Vascular endothelial peptide oxidation leading to hypertension: development of an AOP using *in vitro* assays

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Introduction

An adverse outcome pathway (AOP) is a framework that characterises a chain of mechanistically linked biological events at the molecular, cellular, tissue, organ, whole body and population level following exposure to a given chemical, which ultimately leads to an adverse outcome of interest.
We have recently mapped out an AOP focussing on key events associated with vascular oxidative stress leading to the development of hypertension. As cigarette smoking is a known inducer of vascular oxidative stress and hypertension, we studied the effects of cigarette smoke extract in vascular endothelial cells at the molecular and cellular level of the AOP *in vitro*. The purpose of the work was to determine if exposure to cigarette smoke toxicants led to measurable perturbations in the AOP key events, which could serve as a baseline for the future comparative assessment of next generation tobacco and nicotine products (NGPs).

Methods

Cigarette smoke aqueous extract (CSE) preparation
CSE was prepared by bubbling 3R4F into 10 mL of 1% FBS-containing DMEM growth medium without phenol red as previously described[3]. CSE was pH corrected (7.4) and QC evaluation at 320 nm. Vehicle control medium was made by bubbling filtered air through DMEM containing 1% FBS for one minute. CSE was freshly prepared and used within 15 minutes of preparation. Cells were exposed to CSE (0.625, 1.25, 2.5, 5, 7.5 & 10%) for 2 to 4 hours.

Cell culture
Bovine aortic endothelial cells (BAECs) were cultured in DMEM containing 1 mmol/L glucose supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/ mL streptomycin, 1 % non-essential amino acids, and 0.1% endothelial cell growth factor. Cells were maintained in a humidified incubator at 37° C and 5% CO_2 and used between passages 6-13.

Cytotoxicity assay (MTT)
BAECs were exposed to CSE, (0.625, 1.25, 2.5, 5, 7.5 & 10%) for 2 and 4 hours and cytotoxicity assessed using 5% MTT solution.. Absorbance was measured at 570 nm and cell viability expressed relative to the untreated control cells.

Western blot analysis
Following treatment, BAECs were lysed using ice-cold lysis buffer containing protease and phosphatase inhibitor cocktail. Equal protein amounts of cell lysate were loaded into a (4-20%) graded SDS- polyacrylamide gel. Proteins were electro-blotted on PVDF membranes. The membranes were blocked and incubated with primary antibodies overnight at 4˚C followed by incubation with secondary antibody-HRP linked followed by washing and subsequently developed using the Amersham ECL Western Blotting Detection Reagent.

HPLC determination of GSH and GSSG content
GSSG and GSH was measured in treated BAECs by HPLC .For GSSG determination, free thiols were blocked by incubation with 100 mM N-ethylmaleimide (NEM) and GSSG reduced by incubation with 100 mM DTT.

Immunofluorescence (IF) microscopy
eNOS-GSH protein radical adducts were visualised in treated BAECs by IF following staining mouse monoclonal anti-GSH and rabbit polyclonal anti-eNOS overnight at 4ºC, followed by secondary Alexa fluor-488- and Alexa fluor-568- conjugated antibody. 1µM DAPI was used to counterstain cellular DNA. Images were observed at 60X magnification by confocal microscopy.

Immunoprecipitation
Supernatants from treated BAECs were incubated with agarose-conjugated anti-eNOS antibody and assessed for eNOS by immunoblotting.

Measurement of superoxide from uncoupled eNOS
Cells were exposed to CSE 5% for 2 hours with or without treatment with 100 µM DTT for 15 minutes at the end of exposure followed by 10µM DHE incubation +/- SOD. DAPI was used as counter stain for the nuclei. Images were observed at 60X magnification by confocal microscopy.

Tetrahydrobiopterin and total biopterin quantification.
BH4 and total biopterin levels were assayed using HPLC .

EPR Spin-trapping measurement of NO production.
Fe-MGD spin trap was used to measure NO in treated cells. Spin-trapping measurements of NO were performed using a Bruker EPR spectrometer.

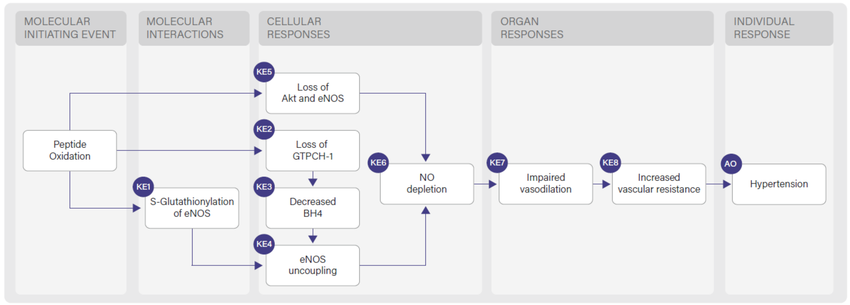
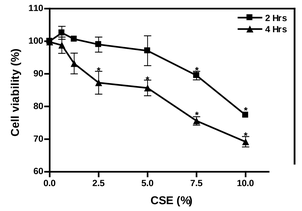


Figure 1. Schematic of the adverse outcome pathway, linking peptide oxidation to hypertension[1].
Abbreviations: KE (key event), AO (adverse outcome), eNOS (endothelial nitric oxide synthase), AKT (Protein kinase B), GTPCH-1 (guanosine triphosphate cyclohydrolase 1), NO (nitric oxide),
BH4 (tetrahydrobiopterin).

https://aopwiki.org/aops/149 [2]

Results

Figure 2. Exposure to cigarette smoke aqueous extract (CSE) reduced Bovine Aortic Endothelial Cell (BAEC) viability via MTT assay. Data presented as mean ± SEM of 3 independent experiments. * Significant difference from control at p<0.05.



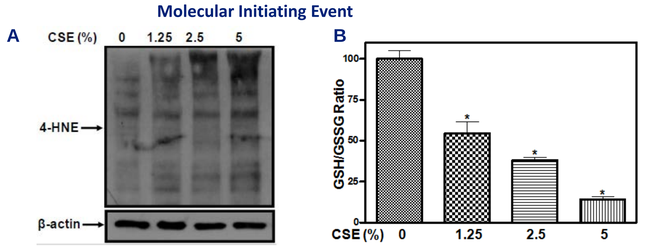


Figure 3: CSE exposure (A) Increased formation of BAEC 4-Hydroxynonenal (4-HNE) protein adducts and (B) Decreased the BAEC GSH/GSSG ratio. BAECs were exposed for 4hrs (4-HNE) and 2hrs (GSH/GSSG) Data presented as % baseline untreated control; mean ± SEM of three independent experiments. * Significant difference from control at $p<0.05$.

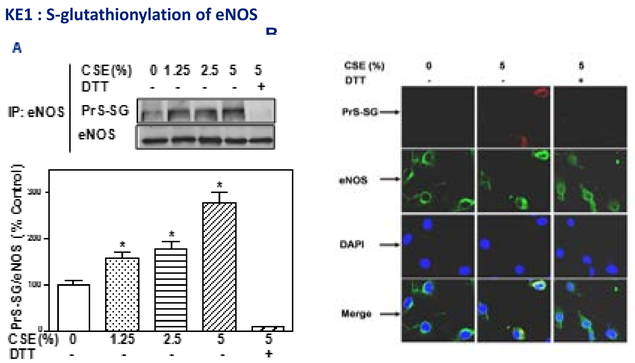


Figure 4. CSE exposure increased eNOS S-glutathionylation, which was reversed by incubation of the eluent with reducing agent Dithiothreitol (DTT). (A) Data represent means ± SEM of three independent experiments. *: Denotes significant difference from control at $p<0.05$. (B) Confocal fluorescence microscopic images

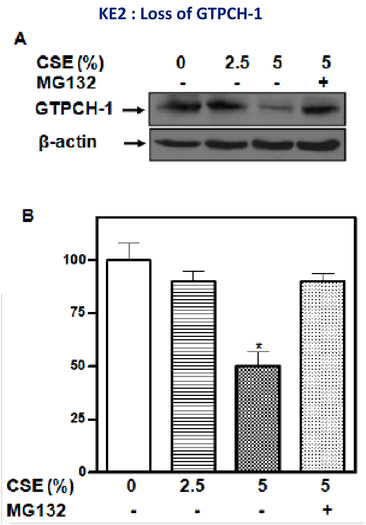


Figure 5. CSE depleted BAEC GTPCH-1 protein levels, which was reversible upon administration of 26S proteasomal inhibitor MG132. BAECs were exposed CSE for 4 hours and harvested for Western blotting against GTPCH, and β-actin. Data represent means ± SEM of three experiments. * p<0.05.

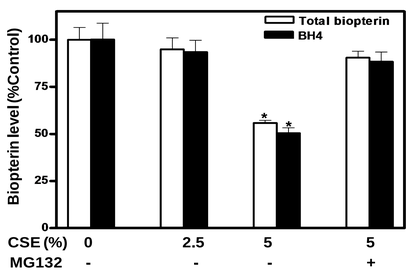


Figure 6: CSE depleted BAEC total biopterin and BH4. BAECs were exposed to CSE for 4 hours, and harvested for HPLC analysis. Data presented as % baseline untreated control; mean ± SEM of three experiments. *: Significant difference from control at p<0.05.

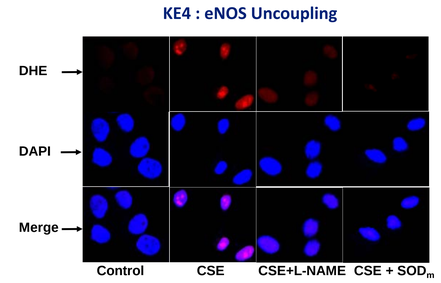


Figure 7: CSE induced BAEC eNOS uncoupling characterized by production of superoxide, which was inhibited by L-NG-nitroarginine methyl ester (L-NAME), and reversible by superoxide dismutase mimetic (SOD_m). BAECs were exposed to 5% CSE alone or with eNOS inhibitor L-NAME or SOD_m for 4 hours, incubated with Dihydroethidium (DHE), and visualized by confocal fluorescence microscopy.

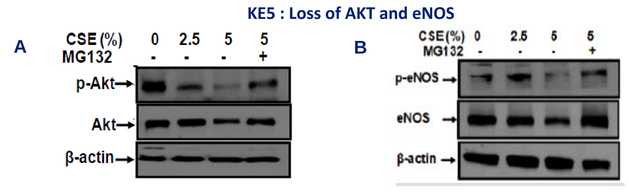


Figure 8: CSE decreased both phosphorylated AKT^{Ser473} and total AKT (A), and phosphorylated $eNOS^{Ser1179}$ and total eNOS protein expression (B), which was reversible upon administration of 26S proteasomal inhibitor MG132.

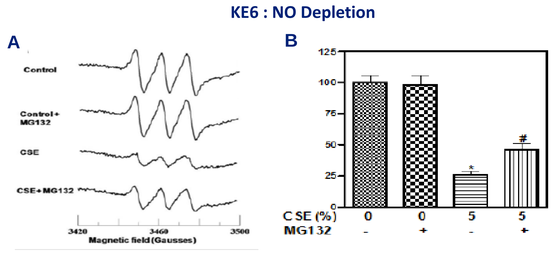


Figure 9: CSE decreased production of BAEC NO, which was partially reversible upon administration of 26S proteasomal inhibitor MG132. BAECs were exposed to 5% CSE for 4 hours, after which the spin trap Fe-MGD was added and NO was measured by EPR spectroscopy (A). Data are presented as % baseline untreated control; mean ± SEM of three experiments. *: Significant difference from control at p<0.05.

Conclusions

- CSE induced measurable perturbations of AOP key events *in vitro*
- The qualitative *in vitro* measures shown here could be suitable for future comparative assessments of NGPs
- Quantitative measurement systems are desirable to improve AOP-based risk assessment

References

•Lowe-FJ, Luettich-K, Talikker-M, Hoang-V, Haswell-LE, Hoeng-J and Gaca-MD. Development of an adverse outcome pathway for the onset of hypertension by oxidative stress-mediated perturbation of endothelial nitric oxide bioavailability. Applied in vitro Toxicology 2017, 3(1): 131-148
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