The effect of long-term maternal smoking on the offspring’s lung health

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ABSTRACT
Maternal smoking during pregnancy contributes to long-term health problems in offspring, especially respiratory disorders which can manifest in either childhood or adulthood. Receptors for advanced glycation end-products (RAGE) are multi-ligand receptors abundantly localized in the lung, capable of responding to by-products of reactive oxygen species and pro-inflammatory responses. RAGE signalling is a key regulator of inflammation in cigarette smoking-related pulmonary diseases. However, the impact of maternal cigarette smoke exposure on lung RAGE signalling in the offspring is unclear. This study aims to investigate the effect of maternal cigarette smoke exposure (SE), as well as MitoQ (mitochondria-targeted antioxidant) treatment during pregnancy on RAGE-mediated signalling pathway in the lung of male offspring.

Female Balb/c mice (8 wk) were divided into a sham group (exposed to air), an SE group (exposed to cigarette smoke) and an SE+MQ group (exposed to cigarette smoke with MitoQ supplement from mating). The lungs from male offspring were collected at 13 weeks.

Results: RAGE and its downstream signaling including NF-κB and MAPK family consisting of ERK1, ERK2, JNK, and phosphorylated-JNK in the lung were significantly increased in the SE offspring. Mitochondrial antioxidant manganese superoxide dismutase (MnSOD) was reduced, while IL-1β and oxidative stress response nuclear factor (erythroid-derived 2)-like 2 (Nrf-2) was significantly increased in the SE offspring. Maternal MitoQ treatment normalised RAGE, IL-1β and Nrf-2 levels in the SE+MQ offspring.

Maternal SE increased RAGE and its signalling elements associated with increased oxidative stress and inflammatory cytokines in offspring’s lungs; whereas maternal MitoQ treatment can partially normalise these changes.
Maternal smoking during pregnancy contributes to various long-term health problems in offspring, especially respiratory disorders (21, 37). Several human studies have indicated that maternal smoking is associated with lung under-development, airflow limitations, increase in the risk of respiratory infections and development of airway hypersensitivity and asthma (7, 62, 64). Several mechanisms have been proposed, including a reduction in the development or physical size of the lung including reduced elastic tissue and the number of alveolar attachments to the airway, an increase in oxidative stress, and alteration to the inflammatory response and immune system (14, 17, 41, 42).

Receptors for advanced glycation end-products (RAGE) are multi-ligand receptors abundantly localized in the lung (16). Recent studies have implied a role of RAGE in cigarette-smoking-related diseases, where RAGE signaling is a key regulator of inflammatory response in pulmonary diseases (13, 48). Cigarette smoke induces the formation of advanced glycation end-products (AGEs), resulting in the development of diseases through the AGEs-RAGE axis (8, 44). Indeed, it has been reported that serum levels of AGEs are elevated in smokers including both current and past smokers (40), and RAGE levels are elevated in pulmonary tissue from mice exposed to cigarette smoke (20, 65). AGEs can interact with RAGE leading to pro-inflammatory responses via several downstream kinases, such as the Mitogen-activated protein kinase (MAPK) family consisting of extracellular signal-regulated kinase-1/2 (ERK1/2), c-JUN N-terminal kinase (JNK) and p38MAPK. The transcription factor nuclear factor-κ-light-chain-enhancer of activated B cells (NF-κB) can also be activated, which results in the expression of a variety of pro-inflammatory mediators and cytokines, including IL-1, IL-6 and TNF-α (30, 32, 45). Thus, the activation of RAGE-mediated signalling pathways is likely to play a key role to mediate inflammatory response in many pulmonary disorders (47). In a previous study, short-term maternal cigarette smoke exposure during embryo days 14.5-18.5 was shown to increased RAGE level in the fetal lung tissue at embryo day 18.5 (63). However, whether such changes are still present at adulthood is unknown.

Pathological responses induced by the AGEs-RAGE axis are mediated by the generation of intracellular reactive oxygen species (ROS), the ensuing oxidative stress (15), and the activation of ROS induced cytokine production and inflammation. Mitochondria are the major cellular source of ROS (59), and as such are a pharmacological target for ROS production. It is well established that
oxidative stress can induce the secretion of inflammatory cytokines and the expression of adhesion
molecules and inflammatory mediators in the lung (3, 24, 27). It has also been shown that maternal
smoking can significantly increase oxidative stress in the offspring, including the lung tissue (4).
Therefore, reducing oxidative stress may reduce pulmonary inflammatory responses in the lungs of
offspring from smoking parents.

Coenzyme Q10 (CoQ10) is a mitochondrial endogenous antioxidant. It has been shown that CoQ10
dietary supplementation (1%) in mice with diet-induced obesity can lower liver markers of
inflammation and oxidative stress (55). Plasma CoQ10 levels are reduced in smokers (1). However,
mitochondrial intake of commercial CoQ10 is very low via oral supplementation, thus a
superphysiological dose was used in the abovementioned study. Mitoquinone mesylate, also known
as MitoQ, is a mitochondria-targeted antioxidant. It consists of a ubiquinone moiety, the same
structure to the ubiquinone found in CoQ10 that is linked to a triphenylphosphonium moiety by a
ten-carbon alkyl chain, which allows its rapid uptake and accumulation in the mitochondria to
restore the antioxidant efficacy of the mitochondrial respiratory complex (26). As such, it has been
reported that MitoQ has a protective role against oxidative damage-related pathologies in metabolic
disease (36) and neurodegenerative diseases (34). Amniotic fluid CoQ10 levels are significantly
lower among women delivering preterm babies, a risk which is increased by maternal smoking (29,
60). Therefore, MitoQ might be a suitable intervention option since it is already marked for human
consumption.

Thus, the main aim of this study was to investigate the long-term impact of maternal cigarette
smoke exposure (SE) on lung RAGE signalling elements in adult offspring. In addition, whether
MitoQ supplementation during gestation can mitigate the adverse impact of maternal SE was also
investigated.

MATERIALS AND METHODS

Animal experiments

The animal experiments were approved by the Animal Care and Ethics Committee at the University
of Technology Sydney (ACEC#2014-638 and #2016-419). All protocols were performed
according to the Australian National Health & Medical Research Council Guide for the Care and
Use of Laboratory Animals. Female Balb/c mice (8 weeks) were housed at 20±2 °C and
maintained on a 12:12 hour light/dark cycle with ad libitum access to standard laboratory chow and
water. After the acclimatisation period, mice were divided into the following three groups: sham (exposed to air), SE (exposed to 2 cigarettes twice daily, 6 weeks before mating and throughout gestation and lactation, as previously described (2)), and SEMQ (SE mothers supplied with MitoQ (1.5 g/L in drinking water) during gestation and lactation). This dose was chosen as it has previously shown to be effective, safe and maintain steady-state tissue concentrations of 1-100 pmol MitoQ/per g of tissue(36, 49) (depending upon the organ analysed; MitoQ accumulates in liver and heart, but is effective in the lung with this dosing regimen (33)). Male breeders and suckling pups stayed in the home cage when mothers were exposed to sham or cigarette smoke. Pups were weaned at postnatal day 20 and maintained without additional intervention. Male offspring were euthanized (4% isoflurane, 1% O₂, Veterinary companies of Australia, Kings Park, NSW) at 13 weeks (mature age) and the lung tissues were collected and stored at -80°C for later analysis.

**Western blotting**

Lungs tissues were homogenized in lysis buffer with phosphatase inhibitors (Thermo Fisher Scientific, CA, USA). Protein concentrations were measured using DC Protein assay (Bio-rad, Hercules, CA, USA). Equal amount of proteins (20 μg) were separated on NuPage® Novex® 4–12% Bis-Tris gels (Thermo Fisher Scientific, CA, USA) and transferred to PVDF membranes. The membranes were blocked with TBS-0.05% Tween 20 (TBS-T) containing 5% BSA or skim milk for 1 h, before incubation with primary antibodies against phospho-Erk1/2 (1:1000, Cell Signaling Technology Inc), Erk1/2 (1:1000, Cell Signaling Technology Inc, MA, USA), phospho-JNK (1:1000, Cell Signaling Technology Inc, MA, USA), JNK (1:500, Cell Signaling Technology Inc, MA, USA), phospho-p38 MAPK (1:1000, Cell Signaling Technology Inc, MA, USA), p38 MAPK (1:1000, Cell Signaling Technology Inc, MA, USA), NF-κB and phospho-NF-κB (1:1000, Cell Signaling Technology Inc, MA, USA), IL-6 (1:1000, Cell Signaling Technology Inc, MA, USA), IL-1β (1:1000, Cell Signaling Technology Inc, MA, USA), RAGE (1:1000, GeneTex Inc, CA, USA), TNF-α (1:1000, Gene Tex Inc, CA, USA), antioxidant response element nuclear factor (erythroid-derived 2)-like 2 (Nrf-2, 1:500, Aviva System Biology, CA, USA), endogenous antioxidant Manganese superoxide dismutase (MnSOD, 1:1000, Santa Cruz Biotechnology, Texas, USA), transforming growth factor-β1 (TGF-β1, 1:500, R&D Systems, MN, USA), and collagen 1A (1:1000, Santa Cruz Biotechnology Inc, Texas, USA) overnight at 4°C, which was followed by secondary antibodies (peroxidase-conjugated goat anti-mouse or anti-rabbit IgG, 1:2000, Santa Cruz Biotechnology Inc). The blots were then incubated in Super Signal West Pico...
Chemiluminescent substrate (Thermo Fisher Scientific, CA, USA) and the membranes were then visualized by an Amersham Imager 600 (GE Healthcare, NSW, Australia). Protein band density was determined using ImageJ software (National Institute of Health, Maryland, USA) for densitometry, and β-actin (1:5000, Santa Cruz Biotechnology, Texas, USA) was used as the housekeeping protein.

**Quantitative real-time PCR**

Total mRNA was isolated from lung tissues using TRIzol Reagent (Life Technologies, CA, USA). First strand cDNA was generated using M-MLV Reverse Transcriptase, RNase H, Point Mutant Kit (Promega, Madison, WI, USA). Real-time PCR was performed using manufacturer pre-optimized and validated TaqMan® primers and probes (Thermo Fisher Scientific, CA, USA). Only RAGE probe sequence is provided by the manufacturer (CCCAGGCGTGAGGAGAGGAAGGCC, NCBI gene references: NM_001271422.1, NM_001271424.1, NM_007425.3; ID: Mm01134790_g1). RAGE probes were labelled with FAM® dye and those for housekeeping 18s rRNA was labelled with VIC® dye. Gene expression was standardized to 18s RNA. The average expression of the control group was assigned as the calibrator against which all other samples were expressed as fold difference.

**Statistical analysis**

The results are presented as the mean ± S.E.M. The data were analysed by one-way ANOVA followed by post hoc Bonferroni test (Prism 7, Graphpad CA, USA). The differences were considered statistically significant at P < 0.05.

**RESULTS**

**Effect on the body weight of offspring**

At postnatal day 1, as expected male offspring from the SE mothers (1.30 ± 0.07g, n=11) were significantly smaller than those from the SHAM mothers (1.49 ± 0.03g, P<0.01, n=17). Smaller body weight was maintained until 13 weeks of age (SE 24.3 ± 0.2g n=21, SHAM 25.3 ± 0.3g n=20, P<0.01), which was consistent with our previous study using the same model (25).

MitoQ supplementation during gestation and lactation significantly reversed in the impact of maternal SE on small birth weight in the male pups (1.65 ± 0.02g, P<0.05 vs SHAM, P<0.01 vs SE,
In adulthood, the body weight of SEMQ offspring was also normalised to the Sham level (25.2 ± 0.2 g, P=0.01 vs SE, n=12).

**Effect on lung RAGE and MAPK signalling elements in offspring**
Maternal smoking increased the amount of RAGE protein in adult offspring’s lungs (P<0.05 vs SHAM; Fig. 1a) which was reduced by MitoQ. At the mRNA level, RAGE was only slightly reduced in the SE offspring at 13 weeks, but increased by MitoQ (Fig.1b). RAGE downstream signalling molecules including total Erk1, Erk2, JNK, and p-JNK protein levels were increased in SE offspring (P<0.05 vs SHAM; Fig.2a-c); however, p-Erk1 and p-Erk2 levels were not different between SHAM and SE group. There was no change in total or phosphorylated p38MAPK between the SHAM and SE groups (Fig. 2d). Maternal MitoQ supplementation during gestation marginally reduced total ERK1 and ERK2, as well as p-ERK1 and p-ERK2 levels, although without statistical significance (Fig 2).

**Effects on lung antioxidant enzyme in offspring**
Manganese superoxide dismutase (MnSOD) is the primary endogenous mitochondrial antioxidant that plays a key role in protecting cells against oxidative stress. As shown in Figure 3, mitochondrial levels of MnSOD were significantly reduced in the SE offspring (P<0.05), which was only slightly enhanced by maternal MitoQ treatment. Furthermore, Nrf-2, a transcription factor which is high sensitivity to oxidative stress, was significantly increased in the SE offspring (P<0.05 vs SHAM; Fig. 4a), which was normalised by maternal MitoQ supplementation (P<0.05 vs SE; Fig. 4b).

**Effects on lung pro-inflammatory mediators in offspring**
RAGE-induced release of pro-inflammatory cytokines is mainly via the activation of NF-κB, a redox-sensitive transcription factor that regulates the transcription of several pro-inflammatory cytokines. There was a nonsignificant trend towards increasing levels of phosphorylated NF-κB in SE offspring, and the total NF-κB level was significantly increased in SE offspring compared to SHAM offspring (P<0.05; Fig.4a,b). As a result, the ratio of phosphorylated NF-κB / total NF-κB was unchanged. Similarly, IL-1β protein level was more than doubled in SE offspring (P<0.05; Fig. 4b), while TNF-α level was increased by 50%, albeit without statistical significance (Fig. 4d),
However, IL-6 levels were similar between the SHAM and SE group (Fig. 4c). Maternal MitoQ supplementation normalised phosphorylated and total NF-κB and IL-1β levels in SEMQ offspring (Fig 4a,b), without any effect on IL-6 and TNF-α (Fig 4c,d).

Effect on lung fibrotic markers in offspring
A prolonged increase in TGF-β1 activity can lead to persistent lung fibrosis resulting in excessive production of collagen-1A (11). Here, neither TGF-β1 nor collagen-1A proteins levels were changed in 13 weeks old SE offspring (Fig.5). Maternal MitoQ treatment also showed no impact on these two proteins (Fig.5).

DISCUSSION
Maternal smoking during pregnancy has been shown to adversely affect fetal lung development and has also been linked to an increased risk of long-term respiratory disorders (21, 37). In this study, we found that SE offspring had reduced body weight at birth which was maintained until adulthood. The protein levels of RAGE and its downstream signalling, including NF-κB and MAPK family consisting of ERK1, ERK2, JNK, and p-JNK, were significantly increased in the lung of SE offspring, with increased inflammatory cytokine IL-1β level. Mitochondrial antioxidant MnSOD levels were reduced, and the oxidative stress response Nrf-2 was significantly increased in the SE offspring. Maternal MitoQ treatment reversed the impact of maternal SE on birth weight and normalised RAGE, NF-κB, IL-1β and Nrf-2 levels in offspring.

RAGE plays an important role in cigarette-smoking-related diseases as a key regulator in maintaining and promoting inflammatory responses (13, 48, 57). It has been shown that the expression of RAGE is increased in pulmonary epithelial cells after exposure to cigarette smoke extract (46), whereas increased inflammatory cytokines have been found in the lung lavage fluid of smokers and mice exposed to cigarette smoke (25, 28). To our knowledge, this is the first study to demonstrate that continuous maternal SE from pre-gestation to lactation leads to increased RAGE expression in the offspring’s lung at adulthood, with increased downstream signalling elements and inflammatory cytokines. The study by Winden et al. only showed RAGE augmentation in the fetal lung following 4 days maternal SE during the pseudoglandular period of lung development (63). This suggests that the changes in RAGE and signaling elements by maternal SE can begin in the intrauterine period, and last long into adulthood. In our study, RAGE mRNA expression was marginally suppressed (non-significantly) in the SE offspring, which may be due to a negative
feedback loop. This suggests that the level of RAGE may be regulated at a transcriptional level. Additional studies will be required to determine the exact mechanism, such as the involvement of transcriptional regulator non-coding RNAs.

The activation of RAGE signaling pathways can influence alveolar remodeling characteristics of pulmonary disease (50). Several studies have indicated that cigarette smoke induces the expression of RAGE and promotes the phosphorylation of ERK1/2, p38 and JNK in primary human gingival epithelial cells and in the lungs of rats exposed to cigarette smoke (52, 65, 66). Therefore, here we investigated the signal transduction pathways and fibrotic markers in lung tissue. In the present study, we demonstrated that maternal SE can increase the expression of RAGE-dependent signalling protein kinases including Erk1, Erk2, and JNK. However, the phosphorylation of these molecules was not significantly increased by maternal SE. Therefore, the impact of maternal SE on the health outcome of the offspring’s lung may prime the lung to be hyperresponsive to certain stimuli, but the pathways themselves are not intrinsically activated. This is consistent with studies in humans, where smoking during pregnancy is a risk factor for the development of lung diseases such as asthma and COPD, but in both of these diseases other environmental stimuli are mostly needed to develop the disease in children.

The two fibrotic markers measured in this study were not affected by maternal SE. This is consistent with the changes in fibrotic markers in the kidney in our previous study using the same model of maternal SE (2). Increased collagen deposition has been found in foetal lung tissue of monkeys due to maternal nicotine administration (51). Such differences may be due to the dose of nicotine administered. In our study the nicotine dose was low (equivalent to a human smoking 1-2 cigarettes/day). The other significant difference is that cigarette smoke is a complex mixture of chemicals which may inhibit or enhance the effects of nicotine.

Cigarette smoke contains free radicals, and itself can stimulate the production of ROS in lung tissues, leading to oxidative damage in both pregnant women and newborns (12, 18). MnSOD is an enzyme present in mitochondria that is one of the first-line enzymes to detoxify the superoxide radicals generated during ATP synthesis (6). Here we showed that the level of mitochondrial MnSOD was reduced in the offspring’s lung in response to maternal SE. This is consistent with our findings in the brain and kidneys of SE offspring in adulthood in our previous studies (10, 56). RAGE activation by multiple ligands such as ceramides, cigarette smoke (39), or intracellular amyloid-β peptide (58) results in mitochondrial damage, likely mediated via mitochondrial ROS
production (22). Given this it is likely that reduced MnSOD in the current experiments is the result of mitochondrial damage.

Additionally, Nrf2 is a transcription factor that responds to oxidative stress and contributes to the induction of several protective enzymes to scavenge excess free radicals during oxidative stress. Nrf2 was found be increased in moderate smokers in response to increased oxidative stress induced by cigarette smoke (19). Here, we found that Nrf2 was significantly increased in the SE offspring at adulthood, in line with their increased oxidative stress markers. Taken together, maternal cigarette smoking during pregnancy may cause long lasting oxidative stress in offspring’s lungs, possibly due to the reduction of protective antioxidative enzymes.

Prolonged oxidative stress can activate the redox-sensitive transcription factor NF-κB (61), which, in turn, results in the transcription of a variety of mRNA encoding pro-inflammatory cytokines (43). In the present study, the level of NF-κB in offspring from SE mothers was higher than those from the SHAM mothers. This is in keeping with increased protein levels of the pro-inflammatory cytokines interleukin-1β and TNF-α, both of which are the downstream targets of NF-κB. It has been reported that chronic production of IL-1β can lead to pulmonary inflammation, emphysema, airway remodelling, and bronchial hyper-reactivity which are the main features of asthma and COPD (23, 31, 35). Therefore, maternal smoking during pregnancy may increase the risk of chronic inflammatory conditions in offspring’s lungs, making them more susceptible to certain pulmonary disorders such as COPD in adulthood, which requires further investigation.

Mitochondrial oxidative damage occurs in many disease states. Therefore, strategies to prevent oxidative-stress-induced damage may provide new therapeutic options for a range of human disorders, including lung diseases (5). MitoQ is, to date, the best-characterised mitochondria-targeted ubiquinone (53), which reduces the potent antioxidant mitoquinol in the mitochondria (26). MitoQ has been used in several organ systems, but as far as we know never before for pulmonary disorders (9, 36, 38, 54). In the present study, we determined the effects of maternal MitoQ supplementation during pregnancy on the health outcome of the lungs in offspring. Although maternal MitoQ treatment in SE mothers did not affect endogenous MnSOD level in the offspring, oxidative stress seems to be reduced, reflected by normalised Nrf-2 level. RAGE levels in the SEMQ offspring were also reduced. The activity of MAP kinase family members did not seem to be involved in the action of MitoQ. However, NF-κB was normalised by maternal intervention, which
can further normalise pro-inflammatory cytokine levels (including IL-1β and TNF-α) in the offspring’s lung. Taken together, our findings suggest that the administration of the mitochondria-targeted antioxidant MitoQ may be beneficial to lung health outcomes in offspring from SE mothers.

In summary, maternal SE can enhance oxidative stress and the expression of RAGE, as well as promoting RAGE-mediated inflammatory responses in offspring’s lungs. Maternal MitoQ supplementation during pregnancy is beneficial in reducing inflammatory and oxidative stress responses caused by maternal SE. Future human translation may be plausible since MitoQ is already marketed as over-the-counter dietary supplement.

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Figure legends:

Figure 1. RAGE in the Lung. Protein expression of RAGE (a) and mRNA expression of RAGE (b) in the lung from male offspring at 13 weeks. Results are expressed as mean ± S.E.M of n=9 mice. Data were analysed by one-way ANOVA followed by post hoc Bonferroni test. * P < 0.05 vs Sham; # P < 0.05 vs SE. SE: smoke exposed; SEMQ: smoke exposed with dietary supplementation of MitoQ.

Figure 2. Lung Erk1, Erk2, JNK and p38MAPK protein expression. Protein expression of phosphorylated and total Erk1 (a), Erk2 (b), JNK (c) and p38MAPK (d) in the lung from male offspring at 13 weeks. Results are expressed as mean ± S.E.M of n=9 mice. Data were analysed by one-way ANOVA followed by post hoc Bonferroni test. * P < 0.05 vs Sham. Erk: extracellular signal-regulated kinase; JNK: c-JUN N-terminal kinase; p38MAPK: p38 Mitogen-activated protein kinase; SE: smoke exposed; SEMQ: smoke exposed with dietary supplementation of MitoQ.

Figure 3. Lung Oxidative stress markers. Mitochondrial MnSOD (a) and total tissue Nrf-2 (b) protein level in the lung from male offspring at 13 weeks. Results are expressed as mean ± S.E.M of n=8 mice. Data were analysed by one-way ANOVA followed by post hoc Bonferroni test. * P < 0.05 vs Sham; # P < 0.05 vs SE. MnSOD: Manganese superoxide dismutase; Nrf-2: Nuclear factor erythroid 2-related factor 2; SE: smoke exposed; SEMQ: smoke exposed with dietary supplementation of MitoQ.

Figure 4. Lung Inflammatory markers. NF-κB (a-c), Interleukin-1β (d), interleukin-6 (e) and TNF-α (f) protein levels in the lung from male offspring at 13 weeks. Results are expressed as mean ± S.E.M of n=9 mice. Data were analysed by one-way ANOVA followed by post hoc Bonferroni test. *P < 0.05 vs Sham. NF-κB: nuclear factor-κB; SE: smoke exposed; SEMQ: smoke exposed with dietary supplementation of MitoQ.

Figure 5. Markers of Lung fibrosis. TGF-β1 (a) and Collagen-1A (b) protein levels in the lung from male offspring at 13 weeks). Results are expressed as mean ± S.E.M of n=9 mice. Data were analysed by one-way ANOVA. SE: smoke exposed; SEMQ: smoke exposed with dietary supplementation of MitoQ; TGF-β1: Transforming growth factor β1.