

Introduction

Neurovascular pathologies such as a stroke or a transient ischemic attack result in the blockage of an artery that exposes of the tissues downstream to hypoxia.

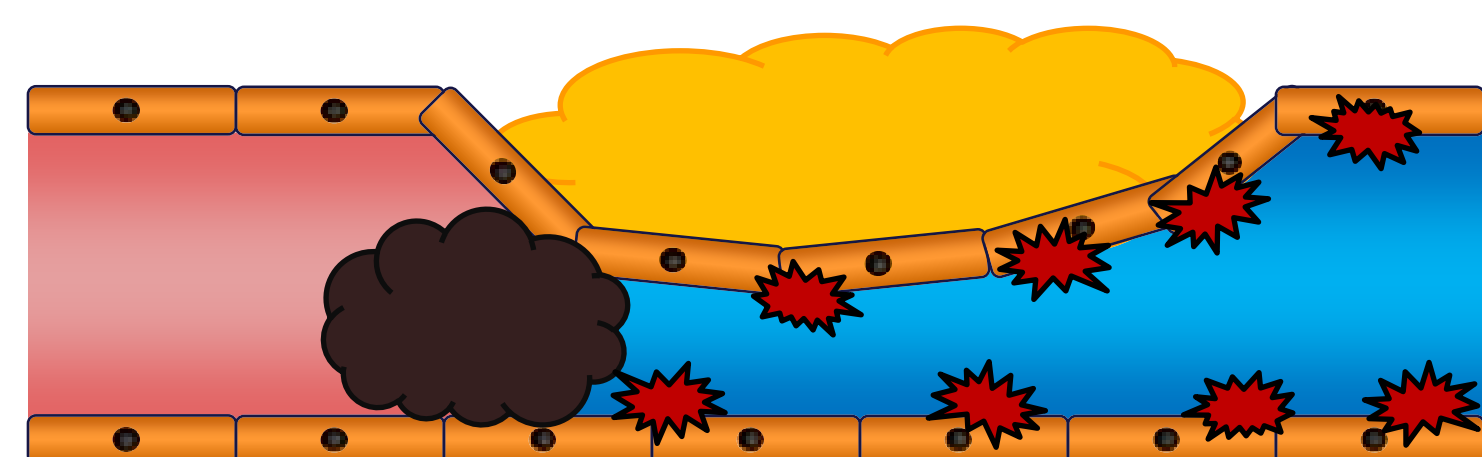


Figure 1: Ischemia/reperfusion injury. Blue shaded regions represent hypoxia in an occluded blood vessel. Due to this blockage, the endothelium lining the lumen is subjected to low oxygen tensions until the occlusion is removed.

The effects of ischemia/reperfusion on neurons has been studied extensively; however, the effects of ischemia/reperfusion on the endothelium are not yet well understood.

Ischemia/reperfusion is known to increase the levels of oxidative stress and lipid peroxidation in the affected tissues.

HNE is a reactive lipid which is produced in ischemia/reperfusion (at concentrations up to 50 μ M), and has been shown to alter bioenergetics in both isolated mitochondria and whole cells.

The Seahorse Bioscience XF24 Analyzer was used to measure the oxygen consumption of intact cells in response to hypoxia and reoxygenation. Using this novel method, we determined the effect of HNE on the cellular bioenergetics of vascular endothelial cells in hypoxia and reoxygenation.

Methods

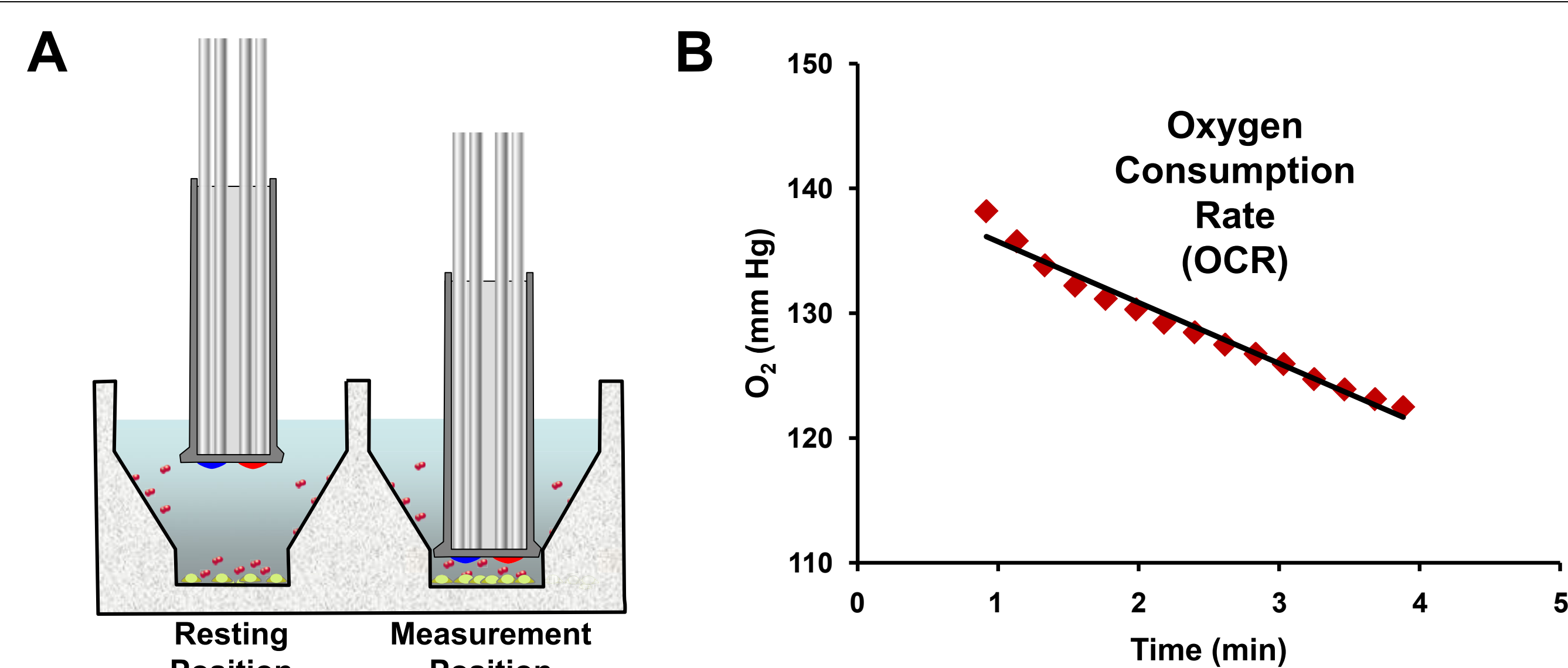


Figure 2: Seahorse XF24 Analyzer technology. The Seahorse Bioscience XF24 utilizes 24 probe heads to analyze cells in culture. Panel A shows a schematic depiction of the measurement chamber utilized in this technique. The first well shows the optics in the resting position. For measurements, the cartridge is lowered in all wells to create a transient 7 μ L chamber. The red dot at the tips of the sensor probes is representative of the fluorescent sensor quenched by oxygen to allow for measurement of this parameter. Monitoring these concentrations over time allows for the calculation of the Oxygen Consumption Rate (OCR) (B).

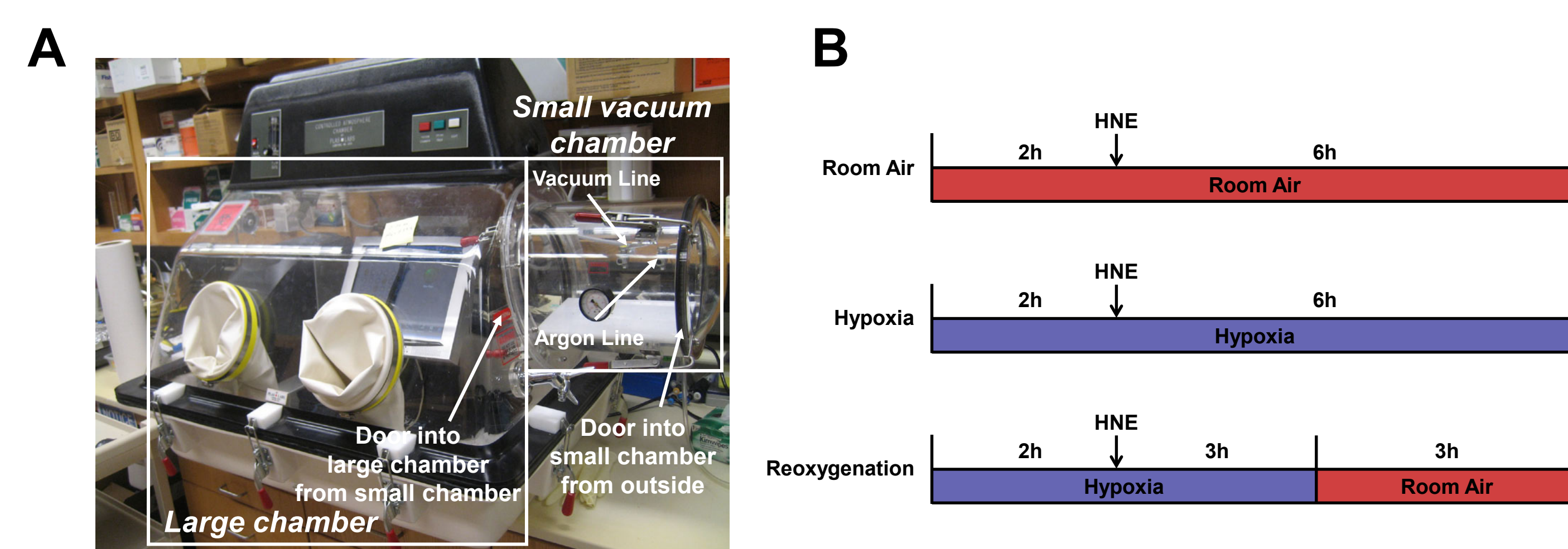


Figure 3: Seahorse Biosciences XF24 Analyzer in hypoxia chamber. The XF24 Analyzer was placed in a hypoxia chamber so that cellular bioenergetics could be measured in cells under hypoxia. The oxygen tension was set to 1% O₂ in the chamber for all experiments under hypoxia (A). The protocol for O₂ exposure to HNE (20 μ M), room air, hypoxia, and reoxygenation (B).

Results

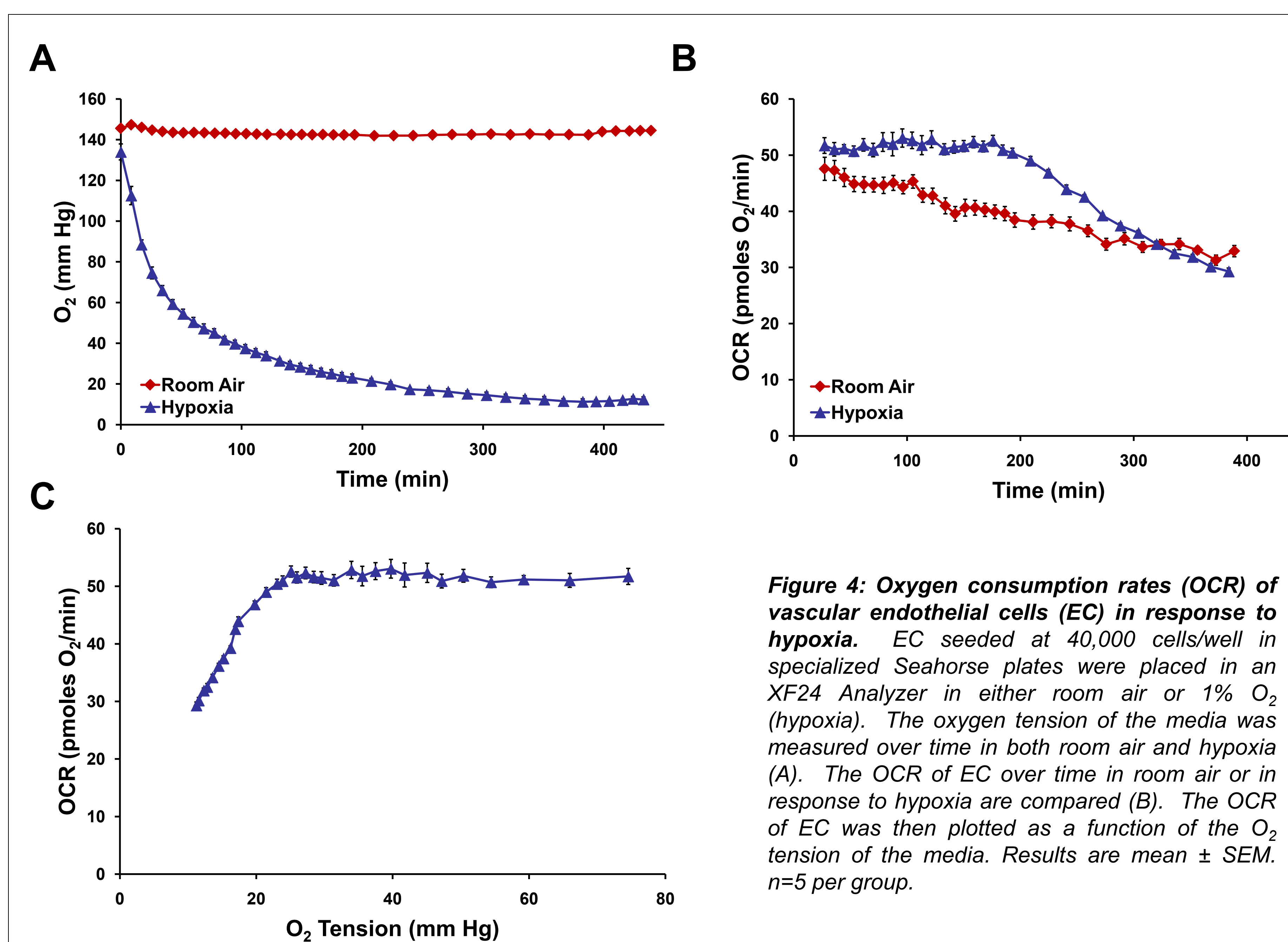


Figure 4: Oxygen consumption rates (OCR) of vascular endothelial cells (EC) in response to hypoxia. EC seeded at 40,000 cells/well in specialized Seahorse plates were placed in an XF24 Analyzer in either room air or 1% O₂ (hypoxia). The oxygen tension of the media was measured over time in both room air and hypoxia (A). The OCR of EC over time in room air or in response to hypoxia are compared (B). The OCR of EC was then plotted as a function of the O₂ tension of the media. Results are mean \pm SEM. n=5 per group.

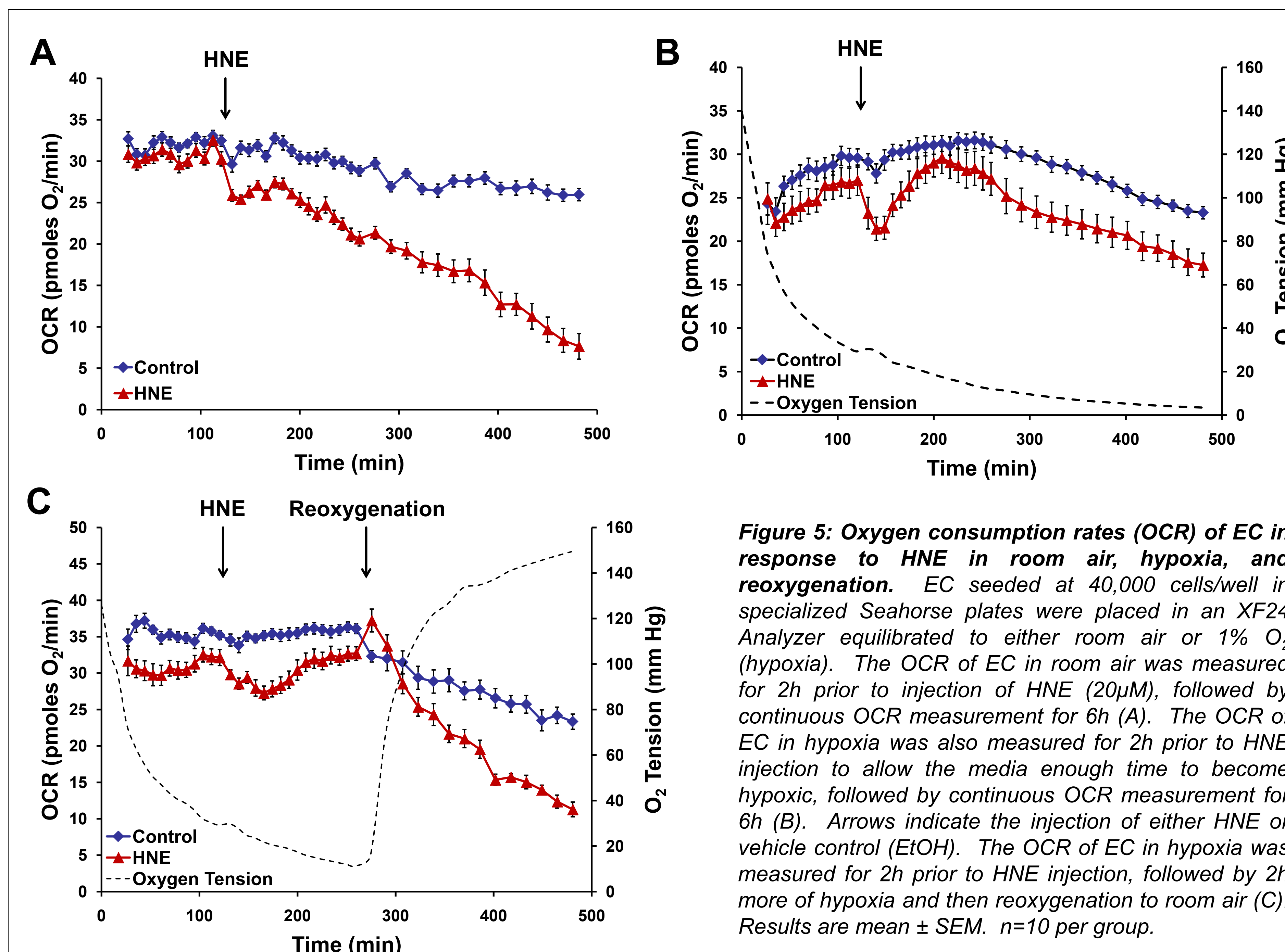


Figure 5: Oxygen consumption rates (OCR) of EC in response to HNE in room air, hypoxia, and reoxygenation. EC seeded at 40,000 cells/well in specialized Seahorse plates were placed in an XF24 Analyzer equilibrated to either room air or 1% O₂ (hypoxia). The OCR of EC in room air was measured for 2h prior to HNE injection, followed by continuous OCR measurement for 6h (A). The OCR of EC in hypoxia was also measured for 2h prior to HNE injection to allow the media enough time to become hypoxic, followed by continuous OCR measurement for 6h (B). Arrows indicate the injection of either HNE or vehicle control (EtOH). The OCR of EC in hypoxia was measured for 2h prior to HNE injection, followed by 2h more of hypoxia and then reoxygenation to room air (C). Results are mean \pm SEM. n=10 per group.

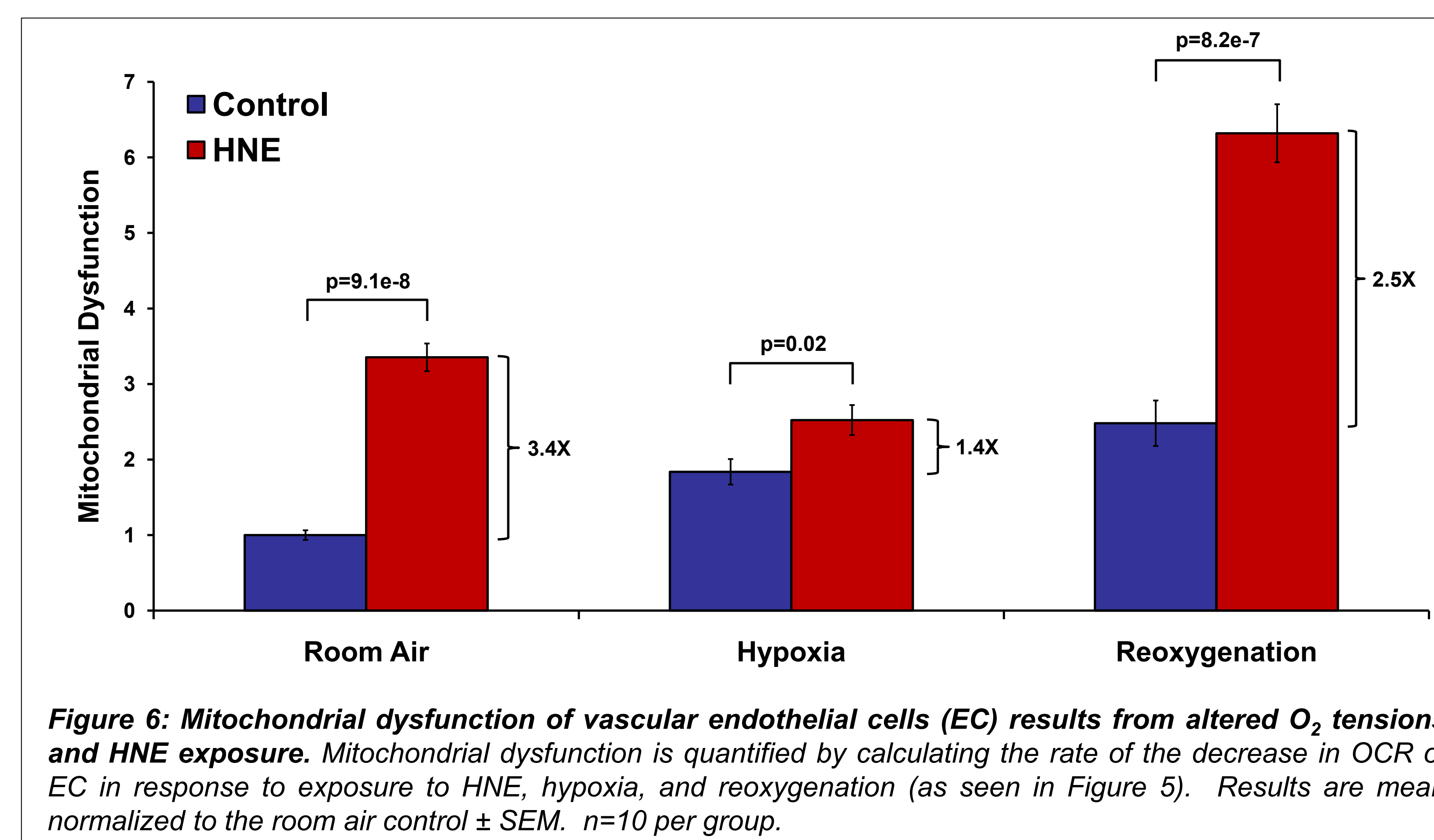


Figure 6: Mitochondrial dysfunction of vascular endothelial cells (EC) results from altered O₂ tensions and HNE exposure. Mitochondrial dysfunction is quantified by calculating the rate of the decrease in OCR of EC in response to exposure to HNE, hypoxia, and reoxygenation (as seen in Figure 5). Results are mean normalized to the room air control \pm SEM. n=10 per group.

Conclusions

- Using the XF24 Analyzer in hypoxia, we can measure the effects of O₂ tension on the OCR of intact cells.
- The OCR of EC varies depending on the O₂ tension of the media. This data can be fit to Michaelis-Menten enzyme kinetics, with an apparent V_{max} of ~55 pmoles O₂/min and an apparent K_m of 10.33 mmHg O₂ (14.54 μ M O₂).
- EC treated with HNE in normoxic conditions exhibited a decrease in O₂ consumption below vehicle control.
- Hypoxia attenuated the HNE-induced decrease in O₂ consumption in EC, particularly at O₂ tensions at which the concentration of O₂ became limiting to mitochondrial respiration.
- When exposed to hypoxia for 4h and then reoxygenated, EC exhibited a decrease in OCR. This is independent of the decrease seen in EC which remain in hypoxia for the full 8h, as they were reoxygenated prior to the point at which O₂ became limiting.
- The exposure of EC to HNE prior to reoxygenation induced a significant increase in mitochondrial damage over the damage caused by reoxygenation alone.
- Taken together, these data suggest that effect of HNE modifications on mitochondrial function is dependent on the concentration of O₂.
- In addition, these data show that hypoxia/reoxygenation can induce mitochondrial dysfunction in endothelial cells in vitro, and that exposure to electrophilic stress, which is known to be produced in ischemic pathologies, exacerbates this mitochondrial dysfunction. This may provide important insight into the biological effects of lipid electrophiles generated in ischemic neurovascular pathologies.