



Oxidative stress, mitochondrial perturbations and fetal programming of renal disease induced by maternal smoking



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ABSTRACT

An adverse in-utero environment is increasingly recognized to predispose to chronic disease in adulthood. Maternal smoking remains the most common modifiable adverse in-utero exposure leading to low birth weight, which is strongly associated with chronic kidney disease (CKD) in later life. In order to investigate underlying mechanisms for such susceptibility, female Balb/c mice were sham or cigarette smoke-exposed (SE) for 6 weeks before mating, throughout gestation and lactation. Offspring kidneys were examined for oxidative stress, expression of mitochondrial proteins, mitochondrial structure as well as renal functional parameters on postnatal day 1, day 20 (weaning) and week 13 (adult age). From birth throughout adulthood, SE offspring had increased renal levels of mitochondrial-derived reactive oxygen species (ROS), which left a footprint on DNA with increased 8-hydroxydeoxyguanosin (8-OHdG) in kidney tubular cells. Mitochondrial structural abnormalities were seen in SE kidneys at day 1 and week 13 along with a reduction in oxidative phosphorylation (OXPHOS) proteins and activity of mitochondrial antioxidant Manganese superoxide dismutase (MnSOD). Smoke exposure also resulted in increased mitochondrial DNA copy number (day 1-week 13) and lysosome density (day 1 and week 13). The appearance of mitochondrial defects preceded the onset of albuminuria at week 13. Thus, mitochondrial damage caused by maternal smoking may play an important role in development of CKD at adult life.

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1. Introduction

The theory of fetal programming which links chronic adulthood diseases to adverse conditions in early life is an intriguing concept, which is increasingly recognized. Low birth weight is a surrogate marker for such unfavorable conditions in-utero and is strongly associated with development of chronic kidney disease (CKD) in later life (White et al., 2009). The prevalence of end-stage kidney disease continues to increase (Coresh et al., 2007) and is not always explained by traditional risk factors. In fact, it often remains unclear why the rate of CKD progression shows substantial variation from patient to patient even among individuals with

similar comorbidities. Although a reduced nephron endowment has been implicated (Brenner et al., 1988; Hoy et al., 2005) the underlying molecular mechanisms for fetal programming of adult onset kidney disease are largely unknown.

Maternal smoking remains the most common modifiable adverse fetal exposure leading to low birth weight and other adverse fetal outcomes (Andres and Day, 2000; Jaddoe et al., 2008). Despite a recent decrease in smoking rates in developed countries, according to the 2010 Pregnancy Risk Assessment and Monitoring System (PRAMS) data from 27 states in the United States approximately 10.7% of women reported smoking during the last three months of pregnancy (Tong et al., 2013). Epidemiological studies have shown that maternal smoking alters the in-utero growth pattern of kidneys and leads to a reduced kidney volume in fetal and postnatal life (Lampl et al., 2005; Taal et al., 2011). Using a mouse model of maternal smoke exposure, we have shown that offspring of SE mothers had delayed glomerular development at an early postnatal period, with adaptively enlarged

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glomerulus size and albuminuria in adulthood (Al-Odat et al., 2014).

Cigarette smoke is a major source of reactive oxygen species (ROS). High concentrations of ROS cause lipid peroxidation, damage to cell membranes, proteins, and DNA. Evidence is mounting that maternal smoking causes an increase in oxidative stress in fetal cord blood and placenta (Aydogan et al., 2013; Sbrana et al., 2011). Mitochondria are the main intracellular source but also a primary target of ROS, which are generated as by-products of ATP synthesis through the oxidative phosphorylation system (OXPHOS). Mitochondria serve a crucial role in development by providing energy for the rapid fetal growth (May-Panloup et al., 2007). Disruption of mitochondrial homeostasis may lead to long-lasting detrimental effects and failure of organ function over time. The role of mitochondrial dysfunction and ROS production is clearly established in a number of chronic adult onset diseases including Parkinson's disease (Jenner, 2001), Alzheimer's disease (Aliev et al., 2003), atherosclerosis (Harrison et al., 2011), diabetes (Green et al., 2004; Nishikawa et al., 2000) as well as aging (Huang and Manton, 2004; Sastre et al., 2000). Evidence for mitochondrial dysfunction in chronic kidney disease is emerging from high throughput genome-based microarray technology (Granata et al., 2009) and a number of experimental studies (Su et al., 2013; Yuan et al., 2012a; Zhang et al., 2007; Zhu et al., 2011). It remains to be shown whether the mitochondrial perturbations are present already at birth as a consequence of an adverse in-utero environment.

The present study was designed to test the hypothesis that maternal smoking causes increased ROS production and mitochondrial perturbations in renal tissue in the offspring at birth and that this effect is sustained into adulthood.

2. Materials and methods

2.1. In vivo experiment

2.1.1. Animal smoking model

The study was approved by the Animal Care and Ethics Committee of the University of Technology, Sydney (ACEC #2011-313A). Female Balb/c mice (Animal Resources Centre, Perth, Australia) were housed at 20 ± 2 °C, and maintained on a 12:12 h light/dark cycle (lights on 06:00 h), with free access to water and standard laboratory chow (11 kJ/g, Gordon's Specialty Stockfeeds, NSW, Australia). Twice daily (5 days/week) they underwent smoke exposure (SE) in a Perspex chamber with smoke generated from 2 cigarettes (nicotine < 1.2 mg, CO < 15 mg) for 6 weeks before mating, throughout gestation and lactation. The control sham-exposed mice were put in an identical chamber for the same period. During lactation the offspring remained in the home cage without SE. Pups were weighed every 5 days and weaned at postnatal day 20. A terminal urine collection was undertaken via direct bladder puncture at the end points: day 1, day 20 (weaning) and week 13 (mature age). Blood was collected via cardiac puncture after mice were anesthetized. Plasma was separated immediately and stored at -20 °C for creatinine measurements. Then animals were sacrificed by cervical dislocation. Kidneys were harvested, snap frozen and stored at -80 °C for further processing. Only male offspring were used for this study.

2.2. Mitochondrial marker

2.2.1. Mitochondrial protein extraction

The protein extraction method was derived from the Calbiochem superoxide dismutase assay kit II (Merck Millipore, Darmstadt, Germany) that was subsequently used to assess superoxide dismutase (SOD) activity. Mitochondrial protein fractions

were obtained by differential centrifugation. Prior to protein extraction, tissue was rinsed with phosphate buffered saline (PBS), pH 7.4, containing 0.16 mg/ml heparin to remove any red blood cells and clots. Tissue was homogenized with a Quiagen TissueRuptor (Quiagen, Limburg, Netherlands) in 1.5 ml of cold 20 mM HEPES buffer, pH 7.2, containing 1 mM EGTA, 210 mM mannitol, 70 mM sucrose and centrifuged at $1500 \times g$ for 5 min at 4 °C. The supernatant was centrifuged for 15 min at $10,000 \times g$ at 4 °C. The pellet containing the mitochondrial fraction was suspended in 20 mM HEPES buffer, pH 7.2 with 1 mM EGTA, 210 mM mannitol and 70 mM sucrose. The purity of the mitochondrial fraction was tested by determining the expression of the mitochondrial specific protein VDAC in cytosolic and mitochondrial extracts. Protein quantification (BioRad, CA, USA) was carried out to determine the protein concentration.

2.2.2. Western blot of mitochondrial proteins

10 µg of protein was mixed with 4× loading buffer, 10× reducing agent (Life Technologies, Vic, Australia) and water to make 20 µl solutions; and heated at 70 °C for 10 min. Samples were then analyzed by SDS gel electrophoresis (Life Technologies, Vic, Australia) and electroblotted to Hybond Nitrocellulose membranes (Amersham Pharmacia Biotech, Bucks, UK). Membranes were blocked in Tris-buffered saline containing 0.2% Tween-20 (TBST) in 5% skim milk for 30 min and then incubated overnight at 4 °C with the following primary antibodies: MitoProfil Total OXPHOS Rodent WB antibody cocktail 1:250 (Abcam Ltd., Cambridge, UK), TOM20 1:500 (Santa Cruz, CA, USA), MnSOD 1:1000 (Millipore, Billerica, MA, USA) in TBST containing 5% skim milk. Membranes were washed with TBST and incubated with horseradish peroxidase conjugated secondary antibody. Proteins were visualized using Luminata Western HRP Substrate (Millipore, MA, USA) in a LAS 4000 image reader (Fujifilm, Tokyo, Japan). All membranes were re-probed with β-actin 1:1000 (Santa Cruz, CA, USA) and results were expressed as percentage of protein expression relative to β-actin. Analysis was performed using Image J software (Java based software program, National Institutes of Health).

2.2.3. Determination of mitochondrial MnSOD activity

The activity of MnSOD was determined in the mitochondrial protein fraction by a standard kit from Calbiochem (Merck Millipore, Darmstadt, Germany) following the manufacturer's instructions. The MnSOD activity was expressed as the amount of enzyme causing a 50% inhibition of formazan dye, employing hypoxanthine and xanthine oxidase to generate superoxide radicals.

2.2.4. Electron microscopy of mitochondria

Kidney tissue was fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.4 and subsequently sliced into 100 nm thick ultrathin sections, then mounted on 300 mesh copper grids for imaging of mitochondria with an FEI Morgagni 268D transmission electron microscope (FEI, Eindhoven, The Netherlands).

2.2.5. Mitochondrial copy number

Genomic DNA was extracted from renal tissue using the DNeasy blood and tissue kit (Qiagen). The content of mtDNA was calculated using real-time quantitative PCR by measuring the threshold cycle ratio (ΔC_t) of the mitochondrial-encoded gene cytochrome c oxidase subunit 1 (COX1) (forward primers 5'-ACTATACTACTAA-CAGACCG-3', reverse primers 5'-GGTTCTTTTCCGGAGTA-3') vs. the nuclear-encoded gene cyclophilin A (forward primers 5'-ACACGCCATAATGGCACTGG-3', reverse primers 5'-CAGTCTGGCAGTGCAGAT-3').

2.3. Oxidative stress markers

2.3.1. Confocal microscopy

Frozen kidney sections were stained and imaged using Leica SP2 confocal laser scanning microscope (Leica, Wetzlar, Germany). Data was generated from three independent experiments, each in triplicates. Fifty images were taken from each slide and averaged before the analysis. All imaging parameters including laser intensities, Photomultiplier tubes voltage, pinhole were kept constant during imaging. For total ROS detection, CellROX Deep Red (Molecular Probes, Australia) was used at 5 μM final concentration, images were collected at 633 nm excitation wavelength and detected in the 640–680 nm emission range. MitoTracker Green (Molecular Probes, Australia) was used to visualize the mitochondria at 200 nM final concentration. Images were collected at 458 nm excitation wavelength and detected in the 480–505 nm emission range. Lysosomes were visualized using Lysotracker Red DND-99 (Molecular Probes, Australia) with 100 nM final concentration. Images were collected at 514 nm excitation wavelength and detected in the 525–550 nm emission range. To calculate the correlation between CellROX and Mitotracker, dual staining using CellRox and Mitotracker was performed and images were taken sequentially using separate confocal channels over a time not greater than 30 s. The image pixel intensity value correlation was then calculated using Pearson's correlation for all pixels excluding any pairs containing zero values.

2.4. Immunohistochemistry

Formalin-fixed paraffin-embedded sections were deparaffinized and boiled for 20 min in 10 mM citrate buffer (pH 6.0). Sections were washed in TBST and exposed to 0.3% H_2O_2 for 5 min to quench endogenous peroxidases. Immunohistochemistry was performed using the following antibody: rabbit anti-8OHdG polyclonal antibody (1:100, BIOSS, Woburn, MA, USA). Concentration-matched rabbit IgG was used as an isotype-negative control. The sections were blocked with Dako proteinblock (Dako, Carpinteria, CA, USA) for 10 min and incubated with primary antibody overnight. The slides were then incubated with horseradish peroxidase anti-rabbit Envision-system followed by a 3,3'-diaminobenzidine (DAB) substrate-chromogen solution (Dako) and counterstained with Harris hematoxylin. The slides were examined using a Leica photomicroscope linked to a DFC 480 digital camera (Leica, Wetzlar, Germany).

The quantitation was performed by capturing 6–10 non-overlapping fields of renal cortex from stained sections. Areas of brown staining reflecting 8-OHdG were highlighted using a selective color tool and the proportional area of the field with their respective color range was quantified using Image J.

2.5. Statistical analysis

All results are expressed as mean \pm standard error (SE) and statistical significance was defined as $P < 0.05$. Experiments were performed at least in three independent experiments or as detailed in the text with n reflecting the number of separate experiments. Statistical comparisons between groups were made by unpaired student *t*-tests or non-parametric Mann–Whitney *U* test. Analyses were performed using the software package, GraphPad Prism version 6 (GraphPad Software Inc., La Jolla, CA, USA). For the confocal study, the data distribution was assessed using a non-parametric test of different source distributions (Kolmogorov–Smirnov) which is sensitive to difference in the shape and position of the empirical distribution functions of the compared samples and is particularly useful where there is doubt regarding the nature of the source population (Lehmann and Romano, 2005). Multiple images were taken for over 100 cells in each tissue in 3 replicates of 3 independent

samples/group. Pearson's correlation method was used to determine the correlation factor and *P* values for the correlation study.

3. Results

3.1. Physiological and renal parameters

The physiological characteristics of mice offspring are summarized in Table 1. In keeping with the known effect of maternal nicotine exposure during gestation we observed a reduced birth weight (day 1) in offspring of smoke-exposed mothers with normalization of weight at weaning (day 20) and adulthood (week 13). Kidney weight was similarly reduced at day 1 in the SE offspring but had normalized by day 20. Serum creatinine remained normal in both groups at adulthood, however offspring of smoke-exposed mothers developed an increased urinary albumin/creatinine ratio at week 13.

3.2. Increased mitochondrial oxidative stress in offspring kidneys from smoke-exposed mothers

Products from cigarette smoke are known inducers of oxidative stress in various tissues and are able to cross the placenta. In order to investigate whether maternal smoking causes oxidative stress in the offspring, frozen renal sections were co-stained with CellRox Red and Mitotracker Green. There was a significant increase of total ROS in SE offspring at day 20 and week 13 ($P < 0.001$; Fig. 1A and C). The correlation coefficient of the mean fluorescent intensities was significantly higher in the kidney of the SE offspring at day 1 and week 13 ($P < 0.01$, Fig. 1B and D) suggesting that at these time points ROS was localized within or within close proximity to the mitochondria.

3.3. Increased DNA oxidation in offspring kidneys from smoke-exposed mothers

As high levels of ROS can cause damage to DNA, we next performed immunohistochemistry for 8-hydroxydeoxyguanosin (8-OHdG), which is formed when DNA is oxidatively modified by ROS. A significant increase of 8-OHdG in paraffin-embedded kidney sections from smoke-exposed offspring was observed at all time points. Both proximal and distal tubules stained positive for 8-OHdG with sparing of the glomeruli. The most intense staining was

Table 1

Characteristics of offspring mice. Data are expressed as mean \pm SEM of 5–10 male mice per group.

	Control	SE
Day 1		
Body weight (g)	1.55 \pm 0.05	1.35 \pm 0.06*
Kidney weight (g)	0.0081 \pm 0.0004	0.0069 \pm 0.0004
Kidney/body weight (%)	0.52 \pm 0.02	0.51 \pm 0.04
Day 20		
Body weight (g)	9.97 \pm 0.16	9.71 \pm 0.14
Kidney weight (g)	0.067 \pm 0.001	0.062 \pm 0.003
Kidney/body weight (%)	0.67 \pm 0.01	0.64 \pm 0.03
Albumin/creatinine ratio ($\mu\text{g}/\text{mg}$)	8.69 \pm 2.00	6.12 \pm 1.45
Serum creatinine ($\mu\text{mol}/\text{l}$)	10.4 \pm 0.7	12.1 \pm 1.2
Week 13		
Body weight (g)	25.5 \pm 0.3	25.1 \pm 0.6
Kidney weight (g)	0.20 \pm 0.01	0.19 \pm 0.01
Kidney/body weight (%)	0.77 \pm 0.01	0.76 \pm 0.02
Albumin/creatinine ratio ($\mu\text{g}/\text{mg}$)	7.00 \pm 2.3	38.0 \pm 6.3*
Serum creatinine ($\mu\text{mol}/\text{l}$)	15.2 \pm 1.3	14.2 \pm 0.5

* $P < 0.05$ vs. control using unpaired *t* tests.

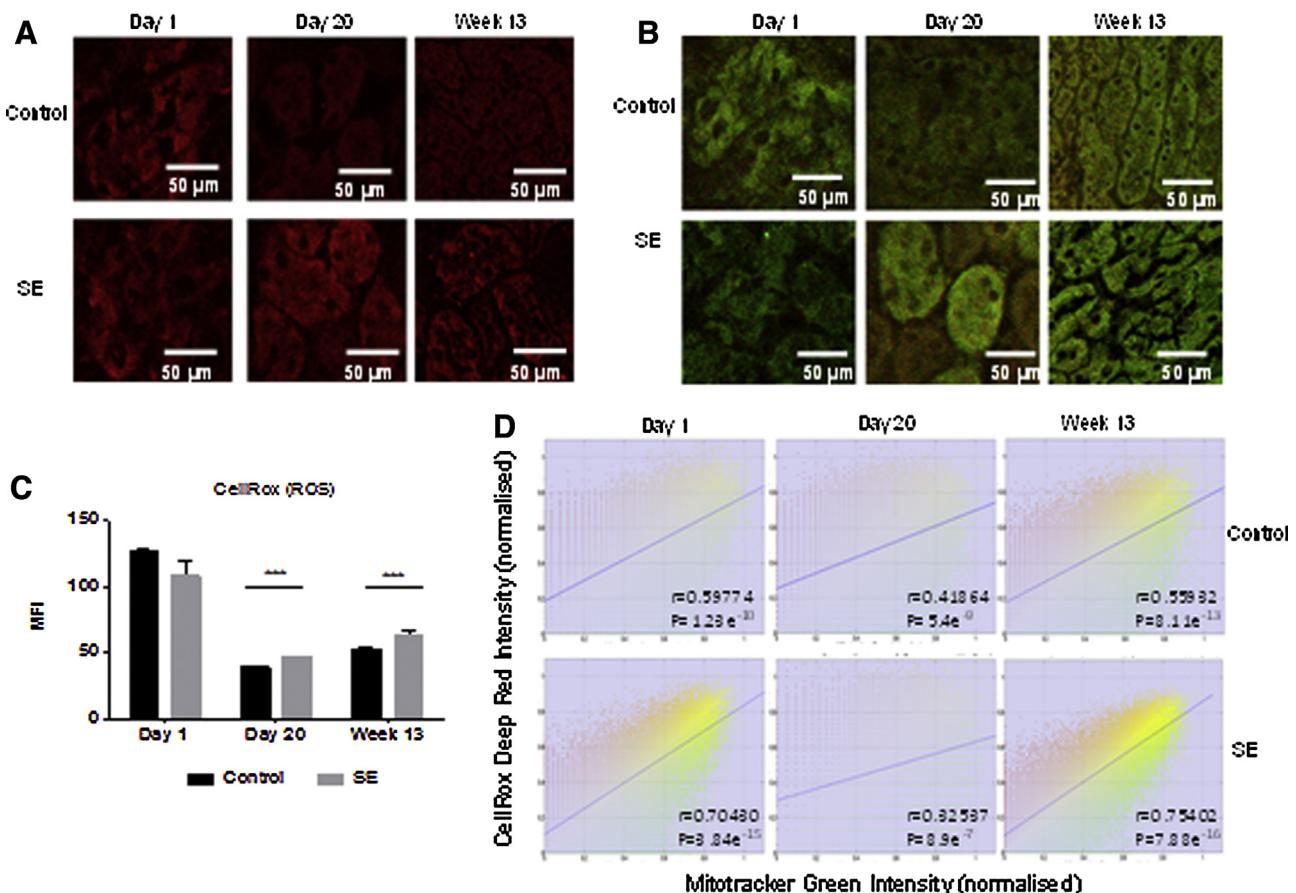


Fig. 1. Detection of oxidative stress. (A) CellROX stain for total ROS in frozen renal sections of offspring mice of smoke-exposed mothers (SE) and control at postnatal day 1, day 20 and week 13. (B) Dual staining for CellROX and Mitotracker showing that most ROS in offspring kidneys from SE mothers, was localized within or within close proximity to the mitochondria at day 1 and week 13. (C) Quantitative representation of Mean Fluorescent Intensity (MFI) for ROS. (D) Pixel intensity scatter plots showing correlation between Mitotracker (green) and CellRox (red) in offspring kidney from control and SE mothers at day 1, day 20 and week 13. Data are expressed as mean \pm SEM, $n = 3$, $^{**}P < 0.01$, $^{***}P < 0.001$ using non-parametric test of different source distributions (Kolmogorov-Smirnov). Correlation factor and Pvalues for the correlation study (Fig. 1D) was determined using Pearson correlation. Colors (green, red and yellow) reflect Mitotracker, CellRox and colocalised pixels. Scale bars represent 50 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

noted in distal tubules with predominantly cytoplasmic staining (Fig. 2).

3.4. Reduced mitochondrial MnSOD in offspring kidneys from smoke-exposed mothers at postnatal day 1 and week 13

SOD is a crucial component of the cellular antioxidant defense. While other isoforms of SOD are located in the cytosol, MnSOD is mostly located in the mitochondria. In order to determine MnSOD activity, mitochondrial protein fractions were obtained by differential centrifugation. MnSOD activity was significantly reduced at day 1 and week 13 ($P < 0.05$) but not at day 20 (Fig. 3).

3.5. Reduced renal OXPHOS protein subunits in offspring kidneys from smoke-exposed mothers

To investigate mitochondrial protein expression, we looked at TOM20, a mitochondrial outer membrane receptor for translocation of cytosolically synthesized mitochondrial pre-proteins, and subunits of the OXPHOS complexes I–V, key components of the mitochondrial respiratory chain for ATP synthesis. TOM20 was significantly reduced in offspring from SE mothers at day 1 and week 13 ($P < 0.01$ and $P < 0.001$ respectively, Fig. 4). There was no change in TOM20 protein levels at day 20. In addition, there was a

significant reduction in subunits of complex I, II, III and V both at postnatal day 1 and week 13 in the SE offspring compared to control (Fig. 5A and C). At day 1 the most marked differences were observed in complex V ($P < 0.01$). However, at weaning age (day 20) there was no significant difference in any of the mitochondrial enzyme subunits, which may suggest a regenerative effect during lactation and consequent to the high levels of antioxidants in breast milk. At mature age (week 13) there was again a significant protein reduction in all of the examined OXPHOS subunits. The most pronounced reduction at week 13 was observed in complex II, IV and V ($P < 0.01$, Fig. 5C).

3.6. Increased mitochondrial DNA copy number in offspring from smoke-exposed mothers

It has been suggested, that abnormal amounts of mitochondrial DNA, either depletion or elevation, are associated with mitochondrial dysfunction. We thus investigated mitochondrial copy number in SE offspring. Our data showed that offspring DNA from SE mothers have increased levels of mitochondrial-encoded COX1/nuclear-encoded cyclophilin at postnatal day 1, day 20 and week 13 ($P < 0.05$ vs. control, Fig. 6). This suggests that maternal smoke exposure increases mitochondrial size/mass due to either increased mitochondrial size or number.

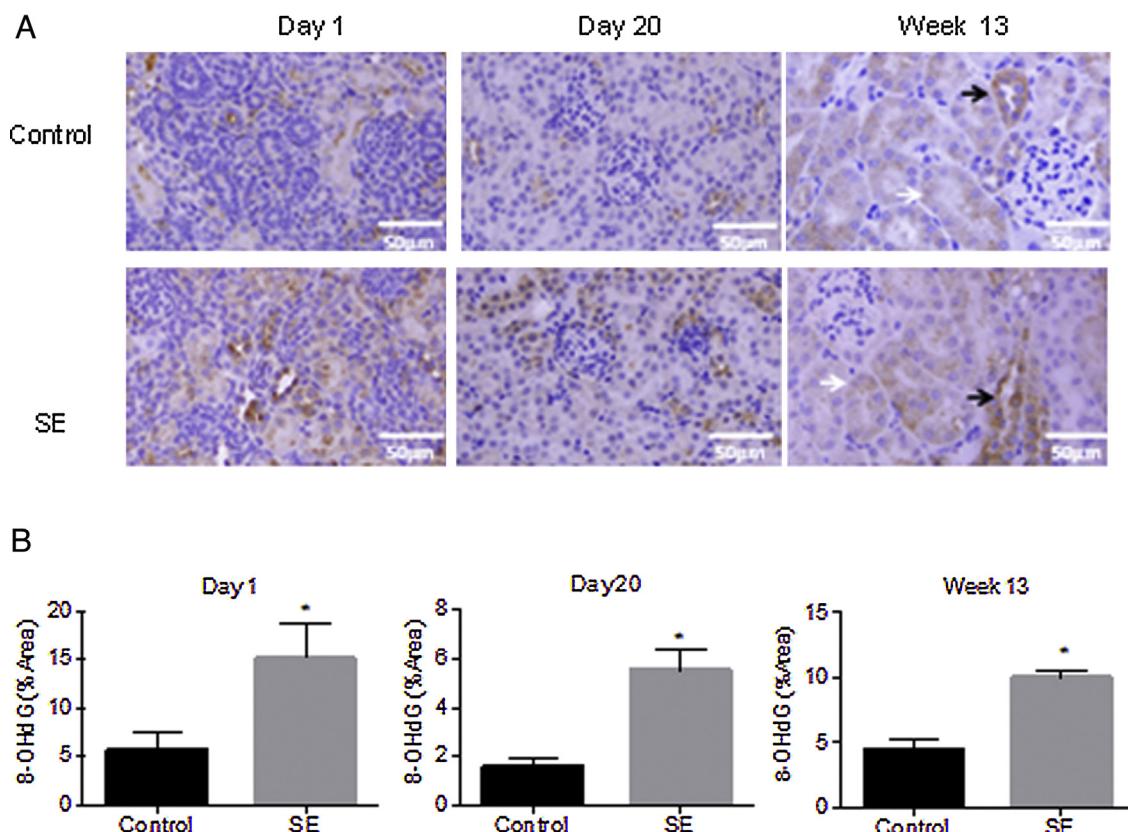


Fig. 2. Immunostaining of 8-OHdG in paraffin sections of renal tissue. (A) Representative images of renal cortex showing 8-OHdG expression at day 1, day 20 and week 13. Black arrows and white arrows showed increased expression of 8-OHdG in representative distal tubules and proximal tubules respectively. Original magnification: 200 \times and (B) quantitation of 8-OHdG. Data is expressed as mean % of stained area \pm SEM, n = 5–6 *P<0.05 vs. control mice using unpaired t tests. Scale bars represent 50 μ m.

3.7. Alteration of mitochondrial ultrastructure in smoke-exposed offspring

Renal mitochondria structure was examined using electron microscopy. Offspring kidneys from control mice had normal mitochondrial morphology as demonstrated by long filamentous mitochondria. In contrast, offspring from SE mothers exhibit mitochondrial enlargement and swelling at day 1 and week 13. Additionally, an increased number of small punctate mitochondria at day 1 and week 13 was also evident suggesting increased mitochondrial fragmentation. However, this effect was not evident in the SE offspring at day 20 (Fig. 7).

3.8. Increased mitochondrial density in offspring kidneys from smoke-exposed mothers

Using Mitotracker, a mitochondrial selective fluorescent probe, we confirmed that the cellular mitochondrial density is significantly increased in offspring kidney from SE mothers at day 1 and week 13 compared to control ($P<0.05$ and $P<0.001$ vs. control,

Fig. 8). However, there was a reduction ($P<0.05$ vs. control) in the mitochondrial density at day 20.

3.9. Increased lysosome density in offspring from smoke-exposed mothers

Defective mitochondria are engulfed in autophagosomes that fuse with lysosomes. In order to investigate whether maternal smoke exposure induces mitophagy, lysosomal density was determined. Lysosome density significantly increased in offspring from SE mothers at week 13 ($P<0.001$ vs. control; Fig. 9). As was evident in the divergent results for the offspring at weaning for the above parameters, the mean cellular lysosome levels were significantly decreased in offspring from SE mothers at day 20 ($P<0.001$ vs. control; Fig. 9).

4. Discussion

The present study demonstrates increased renal oxidative stress, reduced mitochondrial antioxidant activity as well as

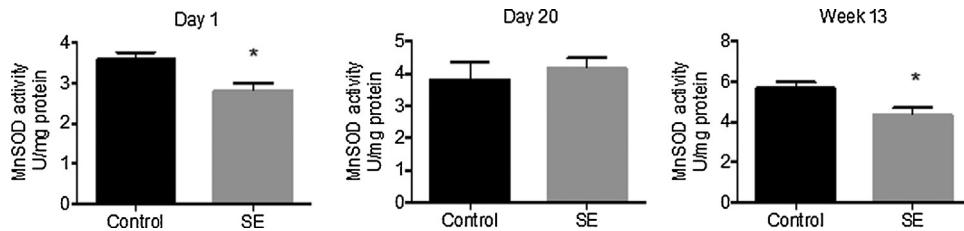


Fig. 3. Mitochondrial MnSOD activity in offspring kidney at postnatal day 1, day 20 and week 13. Results are expressed as mean \pm SEM, n = 6–8; *P<0.05 vs. control using unpaired t tests.

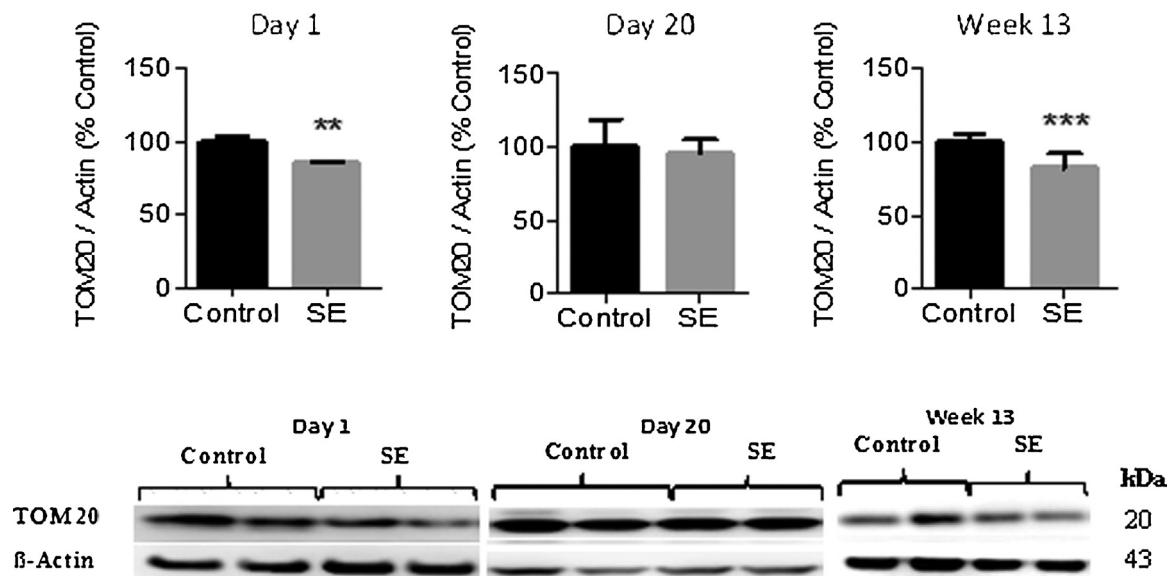


Fig. 4. Renal TOM 20 levels and representative blot in offspring kidney at day 1, day 20 and week 13. Results are normalized to β -actin which was detectable in mitochondrial fractions and did not change with experimental conditions. Results are expressed as mean \pm SEM, $n=6-8$. ** $P<0.01$, *** $P<0.001$ vs. control using unpaired t tests.

mitochondrial structural changes and reduced OXPHOS proteins in offspring of SE mothers at birth and adulthood. This is associated with an increased urinary albumin to creatinine ratio in adulthood. The presence of albuminuria suggests renal pathology independent of glomerular filtration rate, and independently portends an accelerated decline in kidney function in all forms of kidney disease (Iseki, 2013).

Our study suggests that fetal programming of CKD is regulated, at least in part, by maternal tobacco smoke-mediated production of oxidative stress and mitochondrial perturbations. The presence of mitochondrial dysfunction in the placenta of smoking mothers has previously been demonstrated (Bouhours-Nouet et al., 2005). To the best of our knowledge, this is the first study showing enhanced oxidative stress and mitochondrial changes in offspring kidney tissue following maternal cigarette exposure. A previous animal study in Wistar rats similarly demonstrated increased ROS formation and altered mitochondrial structure in adult offspring pancreatic tissue in response to maternal subcutaneous injections of nicotine during gestation and lactation (Bruun et al., 2008). Bruun et al. found that mitochondrial structural defects were accompanied by a modest decline in OXPHOS complex IV activity at adult age. In accordance with our results, Bruun et al. observed mitochondrial structural abnormalities as early as three weeks after birth (weaning age), which progressively worsened even though nicotine exposure was discontinued at weaning. Importantly these changes preceded decreased pancreatic beta cell function and glucose intolerance in adult life highlighting again the role of fetal programming in the development of chronic adulthood diseases. In an atherosclerosis mouse model using Apolipoprotein E null mice, Fetterman et al. demonstrated that in-utero smoke exposure significantly accelerated adult atherosclerosis. Consistent with our findings, the smoke-exposed offspring showed increased oxidative stress and a reduction in MnSOD levels at an adult age, which was associated with increased mitochondrial DNA copy number and the presence of mitochondrial DNA deletions in aorta tissue (Fetterman et al., 2013).

The mitochondrial genome is more susceptible to ROS induced DNA damage than the nuclear genome and exhibits higher rates of DNA mutation due to the lack of histone protection, reduced DNA repair capacity and close proximity to the ROS producing electron transport chain. However, it is suggested that even in the

absence of detectable mitochondrial DNA mutations, alterations in mitochondrial DNA content, either depletion or elevation, may be an indicator of mitochondrial dysfunction (Bai et al., 2004). In this study we detected an increase in mitochondrial DNA content in SE offspring. The mechanism by which mitochondrial DNA increases in response to oxidative stress is not well understood. It is hypothesized that this is a compensatory mechanism for the decline in mitochondrial function by inducing the proliferation of mitochondria and/or mitochondrial DNA amplification (Bai et al., 2004; Lee et al., 2000). An increase in mitochondrial DNA levels have been shown in vitro in response to oxidative stress (Al-Kafaji and Golbahar, 2013; Lee et al., 2000), in age related mitochondrial alterations (Barrientos et al., 1997; Lee et al., 1998) and in smokers (Ballinger et al., 1996; Lee et al., 1998; Masayesva et al., 2006). Masayesva et al. detected not only an age-independent elevation of mitochondrial DNA levels in current smokers but also in former smokers with mean cessation intervals of two decades (Masayesva et al., 2006). The persistence of these changes is consistent with our in-utero smoke exposure model where elevated mitochondrial DNA content was detectable long after birth.

When DNA is oxidatively modified by ROS, the amount of 8-OHdG increases. Our data confirmed that smoke exposure induces oxidative modification of DNA in the offspring from birth and this was persistent till adulthood. The fact that 8-OHdG was increased in the cytoplasm suggests increased oxidative damage to the mitochondrial DNA (Nomoto et al., 2008). Although 8-OHdG was increasingly expressed in the proximal tubule, its level of expression was more intense in distal tubules. A possible explanation is the fact that ROS detoxifying enzymes are more abundant in proximal tubular cells than distal tubules, which may indicate a diminished ability to detoxify reactive metabolites in this part of the nephron and a higher intrinsic susceptibility of distal tubular cells to oxidative injury (Lash and Tokarz, 1990).

Although we detected DNA oxidation and changes in mitochondrial DNA copy number in smoke-exposed offspring persistently from day 1 till week 13, mitochondrial structural changes and OXPHOS as well as TOM20 protein depletion were seen at birth and adulthood without any significant change at day 20. MnSOD protein content and activity mirrored the OXPHOS protein content changes with a significant MnSOD reduction demonstrated at day 1 and week 13 and equally no significant change at weaning age.

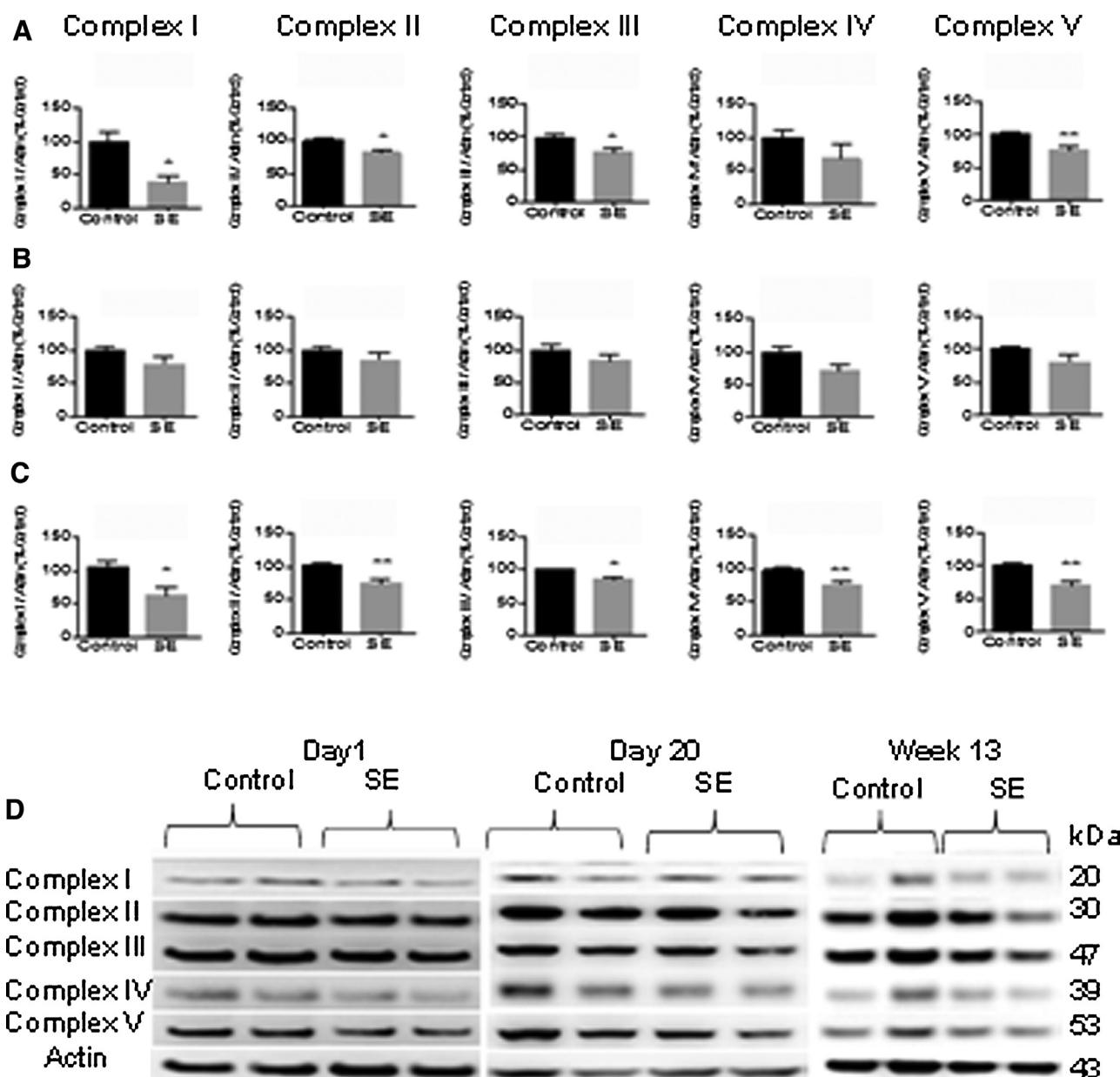


Fig. 5. Renal OXPHOS complex I–V levels in mitochondrial protein fractions of offspring from control and smoke-exposed mothers at postnatal day 1 (A), day 20 (B) and week 13 (C). (D) Representative blots for OXPHOS complex I–V at day 1, day 20 and week 13. Results are normalized to β -actin which was detectable in mitochondrial fractions and did not change with experimental conditions. Results are expressed as mean \pm SEM, $n=6–8$. * $P<0.05$, ** $P<0.01$ vs. control using unpaired t tests.

We propose that the deleterious effects on mitochondria observed at birth and in adulthood may be mitigated at weaning due to the antioxidants in the breast milk (Ermis et al., 2005; Zagierski et al., 2012). Further studies to investigate this hypothesis are needed.

MnSOD is the primary mitochondrial ROS scavenging enzyme that transforms toxic superoxide free radicals to hydrogen peroxide, which is subsequently converted to water by catalases and other peroxidases. MnSOD has also recently been demonstrated

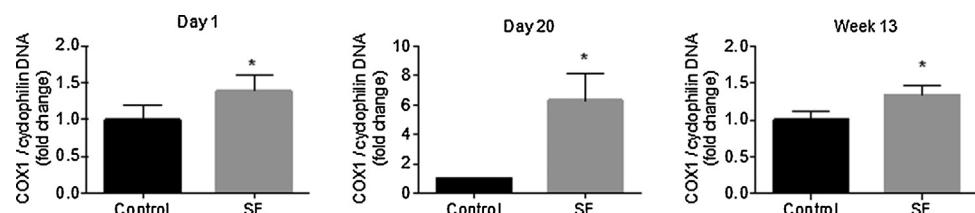


Fig. 6. Mitochondrial DNA copy number shown by the ratio of mitochondrial-encoded COX1 to nuclear-encoded cyclophilin A in offspring renal DNA from control and smoke-exposed mothers at postnatal day 1, day 20 and week 13. Results are expressed as fold increase \pm SEM, $n=4$; * $P<0.05$ vs. control using non-parametric Mann-Whitney U test.

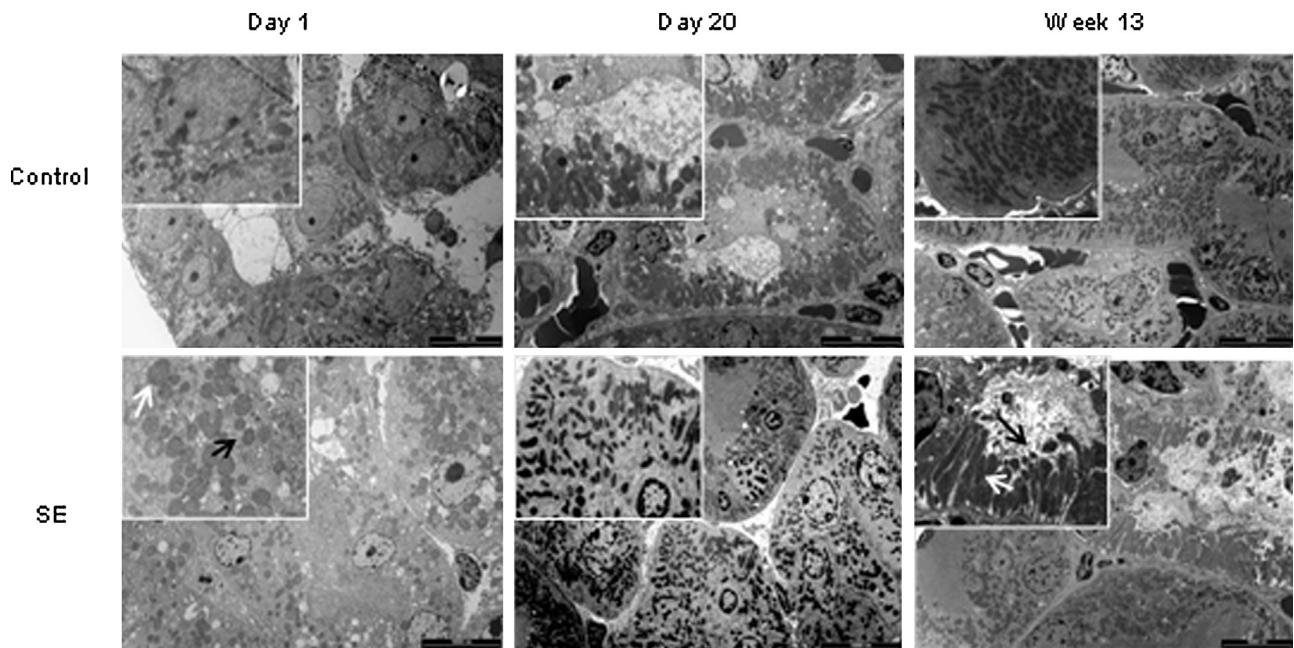


Fig. 7. Electron microscopic detection of mitochondria in offspring renal proximal tubular cells from control and smoke-exposed mothers at day 1, day 20 and week 13. Normal looking (long filamentous) mitochondria are shown in offspring kidney from control mothers at day 1, day 20 and week 13 and in kidneys from SE mothers at day 20. White arrows show enlarged (circular shaped) mitochondria and black arrows show increased number of small punctate mitochondria at day 1 and week 13 in offspring kidney from SE mothers. Insets in the far left corner: high magnification view of mitochondria. Scale bar = 10,000 nm.

to be part of a protein complex necessary for mitochondrial DNA repair (Bakthavatchalu et al., 2012), thus playing a pivotal role in multiple aspects of mitochondrial protection. Cigarette smoke has previously been shown to reduce MnSOD levels in

circulating blood cells (Mandrafino et al., 2010), while in-utero smoke exposure caused a reduction of MnSOD levels in aortic tissue in the offspring (Fetterman et al., 2013). This study is the first to demonstrate reduced MnSOD levels in offspring kidneys

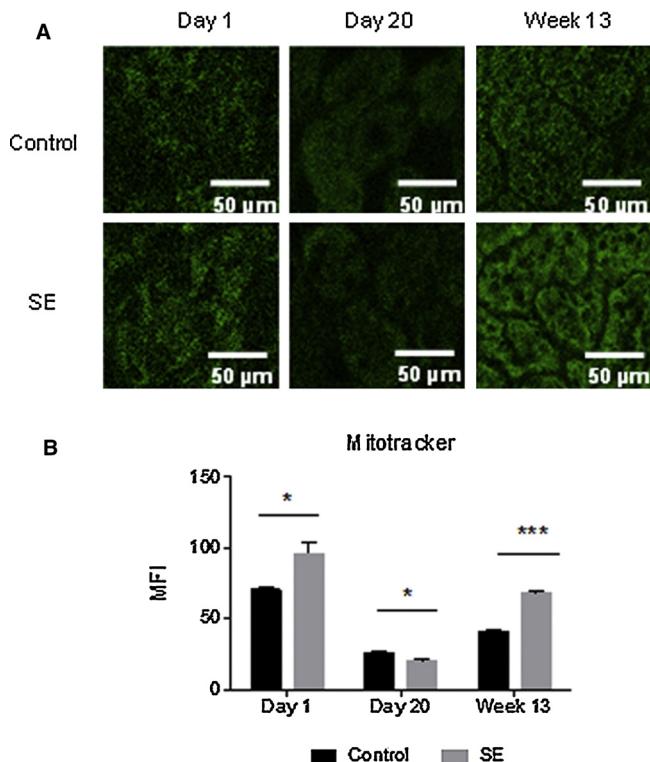


Fig. 8. Mitotracker staining showing mitochondrial mean fluorescence intensity (MFI) in offspring kidney at postnatal day 1, day 20 and week 13. Increased mitochondrial density is shown in offspring kidney from SE mothers at day 1 and week 13. Results are expressed as mean \pm SEM, $n=3$; * $P<0.05$, *** $P<0.001$ vs. control using non-parametric test of different source distributions (Kolmogorov-Smirnov). Scale bars represent 50 μ m.

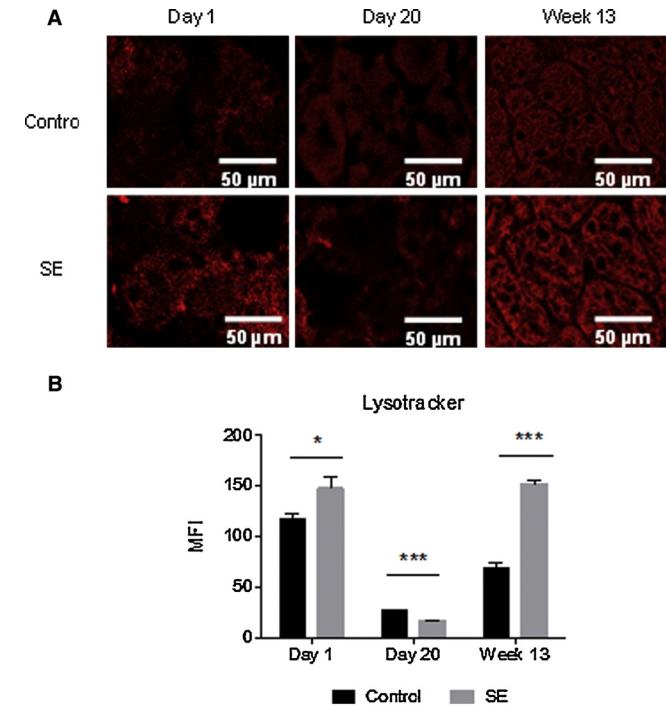


Fig. 9. Mean fluorescence intensity (MFI) for Lysotracker stain in offspring kidney from control and smoke-exposed mothers at postnatal day 1, day 20, and week 13. Increased lysosomal density is shown in offspring kidney from SE mothers at day 1 and week 13. Data is expressed as mean \pm SEM, $n=3$; * $P<0.05$, *** $P<0.001$ vs. control using non-parametric test of different source distributions (Kolmogorov-Smirnov). Scale bars represent 50 μ m.

in response to maternal smoking. Of note is the occurrence of reduced MnSOD levels in offspring long after smoke exposure at adulthood both in our model and in the study of Fetterman et al. (2013) highlighting the long-term toxic effects of cigarette smoke as the basis for fetal programming.

Using electron microscopy we have demonstrated in proximal tubular cells, that offspring from SE mothers exhibit mitochondrial enlargement and had an increased number of small punctate mitochondria at day 1 and week 13 suggestive of increased mitochondrial fragmentation. The presence of structural mitochondrial abnormalities is in accordance with other studies of direct smoke exposure (Hara et al., 2013) and in-utero nicotine exposure (Bruun et al., 2008). Damaged mitochondria are removed by mitophagy, a selective autophagy process, during which autophagosomes enclose whole mitochondria, or selectively target damaged areas and fuse with lysosomes for degradation. Cellular lysosome density was increased in SE offspring at day 1 and week 13. This suggests that smoke exposure induces accumulation of damaged mitochondria and may impair mitophagy but this requires further investigation.

Mitochondria serve a crucial role in development by providing energy for the rapid fetal growth and play key roles in cell signaling (Duchen, 2004; May-Panloup et al., 2007). Environmental exposures that result in mitochondrial perturbations have long-lasting effects and may lead to failure of organ function over time. The kidney is a highly metabolic organ and rich in mitochondria. There is increasing evidence implicating mitochondrial dysfunction in the pathogenesis of chronic kidney disease (CKD) and acute kidney injury (AKI). Genomic analysis of blood samples from CKD patients revealed differential expression of genes encoding OXPHOS and reduced complex IV activity (Granata et al., 2009). Mitochondrial dysfunction has been implicated as an early event in experimentally induced podocyte dysfunction (Yuan et al., 2012b) as well as epithelial to mesenchymal transition (EMT), which is a major mechanism leading to renal tubulointerstitial fibrosis (Yuan et al., 2012a; Zhang et al., 2007). In AKI it is increasingly recognized that cell death is disproportionately low despite often severely impaired renal function (Takasu et al., 2013; Tran et al., 2011). Subtle vacuolization in proximal tubular cells are often the only documented structural lesions and are considered to represent swollen mitochondria (Takasu et al., 2013; Tran et al., 2011). Several studies have shown a reduction of OXPHOS protein and activity in experimental models of AKI (Funk and Schnellmann, 2012; Rasbach and Schnellmann, 2007). Our data suggests that maternal smoking induces mitochondrial perturbations and possibly dysfunction, although this was not directly investigated. Additional functional experiments are needed to validate this finding.

We demonstrated the occurrence of albuminuria at adult age in offspring of smoke exposed mothers. Albuminuria as an early marker for kidney damage is also an independent predictor for an accelerated progression of chronic kidney disease to more advanced stages. The effect of smoking on development of albuminuria is well described (Halimi et al., 2000; Hogan et al., 2007; Pinto-Sietsma et al., 2000) however the effect of prenatal smoke exposure on offspring kidneys is less studied. An experimental study on offspring rats exposed to cigarette-smoke condensate in-utero revealed lower glomerular volume and glomerular cells compared to control (Zarzecki et al., 2012). In contrast to our study this study did not show a difference between smoke exposed offspring and control with respect to birth weight, kidney weight, albuminuria or creatinine clearance. The lack of reduced birth weight may signal lower levels of smoke exposure. The smoke exposure was different to our model with oral mucosa application of cigarette-smoke condensate dissolved in acetone containing nicotine. While there was high nicotine exposure as evidenced by the increased cotinine levels, the amount of other toxins in

condensate may have been less than in our model. A more recent study also found that smoke exposure in utero has pro-fibrotic influences on offspring kidneys (Chen et al., 2015). Although we did not detect any renal histological changes at any time point (data not shown) nor an increase in serum creatinine, we propose that our findings of mitochondrial alterations at birth and adulthood may put offspring at increased risk for renal pathology especially in the setting of additional insults, such as sepsis or toxins. A history of maternal smoke exposure in-utero may thus predispose to more severe forms of AKI or irreversible damage that leads to progression of CKD. The results of this study highlight the importance of optimization of maternal and fetal health as well as smoking prevention. It remains to be shown whether the negative impact on mitochondrial integrity can be reversed or potentially avoided by smoking cessation prior to pregnancy.

Competing interests

None of the authors has a financial disclosure.

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