Mechanisms of Amiodarone and Desethylamiodarone Cytotoxicity in Non-transformed Human Peripheral Lung Epithelial Cells

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ABBREVIATIONS: AM, amiodarone; DEA, desethylamiodarone; RAS, renin-angiotensin system; ann-V, annexin-V-FITC; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; PI, propidium iodide; HPL1A, human peripheral lung epithelial cells; AIPT, AM-induced pulmonary toxicity; ROS, reactive oxygen species; Ang II, angiotensin II; AGTR, angiotensin receptor; PBS, phosphate buffered saline; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; ANOVA, analysis of variance.

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Abstract

Amiodarone (AM) is a potent antidysrhythmic agent which can cause potentially life-threatening pulmonary fibrosis, and N-desethylamiodarone (DEA), an AM metabolite, may contribute to AM toxicity. Apoptotic cell death in non-transformed HPL1A human peripheral lung epithelial cells was assessed by annexin-V-FITC (ann-V) staining and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL), and necrotic cell death by propidium iodide (PI) staining. The percentage of cells that were PI positive increased over 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 over 80% following 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 over 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 pre-treatment 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 appear 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, chronic treatment with AM is associated with several adverse effects, the one of greatest concern being AM-induced pulmonary toxicity (AIPT), since it can progress to potentially life-threatening pulmonary fibrosis. Recent studies have reported that the incidence of AIPT occurs in 5-13% 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 10-23% mortality rate (Oyama et al., 2005; Vrobel et al., 1989).

AM and its major pharmacologically 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). Following chronic 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 does 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 (Reasor and Kacew, 1996). AM and DEA are directly toxic to bovine arterial endothelial cells, alveolar macrophages, interstitial lung fibroblasts (Martin and Howard, 1985), human pulmonary arterial endothelial 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 direct toxicity of AM and DEA, including increased intracellular influx of 
Ca\(^{2+}\) (Powis, et al., 1990), mitochondrial disruption (Bolt et al., 2001 a), free radical and 
ROS formation (Pollak, 1999) and up-regulation of the renin-angiotensin system (RAS) 
(Uhal, et al., 2006).

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 angiotensin 
receptors AGTR1 and AGTR2, plays a role in tissue remodelling in fibrosis (Konigshoff 
et al., 2007). However, the downstream effects of AGTR1 and AGTR2 activation are 
quite different. The classic physiological effects of Ang II, including vasoconstriction, 
ardosterone 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 development, cell differentiation, tissue repair and 
apoptosis (Kaschina and Unger, 2003). Ang II can induce concentration-dependent 
apoptosis in human lung cancer epithelial cells and in primary type II pneumocytes 
isolated from adult Wistar rats, an effect which can be abrogated by the nonselective 
AGTR antagonist saralasin (Wang et al., 1999a). In addition, treatment with the 
angiotensin converting enzyme inhibitor captopril can attenuate apoptosis in human lung 
adenoarcinoma 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 AIPT were administered a lower dose of RAS
inhibitor than were those who did not develop AIPT (Nikaido et al. 2008). Thus, the RAS may play a contributing role in the initiation and/or progression of AIPT.

Surfactant-secreting type II alveolar epithelial cells provide 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 demonstrates alveolar interstitial damage including hyperplasia of type II pneumocytes (Brien et al., 1986). In addition, AM is toxic to epithelial cells in vitro (Bargout et al., 2000), thereby implicating epithelial injury in the initiation of AIPT. In order to better understand the etiology of AIPT, 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 established by immortalization from a normal adult lung specimen, were employed because they retain morphological and biochemical features characteristic of normal adult human peripheral lung epithelial cells (Masuda et al., 1997).
Methods

Reagents

Chemicals and reagents were obtained as follows: AM HCl (98% purity), bovine insulin, fetal bovine serum, hydrocortisone, \( n \)-2-hydroxyethylpiperazine-\( n' \)-2-ethanesulfonic acid (HEPES), angiotensin II (Ang II), captopril, propidium iodide (PI) and trypan blue from Sigma-Aldrich (Oakville, ON); annexin-V-fluorescein isothiocyanate (ann-V-FITC) from BD Bisociences (Mississauga, ON); glacial acetic acid, \( \text{KH}_2\text{PO}_4 \), NaCl, \( \text{NaHCO}_3 \), NaOH and \( \text{Na}_2\text{HPO}_4\cdot7\text{H}_2\text{O} \) from Fisher Scientific Inc. (Nepean, ON); and antibiotic-antimycotic, L-glutamine, Ham’s F-12 nutrient mixture, human transferrin and trypsin-EDTA from Invitrogen Canada Inc. (Burlington, ON). 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 analytical grade and were purchased from standard commercial suppliers. 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/L sodium bicarbonate, 1% fetal bovine serum, 15 mM HEPES buffer, 1x antibiotic-antimycotic, 100 nM 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 3-4 days and cells were sub-cultured approximately every 7 days between passages 8 and 12. Cells were incubated at 37°C under 95% air, 5% CO₂. After reaching 80-90% confluence, cells were removed from tissue culture flasks by washing twice with 10 mL phosphate buffered saline (PBS) (0.2 g/L KH₂PO₄, 0.8 g/L NaCl, 2.16 g/L 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 5-10 minutes (min) and resuspended in HPL1A medium. Cells were seeded at a density of 2.1 x 10⁵ cells/well in 12 well plates or 2.8 x 10⁶ cells/100 mm dish and allowed to acclimatize for approximately 24 h prior to drug treatment.

Quantification of AM and DEA in HPL1A cells

To determine if 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). Briefly, cells in 100 mm dishes were treated with AM for 24 h. Cells were then harvested and centrifuged at 700 x g for 10 min at 4°C. The supernatant was removed and the cell pellet was resuspended in 1.0 mL of 4°C PBS and then centrifuged at 700 x g for 5 min at 4°C. The supernatant was removed, and the resulting cell pellet was immediately 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). Briefly, 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 16,000 x g 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 within-day precision of 7.00% (Brien et al., 1983; Brien et al., 1987). The percent 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 quantifiable detection for DEA was 0.20 μg/mL (0.31 μM). The concentrations 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-FITC and Propidium Iodide Dual Staining**

HPL1A cells were stained with annexin-V-FITC (ann-V) to indicate apoptosis and propidium iodide (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, HPL1A cells in 12-well plates were pre-treated for 2 h with captopril in order to allow captopril to enter the cells and interact with angiotensin converting enzyme prior to the addition of AM or DEA. Following captopril pre-treatment, AM or DEA was added for an additional 24 h. Following 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 215 x g at room temperature. The supernatant was removed and the cell pellet 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 ann-V solution (BD Biosciences cat # 556419) 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 215 x g and the cell pellets resuspended in 0.5 mL PBS. Analysis was completed via flow cytometry (EPICS® ALTRA, Beckman Coulter, Mississauga, ON). 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 GraphPad Prism 5.00 (GraphPad Software, San Diego, CA). Analysis was performed on the mean values of triplicates (values from three individual wells) from three or four independent experiments.

**Modified Tdt-mediated dUTP Nick-End Labeling (TUNEL) Assay**

A modified fluorometric 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. Following 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, centrifuged at 215 x g at 4°C for 5 min and the cell pellet was resuspended in 0.5 mL cold PBS. Cells were fixed using 3.5 mL of 1% paraformaldehyde for 20 min on ice.
Following fixation, cells were centrifuged and resuspended in 0.5 mL of PBS. Ice-cold 70% ethanol (3.5 mL) was then added to the cell suspensions and samples were stored at -20°C overnight.

The following 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 rTdT 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 175 x g 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 175 x g 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

HPL1A cells in 12-well plates were treated with Ang II for 24 h. Following 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 HPL1A cells using the Qiagen 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 (260nm/280nm) 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 ± standard error of 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 GraphPad Prism 5.00 (GraphPad Software, San Diego, CA) and statistical significance was defined as $p < 0.05$. 
Results

Conversion of Amiodarone to Desethylamiodarone in HPL1A Cells

Treatment of HPL1A cells with 5 - 20 μM AM for 24 h resulted in minimal production of DEA; with 5 μM AM, 1.86% ± 0.42% of AM was converted to DEA, while 0.99% ± 0.36% and 0.92% ± 0.35% of AM was converted to DEA after 10 μM and 20 μM treatment respectively. Treatment of HPL1A 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 (ie. < 0.31 μM), confirming that the conversion of AM to DEA occurred during the 24 h incubation and was not artifactual. The recovery of AM from the cell pellet following 24 h incubation was ≥ 85% for the concentrations of AM tested.

AM- and DEA-Induced Necrosis and Apoptosis

Treatment of HPL1A cells with 1-20 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) (Figure 1 A and B). Treatment with 10 μM or 20 μM AM for 24 h increased the percentage of cells that were PI positive over three-fold and six-fold respectively, relative to control (Figure 1 C). However, at 24 h, a significant decrease in ann-V positive cells was observed only with 20 μM AM (Figure 1 C).

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 (Figure 2 A and B). At 24 h, the percentage of cells that were PI positive increased two-fold and over six-fold after incubation with 3.5 and 5 μM DEA respectively, compared to control, while a significant
increase in ann-V positive cells occurred with 3.5 μM DEA (Figure 2 C). 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 μM 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 ~LC44 for each drug (AM LC = 44.9 ± 2.92; DEA LC= 42.4 ± 3.78). Representative dot plots obtained from flow cytometric analysis of ann-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 (Figure 3).

Flow cytometric dot plots from TUNEL staining (Figure 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 – 65% (Figure 4 E and F). In contrast to quantification of apoptosis by ann-V staining, treatment with 1 – 20 μM AM or 1 – 3.5 μM DEA for 24 h caused no change in apoptosis as determined by the percentage of TUNEL positive cells (Figure 4 A, C, E and F), but 5 μM DEA increased TUNEL positive cells from 4.31% ± 2.00% (control) to 26.7% ± 3.72% (Figure 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 hours caused a trend towards an increase in angiotensinogen levels, which fell just short of statistical
significance ($p = 0.0504$, 1-way ANOVA, Figure 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. Following 24 h treatment of HPL1A cells with $100 \text{pM} - 1 \mu M$ 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% - 97.8%. Light microscopic examination also did not reveal any apparent damage to the cells.

To assess effects of Ang II on apoptosis and necrosis induced by AM and DEA, ann-V and PI dual staining was carried out. As expected, the percentage of PI positive cells increased over two-fold after 24 h treatment with $10 \mu M$ AM compared to control, while no change was seen in ann-V positive cells (Figure 6 A). Treatment for 24 h with $3.5 \mu M$ DEA increased PI and ann-V positive cells (Figure 6 B). However, co-incubation of HPL1A cells for 24 h with $10 \mu M$ AM or $3.5 \mu M$ DEA plus $100 \text{pM} - 1 \mu M$ Ang II caused no significant alterations in PI or ann-V positive cells compared to AM or DEA alone (Figure 6 A & 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 (Figures 2 and 6), PI positive cells increased over three-fold after 24 h treatment with $10 \mu M$ AM compared to control, while no change occurred in ann-V positive cells (Figure 7
A). Treatment of cells for 24 h with 3.5 μM DEA increased the percentage of PI and ann-V positive cells (Figure 7 B). Pre-treatment of cells for 2 h with 3 – 6 μM captopril, prior to addition of 10 μM AM or 3.5 μM DEA, caused no significant change in percentages of PI or ann-V positive cells compared to AM alone or DEA alone (Figure 7 A and B).
Discussion

DEA has greater cytotoxic potency than AM and accumulates in tissues to a greater extent than AM following chronic 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 due solely to AM, but also to DEA. After chronic therapy, up to 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 of mRNA and protein of these enzymes in human lung (Mace et al., 1998; Nishimura et al., 2003).

In HPL1A cells, less than 2% of AM was converted to DEA over 24 h. The fact that the percent conversion of AM to DEA decreased with increasing AM concentration was likely due to the direct cytotoxicity of AM at the higher concentrations rather than saturation of biotransforming enzymes, since the $K_M$ value for cytochrome P450 3A production of DEA in liver microsomes is 0.33 mM (Fabre et al., 1993), a concentration well above those used in this study. 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 (Bolt et al., 2001a; Broekhuysen et al., 1969; Nicolescu et al., 2008; Wilson and Lippmann, 1990), 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 hours, but not at 24 hours 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, etc. 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 non-transformed 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 following 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 (Figures 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 (Figure 2C and Figure 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, while DEA induces both necrotic and apoptotic cell death despite being the more potent cytotoxicant. DEA caused an increase in PI positive cells prior to an increase in ann-V positive cells. Since PI enters cells when the plasma membrane becomes permeable during necrosis, this suggests that DEA-induced necrosis occurred prior to 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 Figure 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., 1999b). 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 which is diminished by the ACE inhibitor captopril (Uhal et al., 1998; Wang et al., 1999a). In the present study, treatment of HPL1A cells with AM or DEA caused a non-significant trend towards 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 pharmacotherapy (Nonoguchi et al., 2008) and which are inhibitory in other system (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 well

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within the range found in lungs of patients treated clinically with AM (Brien et al., 1987; Plomp et al., 1984). Therefore, a RAS-independent mechanism appears to be responsible for AM and DEA cytotoxicity in non-transformed human lung epithelial cells, but our findings do not preclude the involvement of Ang II and the RAS in the progression of AIPT in vivo.
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Authorship Contributions

*Participated in research design:* Mulder, Brien, Racz, Massey

*Conducted experiments:* Mulder

*Contributed new reagents or analytical tools:* Takahashi

*Performed data analysis:* Mulder

*Wrote or contributed to the writing of the manuscript:* Mulder, Brien, Massey
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Footnotes

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Legends for Figures

**Figure 1** Percentage of HPL1A cells that were PI positive (necrotic) and ann-V positive (apoptotic), following incubation with AM for: (A) 6; (B) 12; or (C) 24 h. * significantly different from vehicle control, p<0.05 (repeated measures 1-way ANOVA with student Newman-Keuls post hoc test; n=4)

**Figure 2** Percentage of HPL1A cells that were PI positive (necrotic) and ann-V positive (apoptotic), following incubation with DEA for: (A) 6; (B) 12 or (C) 24 h. * significantly different from vehicle control, p<0.05 (repeated measures 1-way ANOVA with student Newman-Keuls post hoc test; n=4)

**Figure 3** Representative dot plots depicting the percentage of HPL1A cells that were ann-V positive (apoptotic) and PI positive (necrotic) after incubation with: (A) vehicle control (24 h); (B) 20 μM AM (24 h); or (C) 3.5 μM DEA (24 h). 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 (Figures 1 and 2).

**Figure 4** 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: (A) vehicle control (24 h); (B) positive control (0.03% H₂O₂, 30 min); (C) 20 μM AM (24 h); or (D) 5 μM DEA (24 h). The percentages of apoptotic cells from triplicates of three independent experiments were transposed to
bar graphs, shown in (E) and (F) for AM and DEA treatment respectively. * significantly different from vehicle control, p<0.05 (repeated measures 1-way ANOVA with student Newman-Keuls post hoc test; n=3)

**Figure 5** Angiotensinogen mRNA levels relative to vehicle control after 24 h treatment with AM or DEA, as detected by quantitative real-time PCR. No statistically significant effect was observed between treatments and vehicle control (repeated measures 1-way ANOVA; n=4)

**Figure 6** Percentage of HPL1A cells that were PI positive (necrotic) and ann-V positive (apoptotic) after 24 h treatment with: (A) Ang II plus 10 μM AM; or (B) Ang II plus 3.5 μM DEA. # significantly different from AM or DEA alone (repeated measures 1-way ANOVA with student Newman-Keuls post hoc test; n=3)

**Figure 7** Percentage of HPL1A cells that were PI positive (necrotic) and ann-V positive (apoptotic) after 2 h pre-treatment with captopril, followed by addition of: (A) 10 μM AM; or (B) 3.5 μM DEA for 24 h. # significantly different from AM or DEA alone (repeated measures 1-way ANOVA with student Newman-Keuls post hoc test; n=3 for (A) and n=4 for (B))
Figure 1

(A) % PI positive cells

Vehicle 1 μM 2.5 μM 5 μM 10 μM 20 μM

AM (6 hours)

(B) % PI positive cells

Vehicle 1 μM 2.5 μM 5 μM 10 μM 20 μM

AM (12 hours)

(C) % PI positive cells

Vehicle 1 μM 2.5 μM 5 μM 10 μM 20 μM

AM (24 hours)
Figure 2

(A) % PI positive cells

Vehicle 1 μM 2 μM 3.5 μM 5 μM

DEA (6 hours)

(B) % PI positive cells

Vehicle 1 μM 2 μM 3.5 μM 5 μM

DEA (12 hours)

(C) % PI positive cells

Vehicle 1 μM 2 μM 3.5 μM 5 μM

DEA (24 hours)

* indicates significant difference from vehicle control.
Figure 3

(A) PI Fluorescence (610 +/- 20nm) vs. AnnexinV Fluorescence (525 +/- 20nm)

(B) PI Fluorescence (610 +/- 20nm) vs. AnnexinV Fluorescence (525 +/- 20nm)

(C) PI Fluorescence (610 +/- 20nm) vs. AnnexinV Fluorescence (525 +/- 20nm)
Figure 5

Angiotensinogen mRNA (relative to vehicle control)

Vehicle  AM2.5 μM  DEA 1 μM  DEA 2 μM
Figure 7

(A) % PI positive cells

Vehicle 6 μM
Captopril 6 μM
AM 10 μM
Captopril + AM 10 μM

(24 hours)

(B) % PI positive cells

Vehicle 6 μM
Captopril 6 μM
DEA 3.5 μM
Captopril + DEA 3.5 μM

(24 hours)

(24 hours)