Vascular Endothelial Oxidative Stress 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).

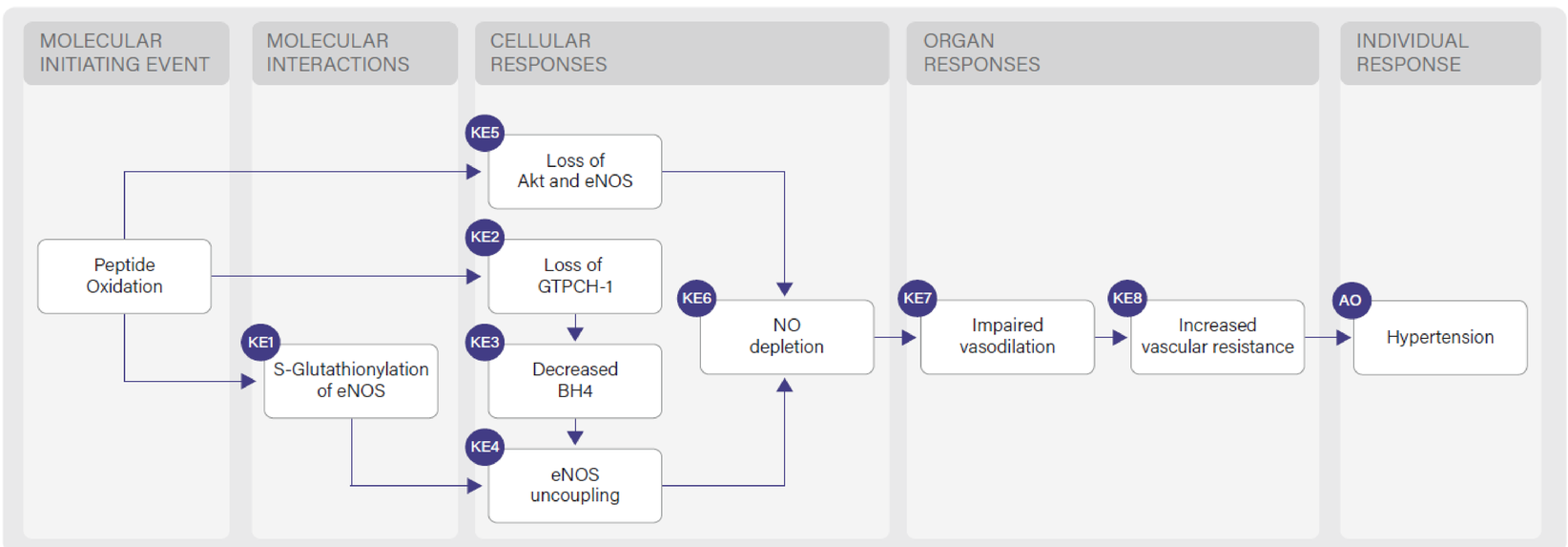


Figure 1. Schematic of the adverse outcome pathway, linking peptide oxidation to hypertension[1].

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

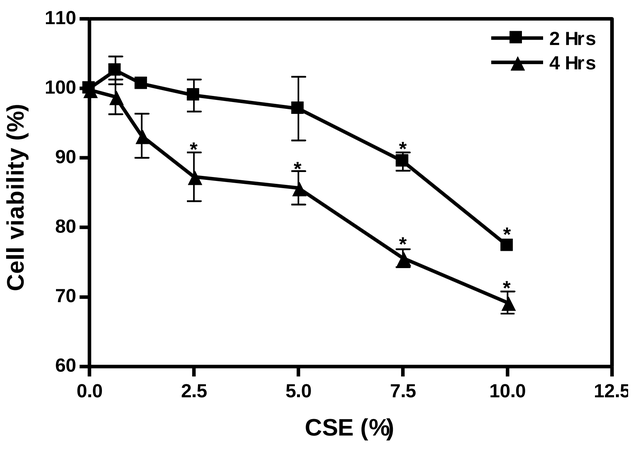


Figure 2. Exposure to cigarette smoke aqueous extract (CSE) reduced Bovine Aortic Endothelial Cell (BAEC) viability via MTT assay. Data presented as mean ± SEM of 3 independent experiments. * Significant difference from control at p<0.05.

Molecular Initiating Event

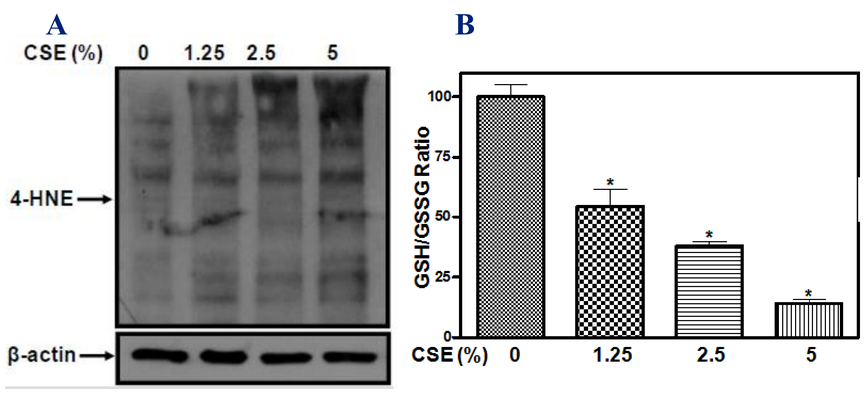
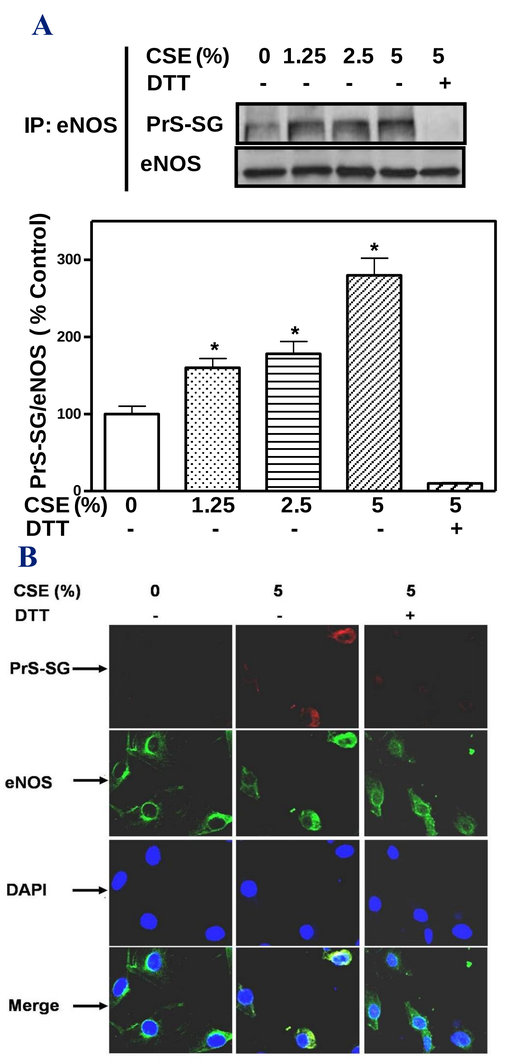


Figure 3: CSE exposure (A) Increased formation of BAEC 4-Hydroxynonenal (4-HNE) protein adducts and (B) Decreased the BAEC GSH/GSSG ratio. BAECs were exposed for 4hrs (4-HNE) and 2hrs (GSH/GSSG) Data presented as % baseline untreated control; mean ± SEM of three independent experiments. * Significant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

KE1 : S-glutathionylation of eNOS



KE2 : Loss of GTPCH-1

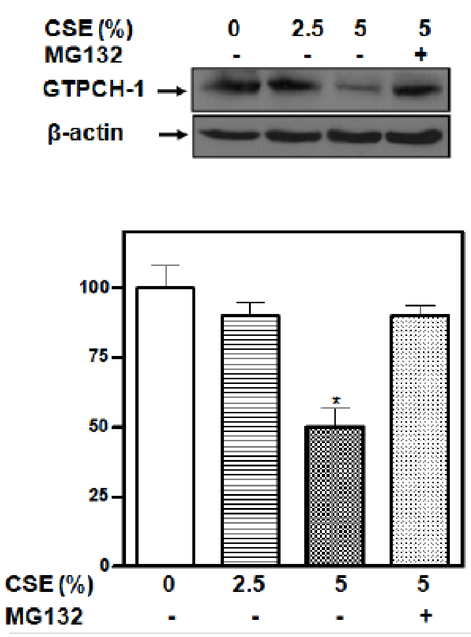


Figure 5. CSE depleted BAEC GTPCH-1 protein levels, which was reversible upon administration of 26S proteasomal inhibitor MG132. BAECs were exposed CSE for 4 hours and harvested for Western blotting against GTPCH, and β-actin. Data represent means ± SEM of three experiments.

* Significant difference from control at p<0.05.

KE3 : Decreased BH4

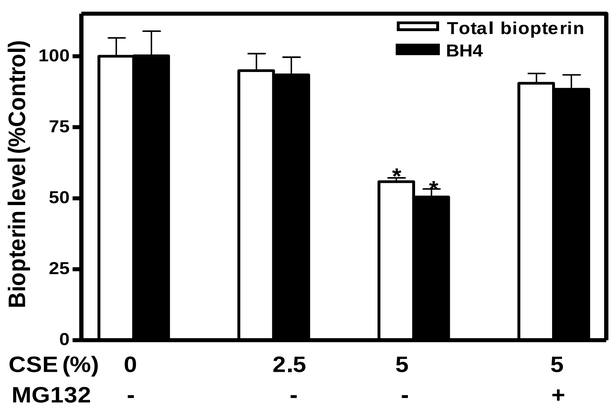


Figure 6. CSE depleted BAEC total biopterin and BH4. BAECs were exposed to CSE for 4 hours, and harvested for HPLC analysis. Data presented as % baseline untreated control; mean ± SEM of three experiments. *: Significant difference from control at p<0.05.

KE4 : eNOS Uncoupling

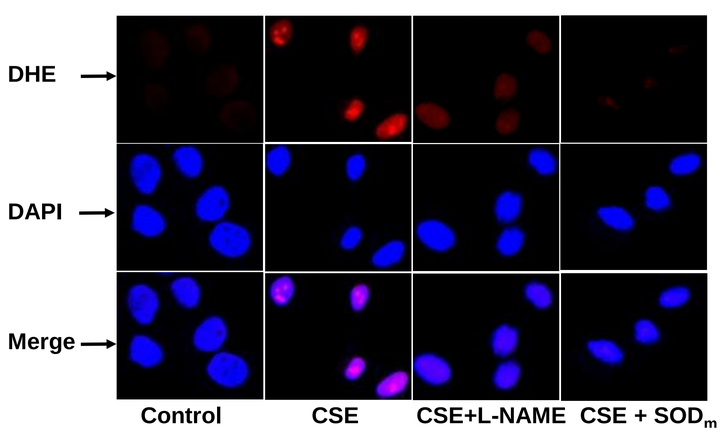


Figure 7. CSE induced BAEC eNOS uncoupling characterized by production of superoxide, which was inhibited by L-NG-nitroarginine methyl ester (L-NAME), and reversible by superoxide dismutase mimetic (SOD_m). BAECs were exposed to 5% CSE alone or with eNOS inhibitor L-NAME or SOD_m for 4 hours, incubated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

Figure 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

KE5 : Loss of AKT and eNOS

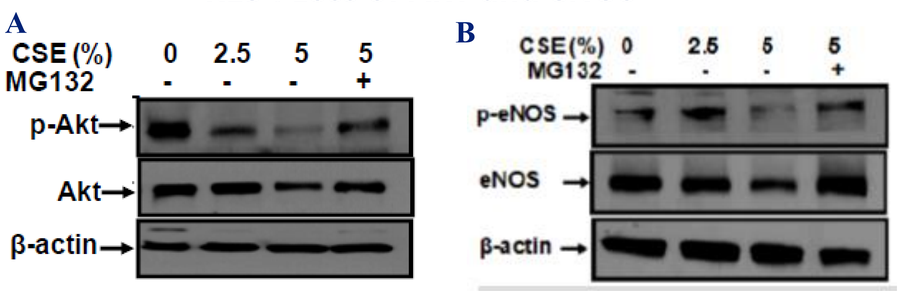


Figure 8. CSE decreased both phosphorylated AKT^{Ser473} and total AKT (A), and phosphorylated $eNOS^{Ser1179}$ and total eNOS protein expression (B), which was reversible upon administration of 26S proteasomal inhibitor MG132.

KE6 : NO Depletion

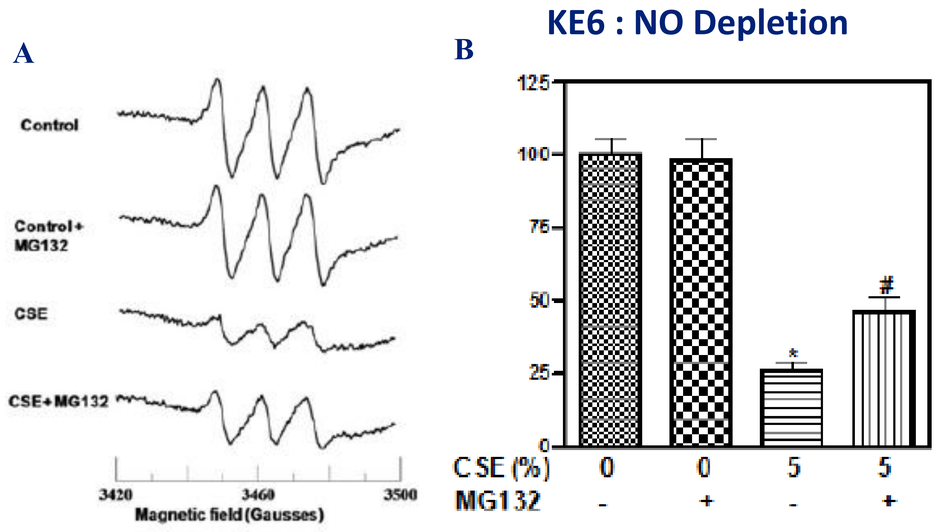


Figure 9. CSE decreased production of BAEC NO, which was partially reversible upon administration of 26S proteasomal inhibitor MG132. BAECs were exposed to 5% CSE for 4 hours, after which the spin trap Fe-MGD was added and NO was measured by EPR spectroscopy (A). Data are presented as % baseline untreated 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

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References

1. 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 (*in-press)*
2. Lowe-FJ *et al*. Peptide Oxidation to Hypertension AOP. https://aopwiki.org/aops/149