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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.

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 TissueRuptur (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 × *g* for 5 min at 4 °C. The supernatant was centrifuged for 15 min at 10,000 × *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 (Quiagen). 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′-ACTATACTACTACTAA-CAGACCG-3′, reverse primers 5′-GGTTCTTTTTTTCCGGAGTA-3′) vs. the nuclear-encoded gene cyclophilin A (forward primers 5′-ACACGCCATAATGGCACTGG-3′, reverse primers 5′-CAGTCTTGGCAGTGCAGAT-3′).