B). Treatment with 10 or 20 µM AM for 24 h increased the percentage of cells that were PI-positive >3- and >6-fold, respectively, relative to control (Fig. 1C). However, at 24 h, a significant decrease in ann-V-positive cells was observed only with 20 µM AM (Fig. 1C).

In contrast, 6- or 12-h exposure to DEA resulted in no change in ann-V staining and an increase in PI-positive cells only with 5 µM DEA (Fig. 2, A and B). At 24 h, the percentage of cells that were PI-positive increased 2- and >6-fold after incubation with 3.5 and 5 µM DEA, respectively, with control, whereas a significant increase in ann-V-positive cells occurred with 3.5 µM DEA (Fig. 2C). The concentration of DEA producing 50% cell viability loss (LC50) after 24-h treatment was significantly lower than that of AM (4.67 ± 2.40 and 12.7 ± 1.42 µM, respectively). The concentrations of AM and DEA used in the subsequent captopril and Ang II experiments were 10 and 3.5 µM, respectively, which are the approximate LC44 for each drug (AM LC44 = 44.9 ± 2.92; DEA LC44 = 42.4 ± 3.78). Representative dot plots obtained from
Flow-cytometric analysis of annexin-V- and PI-stained cells illustrate the shift of AM-treated cells predominantly to necrosis, and the shift of some DEA-treated cells to apoptosis and some DEA-treated cells to necrosis (Fig. 3).

Flow-cytometric dot plots from TUNEL staining (Fig. 4, A–D) show a similar trend for vehicle and AM (no shift in the cell population) and a similar trend for the apoptosis-positive control (hydrogen peroxide) and DEA (upward shift in the cell population, indicating apoptosis). After treatment of HPL1A cells with 0.03% hydrogen peroxide for 30 min, the mean percentage of TUNEL-positive cells ranged from 44 to 65% (Fig. 4, E and F). In contrast to quantification of apoptosis by annexin-V staining, treatment with 1 to 20 µM AM or 1 to 3.5 µM DEA for 24 h caused no change in apoptosis as determined by the percentage of TUNEL-positive cells (Fig. 4, A, C, E, and F); however, 5 µM DEA increased TUNEL-positive cells from 4.31 ± 2.00 (control) to 26.7 ± 3.72% (Fig. 4 A, D, and F).

Angiotensinogen mRNA Levels. Treatment of HPL1A cells with 2.5 µM AM, 1.0 µM DEA, or 2.0 µM DEA for 24 h caused a trend toward an increase in angiotensinogen levels, which fell just short of statistical significance ($p = 0.0504$, one-way ANOVA; Fig. 5). Higher concentrations of AM or DEA caused substantial cytotoxicity, which precluded recovery of high-quality RNA for analysis.

Assessment of Ang II Cytotoxicity. For initial Ang II cytotoxicity experiments, 0.5% trypan blue exclusion (indicative of plasma membrane integrity) was used to rapidly determine whether Ang II affected cell viability. After 24 h of treatment of HPL1A cells with 100 pM to 1 µM Ang II, no significant cell death was observed relative to vehicle control, with the overall range of cell viability for all concentrations tested being 94.5 to 97.8%. Light microscopic examination also did not reveal any apparent damage to the cells.

To assess the effects of Ang II on apoptosis and necrosis induced by AM and DEA, annexin-V and PI dual staining was performed. As expected, the percentage of PI-positive cells increased more than 2-fold after 24-h treatment with 10 µM AM compared with control, whereas no change was seen in annexin-V-positive cells (Fig. 6 A). Treatment for 24 h with 3.5 µM DEA increased PI- and annexin-V-positive cells (Fig. 6B). However, coinubation of HPL1A cells for 24 h with 10 µM AM or 3.5 µM DEA plus 100 pM to 1 µM Ang II...
II caused no significant alterations in PI- or ann-V-positive cells compared with AM or DEA alone (Fig. 6, A and B).

**Evaluation of Protection against AM or DEA Cytotoxicity by Captopril.** The angiotensin-converting enzyme inhibitor captopril was ineffective at attenuating AM- or DEA-induced cytotoxicity. Consistent with previous experiments (Figs. 2 and 6), PI-positive cells increased more than 3-fold after 24-h treatment with 10 \( \mu \text{M} \) AM compared with control, whereas no change occurred in ann-V-positive cells (Fig. 7A). Treatment of cells for 24 h with 3.5 \( \mu \text{M} \) DEA increased the percentage of PI- and ann-V-positive cells (Fig. 7B). Pretreatment of cells for 2 h with 3 to 6 \( \mu \text{M} \) captopril, before the addition of 10 \( \mu \text{M} \) AM or 3.5 \( \mu \text{M} \) DEA, caused no significant change in percentages of PI- or ann-V-positive cells compared with AM alone or DEA alone (Fig. 7).

**Discussion**

DEA has greater cytotoxic potency than AM and accumulates in tissues to a greater extent than AM after long-term treatment of humans with AM (Broekhuysen et al., 1969; Wilson and Lippmann, 1990). Given the high toxicity of DEA and that DEA is a major metabolite of AM, it is possible that many of the initiating processes of AIPT are not solely the result of AM but DEA as well. After long-term therapy, up to...
Fig. 3. Representative dot plots depicting the percentage of HPL1A cells that were ann-V-positive (apoptotic) and PI-positive (necrotic) after incubation with vehicle control (24 h) (A), 20 μM AM (24 h) (B), or 3.5 μM DEA (24 h) (C). Quadrant A3 is unstained cells, quadrant A4 is ann-V-only stained cells, quadrant A1 is PI-only stained cells, and quadrant A2 contains cells stained with both PI and ann-V. The percentages of necrotic (quadrant A1 plus quadrant A2) and apoptotic (quadrant A4) cells from triplicates of three independent experiments were transposed to bar graphs (Figs. 1 and 2).

Fig. 4. A to D, representative dot plots depicting the percentage of HPL1A cells that were TUNEL-negative (live) and TUNEL-positive (apoptotic) (below and above the horizontal line, respectively) after incubation with vehicle control (24 h) (A), positive control (0.03% H2O2, 30 min) (B), 20 μM AM (24 h) (C), or 5 μM DEA (24 h) (D). E and F, the percentages of apoptotic cells from triplicates of three independent experiments were transposed to bar graphs, shown for AM (E) and DEA (F) treatment. * significantly different from vehicle control, p < 0.05 (repeated measures one-way ANOVA with student Newman-Keuls post hoc test; n = 3).
millimolar concentrations of AM and DEA can be found in lung, making the concentrations used in the in vitro studies well within the therapeutically relevant range. In addition, patients who develop amiodarone-induced fibrosis are on the same treatment protocols as those patients who achieve millimolar concentrations of AM and DEA.

In vitro studies investigating cell types in isolation are useful for differentiating the effects of a xenobiotic versus its metabolite(s) if metabolism of the xenobiotic does not occur appreciably in the cells of interest. In humans, AM is metabolized to DEA predominantly by cytochrome P450 enzymes 3A4, 2C8, and 1A1 (Ohyama et al., 2000), which are expressed primarily in the liver, although evidence exists on mRNA and protein of these enzymes in human lung (Macé et al., 1998; Nishimura et al., 2003).

In HPL1A cells, less than 2% AM was converted to DEA over 24 h. The fact that the percentage conversion of AM to DEA decreased with increasing AM concentration was probably attributed to the direct cytotoxicity of AM at the higher concentrations rather than saturation of biotransforming enzymes. The $K_M$ value for cytochrome P450 3A production of DEA in liver microsomes was 0.33 mM (Fabre et al., 1993), a concentration well above those used in this study. Because AM is not converted to DEA to an appreciable extent during the incubation times employed, the HPL1A cell culture model can be used to study the effects of AM and DEA independently on lung epithelial cells.

As observed previously in other systems (Broekhuysen et al., 1969; Wilson and Lippmann, 1990; Bolt et al., 2001; Nicolescu et al., 2008), DEA proved to be a more potent and rapidly acting cytotoxicant than AM in HPL1A cells. Both agents caused HPL1A cell death predominantly by necrosis. Other studies investigating apoptotic and necrotic pathways induced by AM or DEA in other cell types have also found a concentration-dependent increase in necrosis (Bargout et al., 2000; Yano et al., 2008). After AM treatment, we also found a concentration-dependent decrease in apoptosis at 6 and 12 h, but not at 24 h, as reflected by ann-V staining. The percentage of HPL1A cells that underwent apoptosis, however, was small (1.6–12.3%). Therefore, necrosis predominates over apoptosis in AM toxicity. Apoptosis and necrosis can occur in a continuum, whereby cells can undergo processes that have the potential to lead to apoptosis but can ultimately later undergo necrosis, depending on the magnitude of the insult, duration of toxicant exposure, energy...
requirements, and so on. The ann-V-staining results suggest that the pronounced cytotoxicity of the higher concentrations of AM resulted in a shift in the apoptotic-necrotic continuum, resulting in virtually all cells dying via necrosis. This finding in the nontransformed HPL1A human lung epithelium-derived cells contrasts with the results from A549 human lung adenocarcinoma cells and rat alveolar epithelial cells (Bargout et al., 2000; Yano et al., 2008), in which AM caused a concentration-dependent increase in apoptosis. However, in both the previous studies and the present study, both apoptosis and necrosis were observed after exposure to AM or DEA, and necrosis predominated.

Unlike AM, DEA induced apoptosis in HPL1A cells, although the lowest concentration at which apoptosis was detected differed depending on the assay used (Figs. 2C and 4F). This difference can be attributed to the different end points assayed in that ann-V staining indicates phosphatidylserine externalization, an early event in apoptosis relative to DNA fragmentation, which is detected by TUNEL staining (Jetzek-Zader et al., 2007). This is consistent with the fact that the percentage of TUNEL-positive control cells was lower than the percentage of ann-V-positive control cells (Figs. 2C and 4F). If a longer incubation time were to be tested, the percentage of control cells containing fragmented DNA would be expected to increase. The initial induction of ann-V-positive cells was not sustained at higher concentrations of DEA, which is consistent with the marked toxicity associated with 5 μM DEA, a concentration that caused an even greater proportion of the cells to undergo necrosis. These DEA results are also consistent with those of other studies (Bargout et al., 2000; Waldhauser et al., 2006), in which DEA increased both apoptosis and necrosis.

Taken together, the results from the present study suggest that the cytotoxic pathways induced by AM and DEA differ somewhat in HPL1A cells. AM induces necrotic cell death, whereas DEA induces both necrotic and apoptotic cell death despite being the more potent cytotoxicant. DEA caused an increase in PI-positive cells before an increase in ann-V-positive cells. Because PI enters cells when the plasma membrane becomes permeable during necrosis, this suggests that DEA-induced necrosis occurred before apoptosis and that the two cell death processes are occurring simultaneously in different cell populations, which has been proposed previously (Leist et al., 1997; Yano et al., 2008). This concept is best illustrated in Fig. 3, wherein four cell populations can be observed in each dot plot; of significance are the populations of cells that stained only with PI and the distinct populations of cells that stained only with ann-V, indicating that necrosis and apoptosis were occurring simultaneously but in different cells. This phenomenon is not unique to AM and DEA; for example, in ischemia reperfusion and liver damage induced by toxicants, necrotic and apoptotic cell death occur simultaneously (Leist et al., 1997).

A549 cells possess an intrinsic RAS with the ability to generate Ang II de novo (Wang et al., 1999a). Ang II has been reported to induce concentration-dependent apoptosis in A549 cells (0.01–100 μM Ang II) and in rat primary type II pneumocytes (0.005–100 μM Ang II), an effect that is diminished by the angiotensin-converting enzyme inhibitor captopril (Uhal et al., 1998; Wang et al., 1999b). In the present study, treatment of HPL1A cells with AM or DEA caused a nonsignificant trend toward an increase in angiotensinogen mRNA levels. Based on the equivocal effect of AM and DEA on angiotensinogen expression and the evidence supporting RAS involvement in AM toxicity in other systems, the relevance of the RAS was investigated further. The fact that a very broad range of concentrations of Ang II itself was neither cytotoxic nor able to enhance AM or DEA cytotoxicity strongly suggests that a functional intrinsic RAS linked to cell death does not exist in HPL1A cells. Furthermore, the inability of captopril to prevent AM and DEA cytotoxicity at concentrations that occur in plasma during clinical pharma-
cotherapy (Nonoguchi et al., 2008) and that are inhibitory in other systems (Bargout et al., 2000), further precludes the involvement of the RAS in the cell death caused by those two toxicants in HPL1A cells. Hence, a functional RAS is not requisite for cytotoxicity of AM and DEA at concentrations similar to those used in other cell systems, and is well within the range found in lungs of patients treated clinically with AM (Plomp et al., 1984; Brien et al., 1987). Therefore, a RAS-independent mechanism seems to be responsible for AM and DEA cytotoxicity in nontransformed human lung epithelial cells; however, our findings do not preclude the involvement of Ang II and the RAS in the progression of AIP in vivo.

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Authorship Contributions

Participated in research design: Mulder, Brien, Racz, and Massey. Conducted experiments: Mulder. Contributed new reagents or analytic tools: Takahashi. Performed data analysis: Mulder. Wrote or contributed to the writing of the manuscript: Mulder, Brien, and Massey.

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