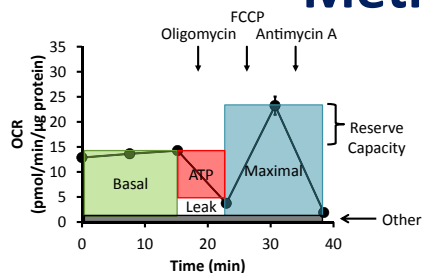


## Introduction

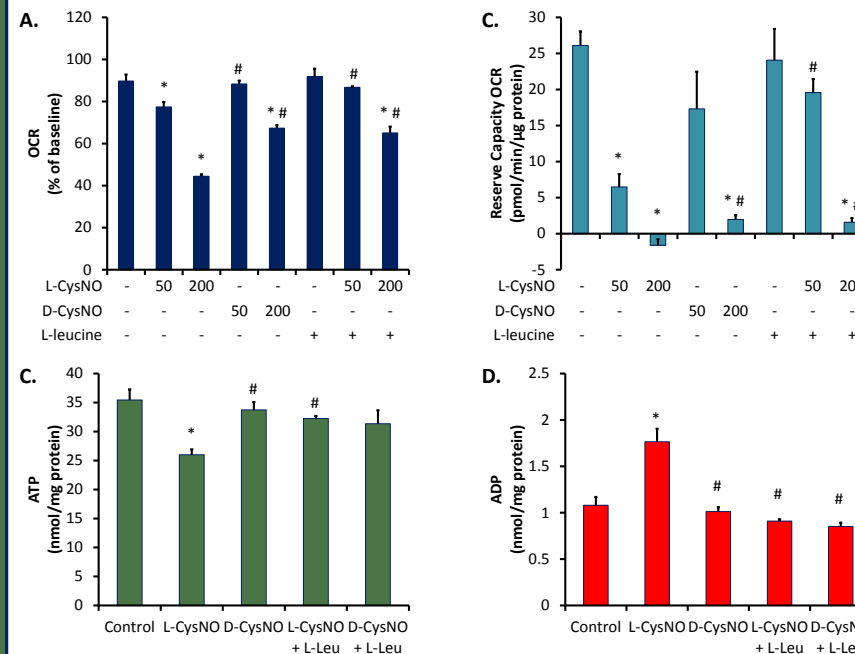
- S-Nitrosation of protein thiols is thought to be an important element of nitric oxide (NO)-dependent signaling mechanisms in vascular physiology and pathology.
- Multiple metabolic enzymes are targets for S-nitrosation (e.g. GAPDH and Complex I), and modification inhibits their activity.
- The intracellular nitrosating agent S-nitroso-L-cysteine (L-CysNO) is transported into cells, initiates S-nitrosation *in vitro*, and has been used as a model for nitrosative stress.
- L-leucine is a competitive inhibitor of L-CysNO uptake, and the D isomer of CysNO (D-CysNO) is not efficiently transported into cells.
- Here, we examine the effects of S-nitrosation on integrated metabolism in endothelial cells using extracellular flux technology.

## Methods



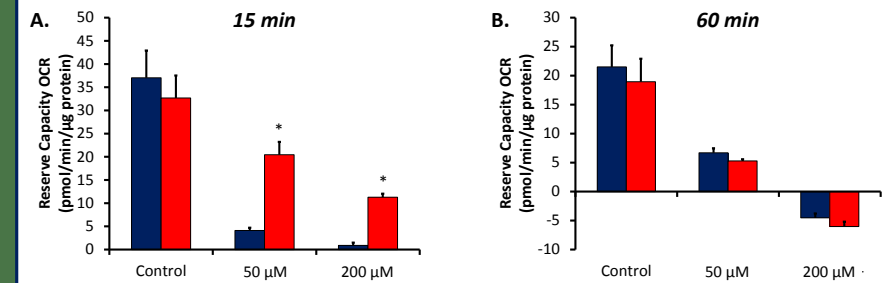
**Figure 1: Measurement of bioenergetic function parameters using extracellular flux technology.** A schematic representation of the mitochondrial function assay is shown. After a baseline oxygen consumption rate (OCR) is established, sequential injections of oligomycin, FCCP, and Antimycin A allow for determination of the multiple mitochondrial function parameters including basal OCR, ATP-linked respiration (ATP), proton leak OCR (Leak), maximal OCR, reserve capacity, and oxygen consumption independent of Complex IV (Other).

## Effect of CysNO uptake on mitochondrial function

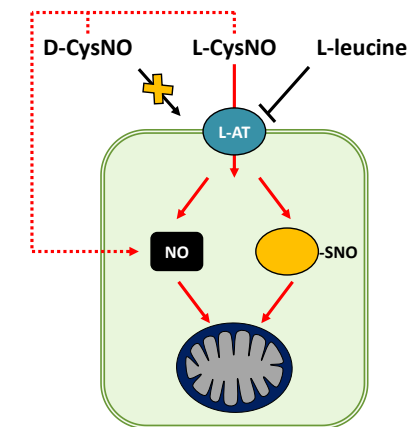


**Figure 3: Effect of CysNO uptake on mitochondrial function and adenine nucleotides in BAEC.** OCR was monitored for 1 h after the addition of L-CysNO (50 and 200 μM), D-CysNO (50 and 200 μM), or L-CysNO (50 and 200 μM) ± L-leucine (8 mM). Sequential injection of oligomycin (1 μg/mL), FCCP (1 μM), and Antimycin A (10 μM) was used to determine mitochondrial function parameters. Basal OCR after 1 h (A) and Reserve Capacity (B) are shown. OCRs were normalized to total protein/well after completion of assay. ATP (C) and ADP (D) were also measured from cells treated with L-CysNO (200 μM) or D-CysNO (200 μM) ± L-leucine (L-Leu; 8 mM). Nucleotide levels were normalized to total protein. Values represent means ± SEM, n=3-4. \* p < 0.05 compared to control. # p < 0.05 compared to concentration-matched L-CysNO.

## CPTIO reversibility of CysNO-dependent inhibition of reserve capacity

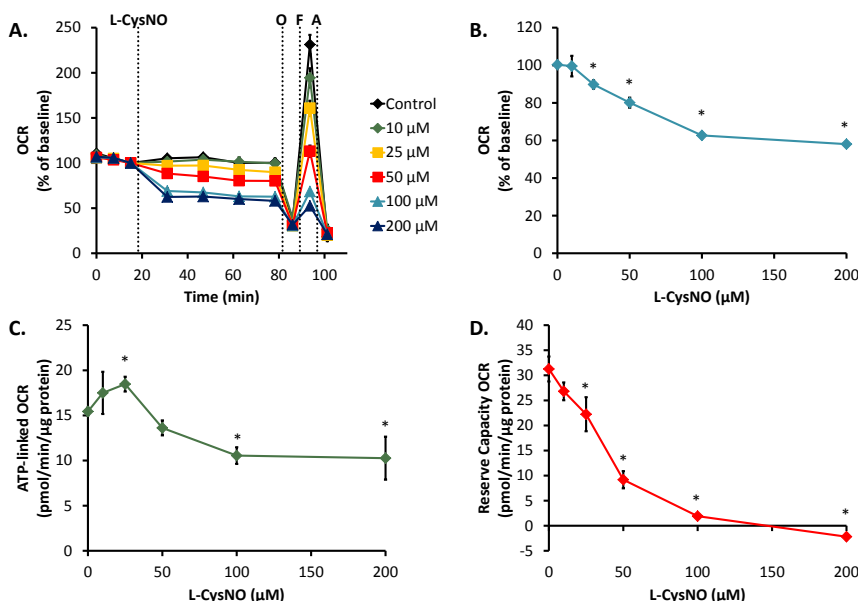


**Figure 5: CPTIO reversibility of CysNO-dependent inhibition of the reserve capacity.** The reserve capacity was measured before (blue) and after (red) injection of the NO scavenger CPTIO (100 μM) 15 min (A) or 60 min (B) after the addition of L-CysNO (50 or 200 μM). OCRs were normalized to total protein/well after completion of assay. Values represent means ± SEM, n=3-5. \* p < 0.05 compared to before CPTIO



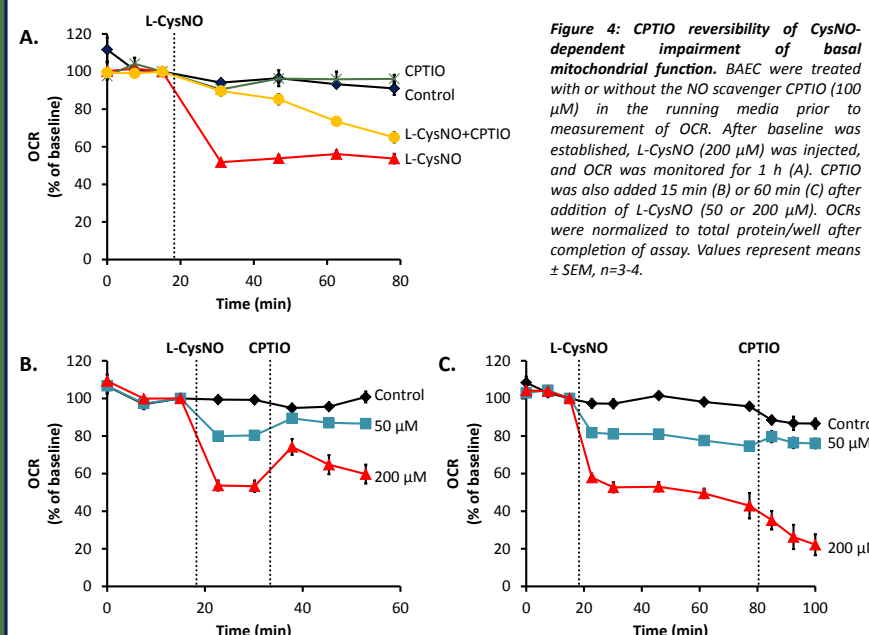
**Figure 6: Regulation of bioenergetics by S-nitrosothiols.** Both glycolytic enzymes and components of the mitochondrial electron transport chain have been reported to be targets of S-nitrosation. Our data indicate that inhibition of mitochondrial function by CysNO occurs through both intracellular S-nitrosation and the release of NO from S-nitrosothiols.

## Regulation of mitochondrial function by CysNO



**Figure 2: Effect of CysNO on mitochondrial function in bovine aortic endothelial cells (BAEC).** After baseline was established, OCR was monitored upon addition of L-CysNO (10-200 μM) for 1 h prior to sequential injection of oligomycin (1 μg/mL; "O"), FCCP (1 μM; "F"), and Antimycin A (10 μM; "A") (A). Basal OCR after 1 h (B), ATP-linked OCR (C), and Reserve Capacity (D) are shown. OCRs were normalized to total protein/well after completion of assay. Values represent means ± SEM, n=3-4. \* p < 0.05 compared to control.

## CPTIO reversibility of CysNO-dependent impairment of basal mitochondrial respiration



**Figure 4: CPTIO reversibility of CysNO-dependent impairment of basal mitochondrial respiration.** BAEC were treated with or without the NO scavenger CPTIO (100 μM) in the running media prior to measurement of OCR. After baseline was established, L-CysNO (200 μM) was injected, and OCR was monitored for 1 h (A). CPTIO was also added 15 min (B) or 60 min (C) after addition of L-CysNO (50 or 200 μM). OCRs were normalized to total protein/well after completion of assay. Values represent means ± SEM, n=3-4.

## Conclusions

- L-CysNO inhibits basal respiration, respiration linked to ATP production, and the reserve respiratory capacity (Figure 2).
- D-CysNO also impairs mitochondrial function though to a lesser extent than the L isomer, and L-leucine partially restores mitochondrial function after L-CysNO treatment (Figure 3).
- Depletion of ATP requires CysNO transport into cells (Figure 3).
- Inhibition of basal respiration and reserve capacity by L-CysNO is partially reversible by CPTIO at an early time point (15 min) and irreversible at a later time point (60 min; Figures 4 and 5).
- These results indicate that release of NO from CysNO accounts for a rapid inhibition of mitochondrial function which is eventually overcome by S-nitrosation-dependent regulation, and these data provide insight into the role of NO and related compounds in vascular (patho)physiology.