Mechanisms for WSCM Induced Cytotoxicity to Human Aortic Endothelial Cells

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Abstract

Chronic exposure to cigarette smoke can lead to endothelial dysfunction and potentially endothelial cell death, which is often associated with atherosclerosis. Here, we used Human Aortic Endothelial Cells (HAECs) from four healthy donors to determine smoke-induced cytotoxicity. HAECs were exposed to whole smoke conditioned media (WSCM) generated from 3R4F reference cigarettes over a range of 0-8000 ng/mL nicotine equivalence (n.e.) concentrations.

Cytotoxicity was evaluated 24 hours post-exposure via neutral red uptake (NRU) and/or reduction of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to formazan for each exposure concentration and compared to vehicle control. The IC50 was then calculated from the concentration-response. Similar IC50 values were observed in both the NRU and MTT assays for the four donors across the dose range of 0-8000 ng/mL n.e. of WSCM.

To examine the mechanism responsible for WSCM-induced cytotoxicity in HAECs at varying n.e. concentrations, necrosis (propidium iodide: PI) and apoptosis (Annexin V) markers were assessed via flow cytometry. Annexin V-positive cell populations increased in a dose-dependent manner while increases in PI-positive cell populations only occurred at the highest doses of WSCM (5000-8000 ng/mL n.e.). To further investigate the induction of apoptotic cells after WSCM exposure, we conducted Western blotting experimentation using caspase-3 cleavage as a marker for apoptosis. Western blots confirmed that apoptosis occurs at the higher concentrations of WSCM which coincide with reduced HAEC survival.

We conclude that WSCM produces comparable cytotoxicity *in vitro* when applied to HAECs from four donors as assessed using both the NRU and MTT assays. Additionally, the mechanism of toxicity appears to be dose-dependent and conserved among the four donors as assessed by distinct measures for apoptosis.

Materials and Methods

- Human Aortic Endothelial Cells (HAECs) were sourced from Lifeline Cell Technology, Lonza and Promocell. All cells were maintained using VascuLife® VEGF Endothelial Complete Medium.
- Whole smoke conditioned media (WSCM) was generated from four Kentucky Reference 3R4F cigarettes smoked using a Borgwaldt RM200 smoking machine under ISO regime bubbled into 20mL of cell media using glass impingers containing 6g of 3mm glass beads. The WSCM was filtered at 0.10 µm with the final solution containing 2% BSA. The final solution was analyzed for nicotine concentration using GC-FID.
- Cells were exposed to 3R4F WSCM for a duration of 24hrs. The NRU and MTT assays were subsequently conducted. Vehicle control cells were exposed to cell media and positive control cells were exposed to 100 µg/mL sodium dodecyl sulfate (SDS) or 68 µg/mL H_2O_2. Phototox (validated software referenced in OECD guideline 432) was used to calculate the IC50 values.
- Apoptosis was evaluated using an Annexin V-FITC apoptosis detection kit (Sigma-Aldrich) following manufacturer's instructions with subsequent fluorescence measured using a FACSCanto™ II flow cytometer. For each cell donor and each cell exposure, the percentage of cells in each graph quadrant was determined. These data were used to calculate the increase or decrease in the percentage of cells in the exposure group compared to the concurrent vehicle control. The mean change was calculated from each quadrant and exposure condition from three independent experiments for each donor and graphed.
- For western blotting, protein was loaded onto 4-12% Tris-Bis Gels (Invitrogen) and subjected to electrophoresis at 200V (120mA) for 1hr and transferred onto AmershamTM Protran® Nitrocellulose membranes (GE Healthcare) at 30V (125mA) for 1.5hrs. Membranes were blocked with 5% Blotting-Grade Blocker (BioRad) in TBS-T for 0.5hrs and then stored in 1% BSA TBS-T overnight at 4-8°C. Primary antibody incubations with rabbit anti-caspase-3 (1:1,000; Cell Signaling Technology®) and mouse anti-β-actin mAb (1:5,000; Cell Signaling Technology®) were conducted in TBS-T with 1% BSA for 4hrs at room temperature. Secondary antibody incubations with horseradish peroxidase conjugates (1:2500 Donkey anti-rabbit; 1:10,000 Donkey anti-mouse) were conducted in TBS-T for 1hr at room temperature. Membranes were visualised with SignalFireTM ECL (Cell Signaling Technology®) working solution and imaged using a LI-COR C-DiGit® Blot Scanner for chemiluminescence detection. Protein levels were quantified using Image StudioTM 5.x. Graphs represent caspase-3 quantification relative to β-actin levels.

Conclusions

- WSCM produces comparable cytotoxicity *in vitro* when applied to HAECs from four donors as assessed using both the NRU and MTT assays (Figure 1 and Table 1).
- The mechanism of toxicity appears to be dose-dependent and conserved among the four donors as assessed by distinct measures for apoptosis. (Figure 2 and 3).

Results

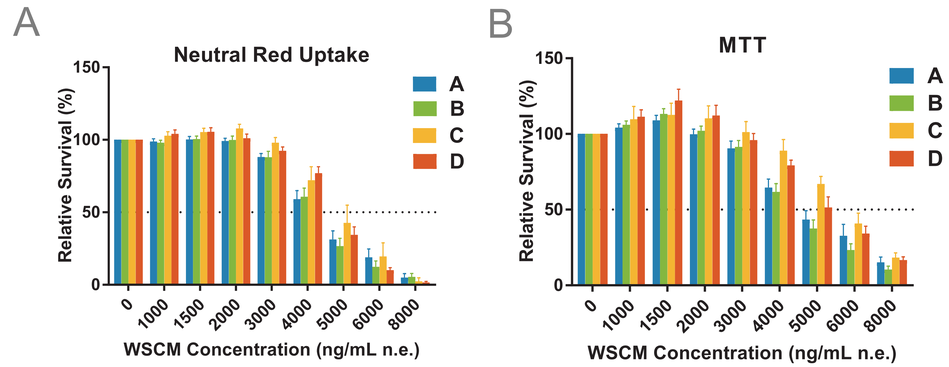

Figure 1. The relative survival of HAECs (% of control) after exposure to a range of WSCM (0-8000 ng/mL n.e.) was determined by two cytotoxicity assays: A. Neutral Red Uptake assay performed in four donor lines (A-D). B. MTT assay performed in four donor lines (A-D). Nicotine Equivalence (n.e.).

	A		B		C		D	
	NRU	MTT	NRU	MTT	NRU	MTT	NRU	MTT
n	24	19	18	19	9	8	9	8
IC50	4582	4885	4587	4584	4993	5749	4691	5228
SD	1074	1341	951	806	1239	783	402	593
Low	3435	3273	3402	3649	3575	4794	4050	4415
High	6805	7288	7838	6483	7315	7053	5227	6034

Table 1. IC50 Means, SD, Low and High Values (ng/mL n.e.).

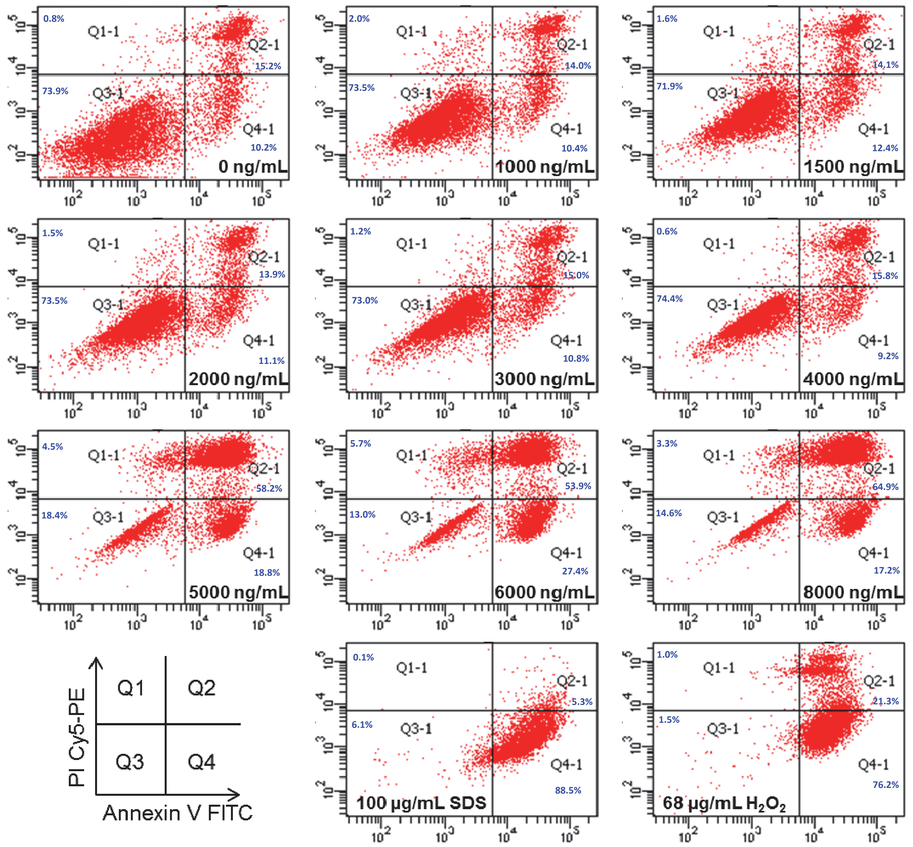

Figure 2. Annexin V (AV)-Propidium Iodide (PI) representative flow cytometry data. The inset concentrations show the nicotine equivalent concentration of WSCM or control. Percentages indicate mean cell population in the respective quadrants. Inlay diagram indicates quadrant designations: Q1 (PI positive; AV negative), Q2 (PI positive; AV positive), Q3 (PI negative; AV negative), Q4 (PI negative; AV positive).

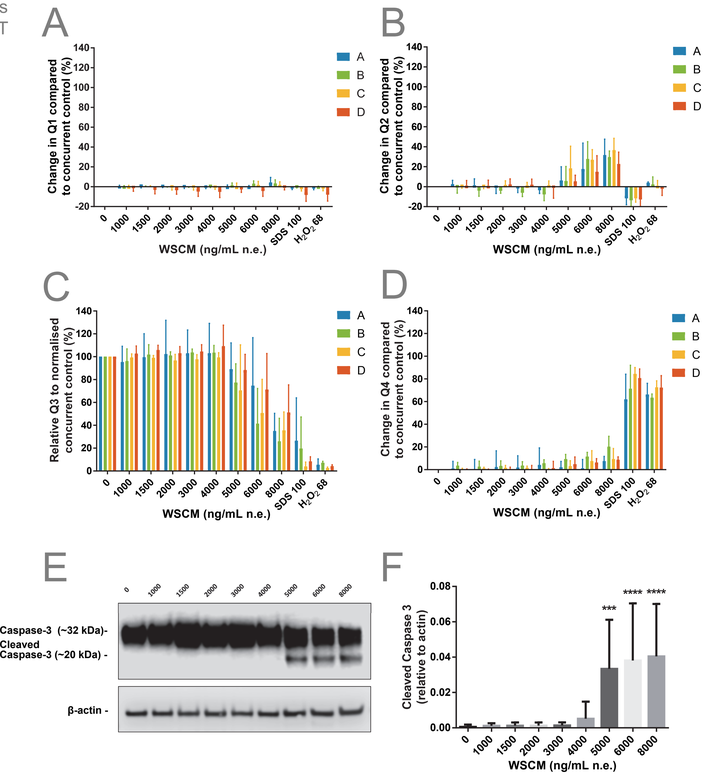

Figure 3. Mechanistic flow cytometry and Western blot data. Each graph (A-D) shows WSCM (1000-8000 ng/mL n.e) or control exposure relative to the concurrent vehicle control for each of the four quadrants as shown in Figure 2. Graphs A, B and D show percentage increase or decrease in the percentage of cells found in quadrant 1 (PI+AV-, necrosis), quadrant 2 (PI+AV+, late-stage apoptosis) and quadrant 4 (PI-AV+, early-stage apoptosis), respectively. Graph C shows the percentage of PI-AV- cells from quadrant 3 where the concurrent vehicle has been normalized to 100%. Representative Western blot (E) showing caspase-3 cleavage following WSCM (ng/mL n.e.) exposures, quantified ratio relative to β-actin loading control (F). *** p≤0.001, **** p≤0.0001.

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