APPENDIX AVAILABLE ON REQUEST

Research Report 159

Role of Neprilysin in Airway Inflammation Induced by Diesel Exhaust Emissions

Simon S. Wong et al.

Appendix A. Uptake of DEP into Human BEAS-2B Cells Observed Using Transmission Electron Microscopy

Note: Appendices Available on the Web may appear in a different order than in the original Investigators' Report, and some remnants of their original names may be apparent. HEI has not changed the content of these documents, only the letter identifier.

Correspondence may be addressed to Dr. Simon S. Wong, Department of Pediatrics, College of Medicine, University of Arizona, 1501 N. Campbell Ave., Tucson, AZ 85724; e-mail: shengjun@email.arizona.edu

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Appendix A. Uptake of DEP into Human BEAS-2B Cells Observed Using Transmission Electron Microscopy

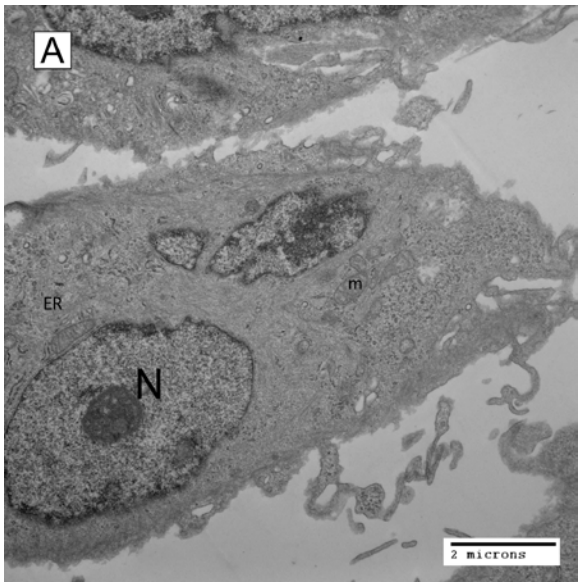
Uptake of DEP

Methods: Cultured BEAS-2B cells exposed to 0, 1, and 10 ug/cm^2 DEP (SRM 2975) was fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH7.4) for 30 mins, washed with buffer, and post-fixed 1% osmium teroxide. Cells were removed from the culture wells, pelleted and stained with 2% aqueous uranyl acetate. Following dehydration through alcohol series, pelleted cells were embedded in Spurr's resin. Ultrathin (50nm) sections were cut on an Ultracut UCT ultramicrotome onto uncoated 150 mesh copper grids and stained with 2% lead citrate. Sections were viewed in a CM12 transmission electron microscope (TEM) and images captured with a Macrofire digital camera and saved as tiff files.

Results: To understand that effects are required by particles taken-up, we conducted the experiments for uptake of DEP with transmission electron microscopy (TEM). The results showed that cultured BEAS-2B cells without DEP treatment presented normal mitochondria and endoplasmic reticulum as well as numerous keratin filaments (Figure 1). Cells treated with 1 or 10 µg/cm^2 DEP for 24 hrs showed that the particles can stick to the cell membrane suggesting an endocytosis of the DEP (Figures 2 and 3). Observations with 140,000X magnification revealed that single particle, loose clusters, and low density agglomerates in phagosomes (Figure 2C). The phagosomes were frequently found to be lodged in large cytoplasmic vacuoles, but occasionally contacted with the nuclear envelope, resulting in deformation of the nuleus. There were notable differences of size of phagosomes between the low and high concentrations of DEP treatments. Both DEP treatments showed obvious disruption to mitochondrial cristae and had a vacuolar appearance. TEM confirmed that DEP was phagocytized with in a particles-filled vesicle

fusing with different size of phagosomes (Figure 3B). It appeared that these ultrastructure alterations were attributed to endocytosis of DEP that was accumulated in large phagosomes, probably resulting from the fusion of several smaller vesicles. Therefore, ultrastructure of cells containing large amounts of DEP was clearly damaged, whereas cells containing small amounts of DEP seem to be mild changed. Necrotic cells that had widespread loss of cytoplasmic density and pronounced swelling of the SER were occasionally observed following 10 μg/cm^2 DEP treatment (Figure 3C).

Discussion: We also carried out the uptake experiment of DEP by the epithelial cells in vitro. It appeared that ultrastructural alterations were attributed to endocytosis of DEP that was accumulated in large phagosomes, probably resulting from the fusion of several smaller vesicles. Obviously, ultrastructures of cells containing large amounts of DEP were obviously changed, whereas cells containing small amounts of DEP seem to be mild changed. These ultrastructure alterations, especially particles-filled vesicle fusing with different size of phagosomes, could likely affect the molecular or cellular processes of down-regulation of the membrane-bound NEP by DEP. It is documented that the mechanisms underlying phagocytosis are complex involving numerous signaling molecules including motor proteins, kinases, adaptor molecules, and lipid-modifying enzymes in these processes.



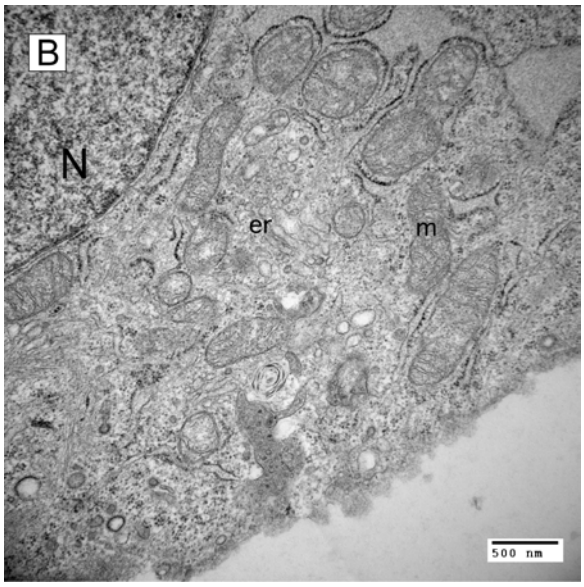
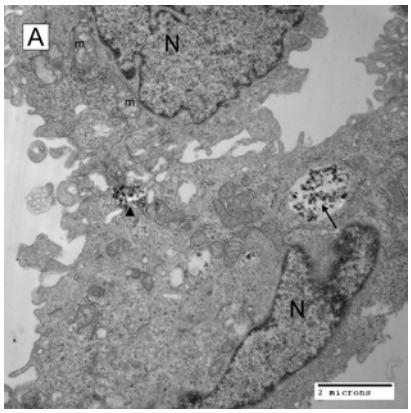
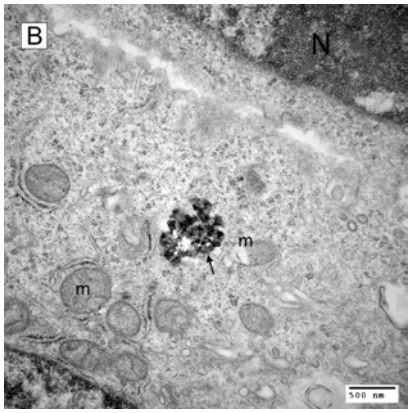


Figure 1. Transmission electronic micrographs (SEM) of BEAS-2B cells in controls (24 h). **A**: Normal the nuclear envelope (N), mitochondria (m), and endoplasmic reticulum (ER) of non-treated cells (88,000X magnification); **B**: Normal the nuclear envelope (N), mitochondria (m), and endoplasmic reticulum (ER) in non-treated cells.





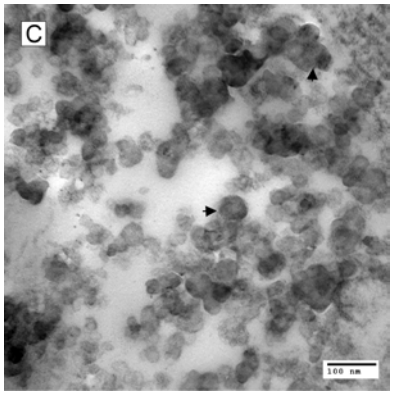


Figure 2. Transmission electronic micrographs (SEM) of BEAS-2B cells exposed to 1 µg/cm^2 of DEP for 24 h. **A**: Note the presence of a DEP-filled vesicle (arrows) and a swollen mitochondria (m) and endoplasmic reticulum (ER) (88,000X magnification); **B**: Note the presence of a DEP-filled vesicle (140,000X magnification); **C**: Note single particle, loose clusters, and low density agglomerates in phagosomes (250,000X magnification).

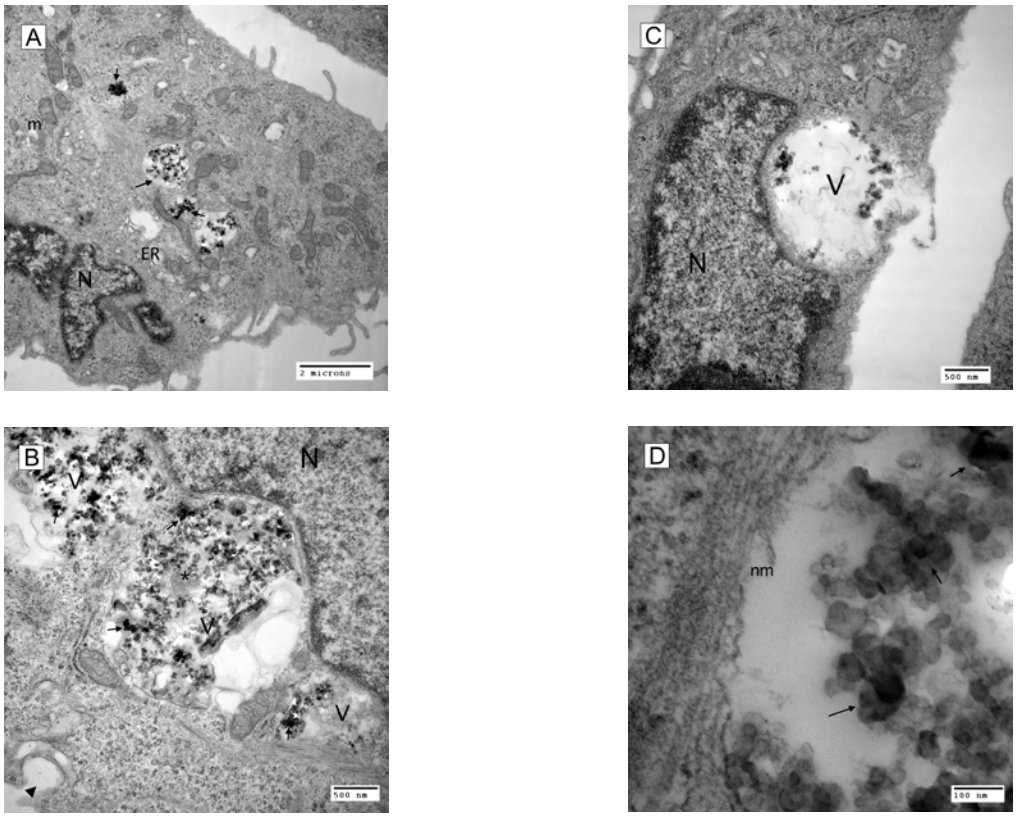


Figure 3. Transmission electronic micrographs (SEM) of BEAS-2B cells exposed to 10 µg/cm^2 of DEP for 24 h. **A**: DEP being hold by cell membranes of two epithelia (arrow head), and a large DEP-filled vesicle (arrow), and obvious swollen mitochondria (m) (88,000X magnification); **B**: Note three DEP-filled vesicles (V) fusing with a large phagosome (140,000X magnification); **C**: Accumulation of DEP in cytoplasmic vacuoles contacted closely with the nuclear envelope, resulting in disruption of the nuclear membrane (250,000X6 magnification). **D**: Note a necrotic cell that had widespread loss of cytoplasmic density and pronounced swelling of the ER (250,000X magnification).