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Impaired lung mitochondrial respiration following perinatal nicotine exposure in rats

Daniel T. Cannon[1,2], Jie Liu[3], Reiko Sakurai[3], Harry B. Rossiter[1,4], Virender K Rehan[3]

[1] Division of Respiratory & Critical Care Physiology & Medicine, Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center

[2] School of Exercise & Nutritional Sciences, San Diego State University

[3] Division of Neonatology, Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center

[4] School of Biomedical Sciences, Faculty of Biological Sciences, University of Leeds

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Correspondence:
DT Cannon
San Diego State University, MC 7251
5500 Campanile Drive
San Diego, CA 92182

dcannon@mail.sdsu.edu
619 594 8283

Abstract

Perinatal smoke/nicotine exposure predisposes to chronic lung disease and morbidity. Mitochondrial abnormalities may contribute as the PPARγ pathway is involved in structural and functional airway deficits after perinatal nicotine exposure. We hypothesized perinatal nicotine exposure results in lung mitochondrial dysfunction that can be rescued by rosiglitazone (RGZ; PPARγ receptor agonist). Sprague-Dawley dams received placebo (CON), nicotine (NIC, 1 $mg.kg^{-1}$), or NIC+RGZ (3 $mg.kg^{-1}$) daily from embryonic day 6 to postnatal day 21. Parenchymal lung (~10mg) was taken from adult male offspring for mitochondrial assessment *in situ*. ADP-stimulated O_2 consumption was less in NIC and NIC+RGZ compared to CON ($F[2,14]=17.8$; 4.5±0.8 and 4.1±1.4 vs. 8.8±2.5 $pmol.s.mg^{-1}$; $p<0.05$). The respiratory control ratio for ADP, an index of mitochondrial coupling, was reduced in NIC and remediated in NIC+RGZ ($F[2,14]=3.8$; $p<0.05$). Reduced mitochondrial oxidative capacity and abnormal coupling was evident after perinatal nicotine exposure. Rosiglitazone improved mitochondrial function through tighter coupling of oxidative phosphorylation.

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Introduction

Perinatal tobacco smoke and nicotine exposure predisposes to low birth weight, chronic lung disease, and increased morbidity and mortality [1]. This is of particular concern in population-dense regions at the outset of tobacco-related disease epidemics [2], or where nicotine delivery via e-cigarettes is growing in popularity, especially among young people [3,4]. We have shown that epigenetic silencing of peroxisome proliferator-activated receptor γ (PPAR-γ) results in morphological and functional airway deficits that accompany smoke and nicotine exposure *in utero* [5,6]. Encouragingly, PPAR-γ receptor agonists are effective in augmenting structural and functional lung maturation and repair, through either peri- or postnatal administration [7,8]. As PPAR-γ is an important regulator of mitochondrial biogenesis, we used the same rat model of perinatal nicotine exposure to investigate the effects of perinatal nicotine exposure on lung mitochondrial respiration *in situ*. Since PPAR-γ receptor agonist rosiglitazone (RGZ) ameliorates nicotine-induced alterations in pulmonary compliance, resistance, and airway reactivity [9], we examined potential RGZ-mediated rescue of lung mitochondrial oxidative capacity as a possible protective mechanism against perinatal nicotine-induced lung damage. We hypothesized perinatal nicotine exposure results in lung mitochondrial dysfunction that can be rescued by RGZ.

Methods

First-time pregnant Sprague-Dawley dams received placebo (CON), nicotine (NIC, 1 $mg.kg^{-1}$), or NIC+RGZ (3 $mg.kg^{-1}$) daily from embryonic day 6 to postnatal day 21. Postpartum, pups were nursed *ad libitum* until weaning on postnatal day 21. Initially, respirometry was performed on mitochondria isolated from lung [10,11], however the isolation procedures consistently resulted in damage to the outer mitochondrial membrane [11]. Following these pilot studies, high-resolution respirometry was performed on parenchymal tissue dissected from the base of the lung (~10mg) of adult males at 5 months of age (CON n=6, NIC n=6, NIC+RGZ n=5). High-resolution respirometry provides measurement of the rate of mitochondrial O_2 consumption *in situ* via measurement of [O_2] in stirred media with a

47 polarographic O_2 sensor. The titration protocol described below allows for the respiratory
48 states to be assessed either in absolute (O_2 consumption per tissue mass) or as flux control
49 ratios.
50
51 Following dissection, tissues were placed immediately in preservation solution at 4°C until
52 measurement could be made (~30 min to 4 hr after euthanasia). Preservation medium
53 (BIOPS) contained 10 mM Ca^{2+}EGTA buffer, 20 mM imidazole, 50 mM K^+-4-
54 morpholineothanesulfonic acid (MES), 0.5 mM dithiothreitol, 6.56 mM $MgCl_2$, 5.77 mM ATP,
55 15 mM phosphocreatine and a pH of 7.1. Tissue samples (~10 mg) were weighed using a
56 microbalance and transferred into a calibrated respirometer (Oxygraph 2k, OROBOROS
57 INSTRUMENTS, Innsbruck, AT) containing 2 ml of media in each chamber. Respirometry
58 was performed in duplicate at 37°C in stirred media (MiR05) containing 0.5 mM EGTA, 3 mM
59 $MgCl_2$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM
60 sucrose, and 1 g/l BSA essentially fatty acid free, adjusted to pH 7.1. [O_2] in the media was
61 kept between 300-500 µM.
62
63 A substrate-uncoupler-inhibitor-titration (SUIT) protocol [12,13] included: 10 mM glutamate
64 and 2 mM malate to support electron entry through complex I (GM; '*LEAK*' state), 5 mM ADP
65 to stimulate oxidative phosphorylation, 10 mM succinate to maximize convergent electron
66 flux at the Q-junction (ADP+S), 10 µM cytochrome-c to test for outer mitochondrial
67 membrane integrity (cyt-c), carbonyl cyanide p-trifluoro-methoxyphenyl hydrazine (FCCP)
68 titrated in 0.5 uM steps to achieve maximal uncoupled respiration for measurement of
69 electron transport system capacity, 0.5 µM rotenone to inhibit complex I (Rot), and 2.5 µM
70 antimycin A + 0.5 mM N,N,N',N'-Tetramethyl-p-phenylenediamine dihydrochloride to inhibit
71 complex III and measure complex IV maximal flux (A+TMPD). Flux control ratios were
72 calculated, where appropriate, with the reference value of electron transport system capacity
73 (FCCP titration). The respiratory control ratio (RCR) for ADP was calculated as (ADP+GM /
74 GM). The substrate control ratio for succinate was calculated as (ADP+S / ADP+GM).

Differences between CON, NIC, and NIC+RGZ groups were tested with a one-factor ANOVA and Bonferroni post-hoc t-tests where appropriate. Data are presented as mean±SD.

Results

Body mass of the pups was not different at 5 months among treatment conditions (CON 637±65, NIC 624±49, NIC+RGZ 582±32 g; p=n.s.). ADP-stimulated O_2 consumption (J_{O2}) with GM was less in NIC and NIC+RGZ compared to CON (F[2,14]=9.4; 3.2±0.9 and 3.2±1.3 vs. 5.7±1.2 pmol.s.mg^{-1}; p<0.05; ADP in Figure 1). Maximal ADP-stimulated O_2 consumption (J_{O2}) with GM and S was less in NIC and NIC+RGZ compared to CON (F[2,14]=17.8; 4.5±0.8 and 4.1±1.4 vs. 8.8±2.5 pmol.s.mg^{-1}; p<0.05; ADP+S in Figure 1). Uncoupled J_{O2} was ~60% less in NIC and NIC+RGZ compared to CON (F[2,14]=10.8; 6.4±1.5 and 6.8±2.5 vs. 15.4±5.7 pmol.s.mg^{-1}; p<0.05, FCCP in Figure 1), with excess complex IV capacity in all cases (A+TMPD Figure 1).

The flux control ratio for GM (*LEAK* respiratory state) was elevated in NIC and rescued in NIC+RGZ (F[2,14]=3.6, p=0.055; GM in Figure 2). Flux control ratios of other respiratory states where unaffected by NIC or NIC+RGZ (Figure 2). The respiratory control ratio for ADP was reduced in NIC, and remediated in NIC+RGZ (F[2,14]=3.8; p<0.05; RCR for ADP in Figure 3). The substrate control ratio for succinate was not different across the conditions (F[2,14)=0.6; p>0.5; SCR for Succinate in Figure 3).

Discussion

Mitochondrial respiration in parenchymal lung tissue from perinatal nicotine-exposed pups was reduced by >50% across the respiratory states. When the respiratory states were normalized to electron transport system capacity, maximal ADP-stimulated respiration was similar across conditions, except for *LEAK* respiration. Thus, the large suppression of maximal mitochondrial respiration following perinatal nicotine exposure was most likely due

to reduced mitochondrial density, rather than due to functional changes of the mitochondrial electron transport system *per se*. This reduction of total oxidative capacity in the lung mitochondria fits with our recent report on the epigenetic silencing of PPAR-γ through PPAR-γ promoter methylation controlled by DNA methyltransferase 1 (DNMT1) and methyl CpG binding protein 2 (MeCP2) [6]. The respiratory control ratio for ADP, an index of coupling, was reduced following perinatal nicotine exposure. Mild uncoupling following nicotine exposure, potentially to mitigate the effects of reactive O_2 species (ROS) production, was improved with simultaneous rosiglitazone administration.

Although PPAR-γ agonists are known to increase mitochondrial biogenesis [14], and RGZ protects against the development of an asthma phenotype following perinatal nicotine exposure [9], lung mitochondrial oxidative capacity in the adult lung was unaffected by perinatal RGZ treatment. However, nicotine exposure was accompanied by reduced mitochondrial coupling, as reflected by the greater GM flux control ratio in NIC exposure group and lower RCR for ADP: an effect that was attenuated by RGZ (Figures 2 and 3). Increased transmembrane proton flux to compensate for an increased proton leak (or *LEAK* state; [13]) is the predominant component of this greater non-phosphorylating respiratory rate. This physiological uncoupling, or pathological dyscoupling, of respiration in parenchymal mitochondria with perinatal nicotine exposure may be a protective feedback response to excessive mitochondrial hydrogen peroxide or superoxide production [15]. Rescue effects of RGZ on alveolar development and airway hyper-reactivity [9], may operate in part through reduced oxidative stress, and therefore less reliance on *LEAK* state dyscoupling to mitigate the deleterious effects of reactive oxygen species.

In conclusion, perinatal nicotine exposure reduced mitochondrial oxidative capacity in adult parenchymal lung by more than 50%, and exacerbated non-phosphorylating respiration. Rosiglitazone did not rescue oxidative capacity, but may have helped preserve inner mitochondrial membrane integrity. Whether perinatal nicotine exposure (via tobacco smoke

or e-cigarette delivery) predisposes offspring towards chronic lung disease by increased reactive oxygen species production, and/or through development deficits following low lung tissue mitochondrial density remains to be confirmed.

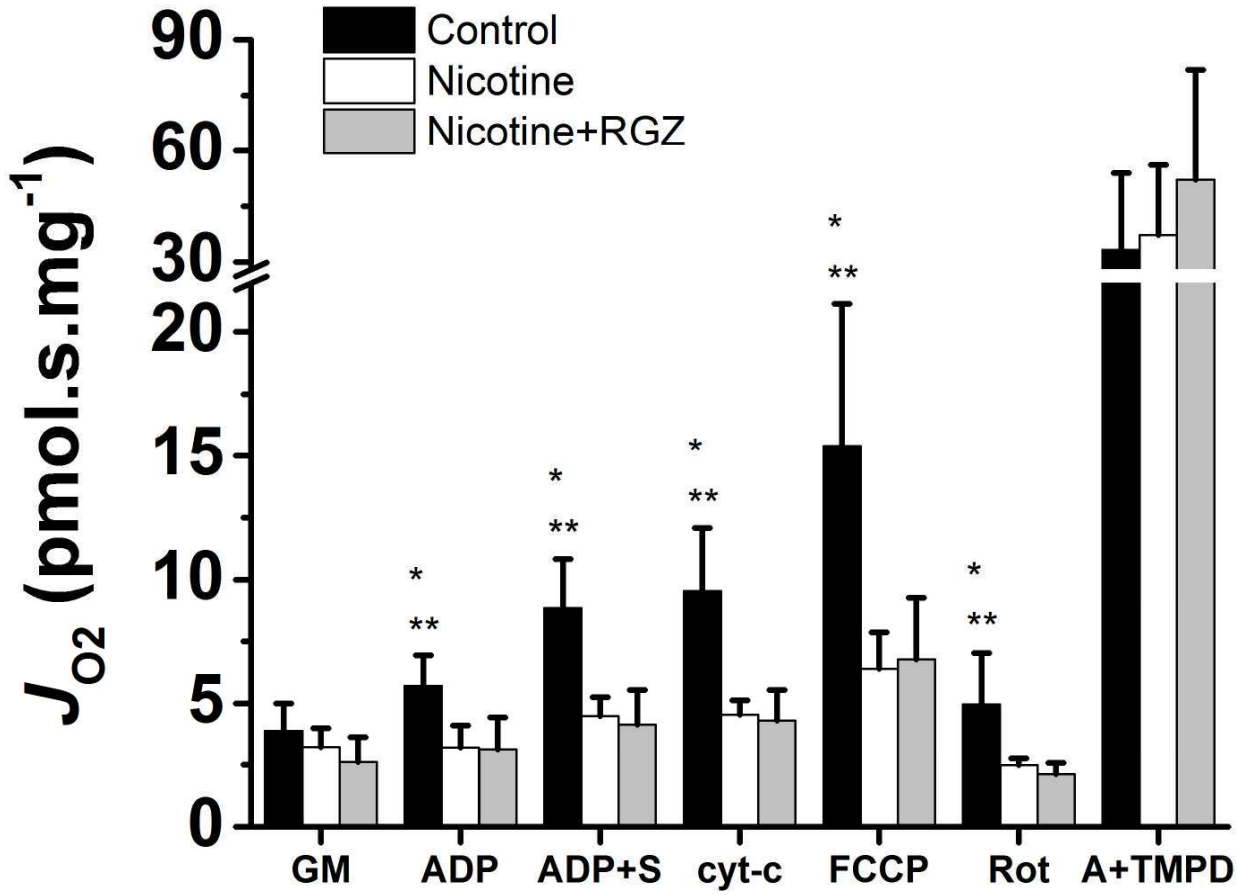


Figure 1. Rate of oxygen consumption (J_{O2}) during a high-resolution respirometry substrate-uncoupler-inhibitor-titration (SUIT) protocol. **GM**: glutamate+malate. **ADP**: ADP. **ADP+S**: ADP+succinate. **cyt-c**: exogenous cytochrome-c. **FCCP**: Carbonyl cyanide p-trifluoro-methoxyphenyl hydrazone. **Rot**: Rotenone. **A+TMPD**: Antimycin A + N,N,N',N'-Tetramethyl-p-phenylenediamine dihydrochloride. Error bars are SD. *Different to NIC. **Different compared to NIC+RGZ.

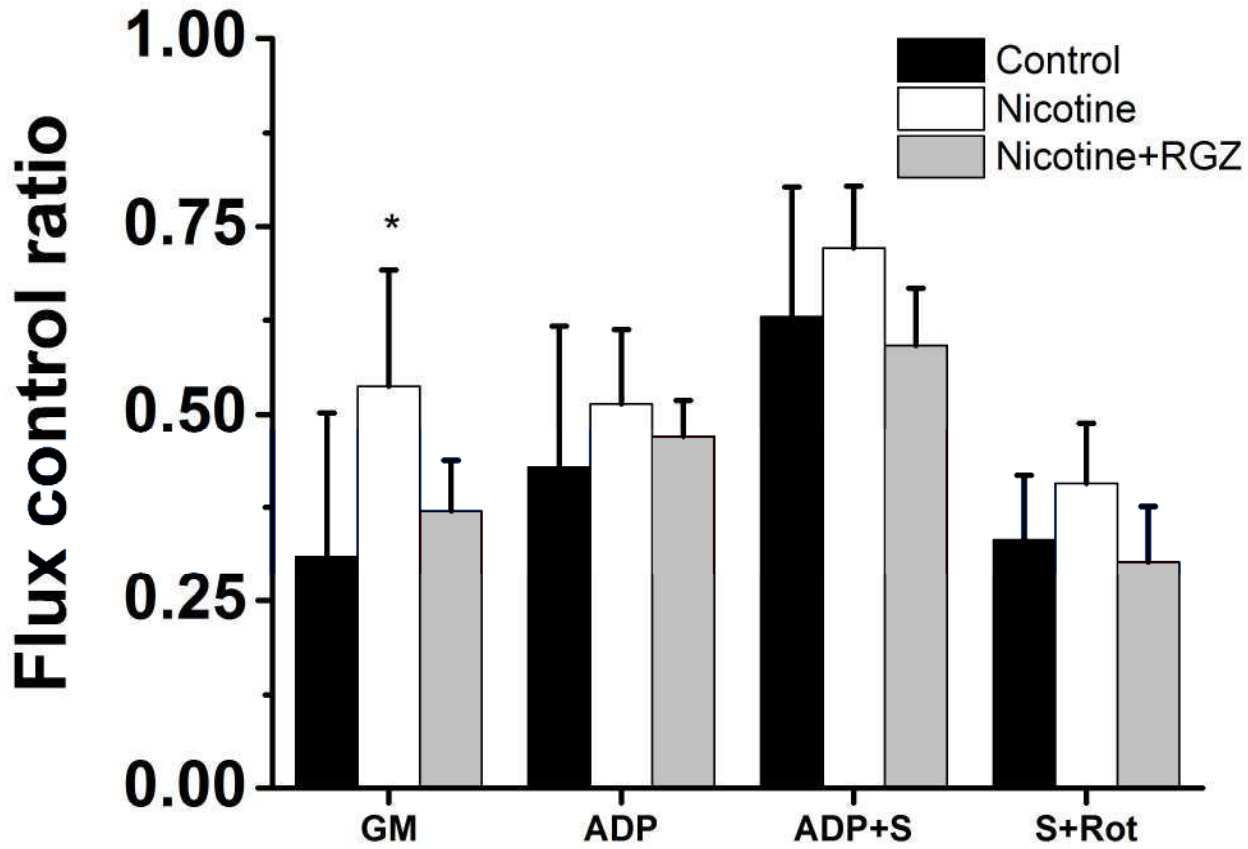
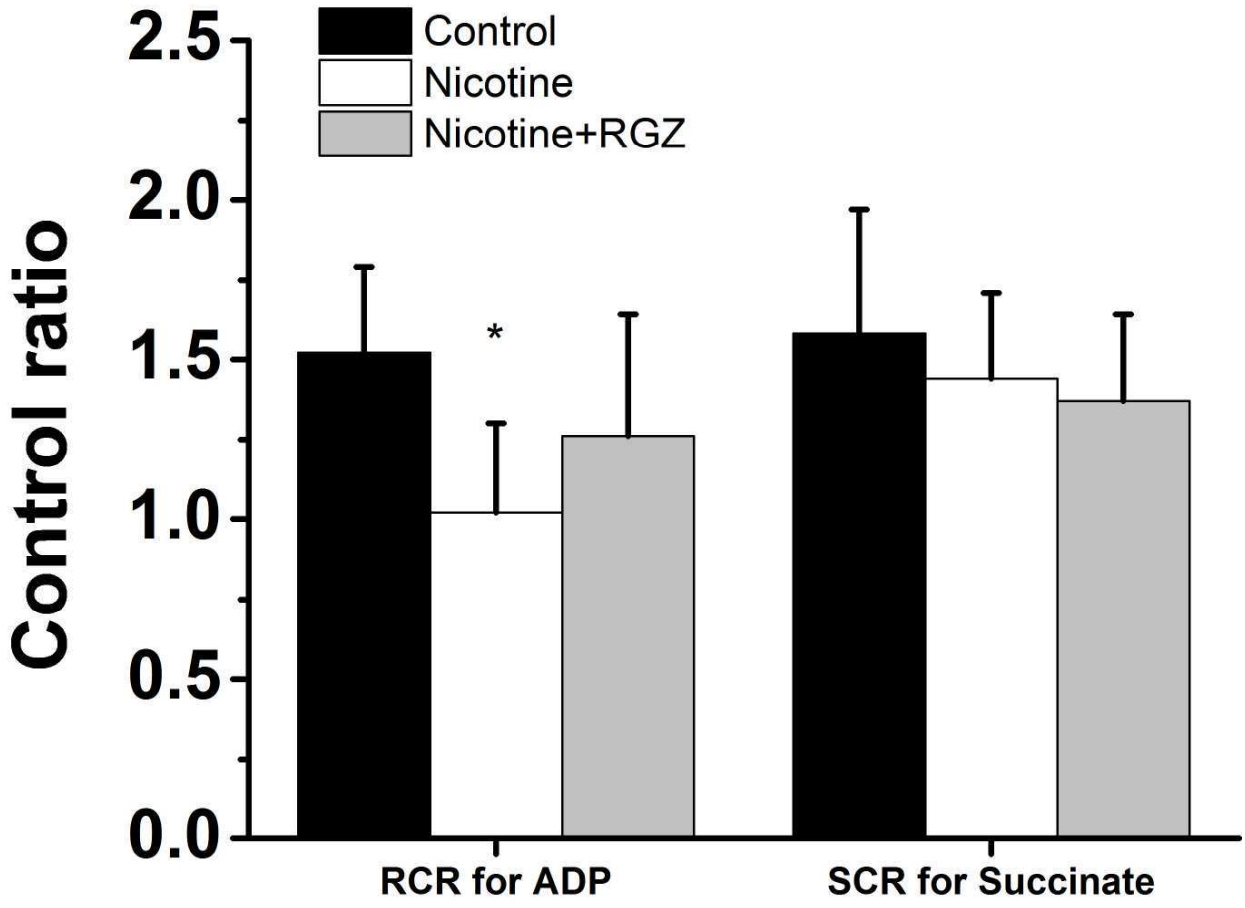


Figure 2. Flux control ratios during a high-resolution respirometry substrate-uncoupler-inhibitor-titration (SUIT) protocol. **GM**: glutamate+malate. **ADP**: ADP. **ADP+S**: ADP+succinate. **S+Rot**: Succinate+rotenone. **A+TMPD**: Antimycin A + N,N,N',N'-Tetramethyl-p-phenylenediamine dihydrochloride. Error bars are SD. *Different compared to CON.



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151 **Figure 3**. Respiratory and substrate control ratios during a high-resolution respirometry
152 substrate-uncoupler-inhibitor-titration (SUIT) protocol. **RCR for ADP** = (ADP+GM / GM).
153 **SCR for Succinate** = (ADP+S / ADP+GM). Error bars are SD. *Different compared to CON.

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Conflict of interest: None.

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