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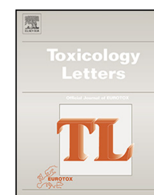

Suppressed mitochondrial biogenesis in folic acid-induced acute kidney injury and early fibrosis

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HIGHLIGHTS

- Folic acid causes robust acute kidney injury with functional recovery in six days.
- Renal mitochondrial biogenesis is suppressed from 1 to 14 d after injury.
- Renal fibrosis develops two weeks after folic acid treatment.
- We report the first connection between AKI, mitochondrial biogenesis, and fibrosis.

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ABSTRACT

Acute kidney injury (AKI) is a disease with mitochondrial dysfunction and a newly established risk factor for the development of chronic kidney disease (CKD) and fibrosis. We examined mitochondrial homeostasis in the folic acid (FA)-induced AKI model that develops early fibrosis over a rapid time course. Mice given a single dose of FA had elevated serum creatinine (3-fold) and urine glucose (2.2-fold) 1 and 2 d after injection that resolved by 4 d. In contrast, peroxisome proliferator gamma coactivator 1α (PGC-1α) and mitochondrial transcription factor A (TFAM), critical transcriptional regulators of mitochondrial biogenesis (MB), were down-regulated ~80% 1 d after FA injection and remained depressed through 14 d. Multiple electron transport chain and ATP synthesis genes were also down-regulated from 1 to 14 d after FA, including NADH dehydrogenase (ubiquinone) 1 beta subcomplex 8 (NDUFβ8), ATP synthase subunit β (ATPS-β), and cytochrome C oxidase subunit I (COXI). Mitochondrial DNA copy number was reduced ~50% from 2 to 14 d after FA injection. Protein levels of early fibrosis markers α-smooth muscle actin and transforming growth factor β1 were elevated at 6 and 14 d after FA. Picrosirius red staining and collagen 1A2 (COL1A2) IHC revealed staining for mature collagen deposition at 14 d. We propose that mitochondrial dysfunction induced by AKI is a persistent cellular injury that promotes progression to fibrosis and CKD, and that this model can be used to test mitochondrial therapeutics that limit progression to fibrosis and CKD.

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1. Introduction

Acute kidney injury (AKI) is defined as a rapid and reversible decline in renal function. AKI affects 40–60% of intensive-care unit patients and incidence rates remain unchanged (Bellomo et al., 2012; Thadhani et al., 1996). Drugs, toxicants, ischemia/reperfusion (I/R), and sepsis are common causes of AKI and lead to reduced glomerular filtration and tubular necrosis. One mechanism of drug and toxicant-induced renal injury is crystal formation in the lumen, as observed with uric acid, acyclovir, and calcium oxalate, the molecule responsible for renal toxicity of ethylene glycol (Thadhani et al., 1996). When given intraperitoneally at high doses (e.g. 250 mg/kg), folic acid (FA) causes AKI in rodents (Brade et al., 1970; Long et al., 2008). The mechanism of FA nephropathy may be due to the formation of luminal crystals at these doses (Schmidt et al., 1973); however, FA also has direct toxicity on the tubular epithelium at high doses (Fink et al., 1987). The Reference Daily Intake for adult humans provided by the Food and Drug Administration is 400 μg. While renal toxicity of FA has not been reported in humans, FA-induced nephrotoxicity has been used as a model of AKI.

CKD is defined as a consistent reduction in the glomerular filtration rate and typically progresses to end stage renal disease (ESRD)

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(Levey and Coresh, 2012). CKD affects over 750,000 people in the US and the incidence is rising due to increasing rates of diabetes and hypertension (Levey and Coresh, 2012). Tubulointerstitial fibrosis (TIF) is the best predictive marker of progression to ESRD (Fine and Norman, 2008) and development of TIF is observed following I/R- and toxicant-induced AKI (Kim et al., 2009; Yang et al., 2010). Fibrotic lesions damage the peritubular vasculature (Choi et al., 2000; Yuan et al., 2003), and lead to a state of chronic hypoxia in the nephron, promoting sustained oxidative stress (Kim et al., 2009; Seok et al., 2008).

Recent advances in nephrology research demonstrate a causal role for AKI in CKD development. For example, AKI is a major risk factor for CKD, increasing the risk of progression to CKD as much as 28-fold (Lo et al., 2009). In addition, the severity of injury in the acute phase of AKI is a highly effective indicator of progression to CKD (Chawla et al., 2011). In mice, CKD and fibrosis have been shown to follow AKI caused by FA, I/R, and aristolochic acid (Leelahavanichkul et al., 2010; Yang et al., 2010). CKD progression in the models is dependent upon the extent of AKI (Yang et al., 2010) and preventable with therapeutic intervention during the acute phase (Kapitsinou et al., 2012). These studies form the basis of our analysis of mitochondrial dysfunction in the AKI to CKD continuum.

Mitochondrial dysfunction is a recognized pathogenic element of AKI and cause of tubular cell dysfunction and death. Increased reactive oxygen species (ROS) and reactive nitrogen species (RNS) production, decreased ATP production, and cytochrome *c* release are frequently associated with epithelial cell injury in AKI (Plotnikov et al., 2007; Szeto et al., 2011; Zager et al., 2004). Our group recently reported persistent disruption of mitochondrial homeostasis and suppression of mitochondrial biogenesis (MB) following I/R- and glycerol-induced AKI (Funk and Schnellmann, 2011). In both models, renal mitochondrial proteins cytochrome *c* oxidase subunit I (COXI), ATP synthase subunit β (ATPS-β) and NADH dehydrogenase (ubiquinone) 1 beta subcomplex 8 (NDUFβ8) were depleted, indicative of mitochondrial damage and suppressed MB (Funk and Schnellmann, 2011). Over-expression of PGC-1α, the master regulator or MB, in renal proximal tubule cells restored mitochondrial and cellular functions after oxidant exposure, demonstrating the importance of MB in recovery from cellular injury (Rasbach and Schnellmann, 2007). While the mechanisms of maladaptive repair of the tubular epithelium after AKI are still unclear, it can lead to TIF through paracrine activation of resident fibroblasts and epithelial-mesenchymal transition (EMT) of renal epithelial cells (Iwano et al., 2002; Lan et al., 2012). Interestingly, mitochondrial-derived ROS can induce EMT in renal tubular cells in vitro, and restoration of functional mitochondria and antioxidant mechanisms by induction of PGC-1α attenuates this transition (Hallman et al., 2008; Yuan et al., 2012). However, little is known about the role of mitochondrial function in renal fibrosis. Here we report that persistent mitochondrial dysfunction is linked to early renal fibrosis in a model of FA-induced AKI.

2. Methods

2.1. Animal model

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina and all efforts were made to minimize animal suffering. Male CD-1 mice (8–10 weeks of age, Harlan Laboratories) were injected with a single intraperitoneal dose of 250 mg/kg FA (Sigma) dissolved in 300 mM $NaHCO_3$ as previously described (Wen et al., 2012). At 0, 1, 2, 4, 6, 10, and 14 d after treatment, serum and urine samples were collected. Urine was collected overnight from mice housed in metabolic cages (18 h collection period). Serum and urine creatinine, blood urea nitrogen (BUN), and urine glucose levels were measured using Quantichrom Assay Kits (BioAssay Sytems). Urine glucose was normalized to urine creatinine. Urine osmolality was measured using the 5004 Micro-Osmette (Precision Systems). Mice were euthanized 0, 1, 2, 6, and 14 d after treatment by CO_2 asphyxiation. These time points were chosen to capture the acute, recovery and chronic phases of FA-induced AKI. Kidneys were removed and preserved either by snap freezing or formalin fixation and paraffin embedding.

2.2. Histological analysis

Kidneys were sectioned and stained with Periodic Acid Schiff (PAS) for the evaluation of histology. Fibrillar collagen content was evaluated using picrosirius red staining. Briefly, slides were deparaffinized and rehydrated, stained with Weigert's iron hematoxylin for 10 min, and washed with water for 10 min. Slides were then stained with picrosirius red (0.1% direct red 80 CI#35780 in saturated aqueous picric acid) for 1 h, washed twice in 1% glacial acetic acid, dehydrated, cleared with xylene, mounted with *Permount* and examined under plane polarized light. Immunohistochemistry was performed as previously described (Korrapati et al., 2012) using the collagen 1A2 (COL1A2) antibody (Santa Cruz).

2.3. mRNA analysis

Total RNA was isolated from renal cortex with Trizol (Invitrogen) and reverse transcription was performed using the iScript Advanced cDNA Synthesis Kit (Bio-Rad) with 2 μg RNA. qPCR was performed using SsoAdvanced SYBR Green Supermix (Bio-Rad). mRNA expression of all genes was calculated using the 2-$\Delta\Delta$CT method normalized to β-actin. Primer sequences for PGC-1α, TFAM, NDUFβ8, COX1, and ATPS-β were described previously (Funk and Schnellmann, 2011). Additional primer sequences were as follows: COL1A2: sense 5′-TGTTGGCCCATCTGGTAAAGA-3′, antisense 5′-CAGGGAATCCGATGTTGCC-3′; β-actin: sense 5′- GGGATGTTTGCTCCAACCAA-3′,antisense 5′-GCGCTTTTGACTCAAGGATTTAA-3′.

2.4. Immunoblot analysis

Protein lysates were isolated from renal cortex using RIPA buffer containing mammalian protease inhibitor cocktail, 1 mM sodium orthovanadate, and 10 mM sodium fluoride (Sigma). Proteins were separated on a 4–15% SDS-PAGE gel and transferred to a nitrocellulose membrane before blocking in 2.5% BSA. Membranes were incubated with primary antibody at 4 °C overnight. Primary antibodies used were α-smooth muscle actin (α-SMA) (1:1000, Sigma), transforming growth factor β_1 (TGF-β_1) (1:1000, Abcam), neutrophil gelatinase-associated lipocalin 2 (NGAL) (1:1000, Abcam) and β-actin (1:500, Santa Cruz). Membranes were then incubated with an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h. Bound antibody was imaged following chemiluminescent visualization using a GE ImageQuant LAS4000 standalone imaging system. Densitometry of Western blots was performed using NIH ImageJ software.

2.5. Mitochondrial DNA content

qPCR was used to determine relative quantities of mitochondrial DNA content in renal cortex as previously described (Funk et al., 2010). Briefly, the DNeasy Blood and Tissue kit (QIAGEN, Valencia, CA) was used for genomic DNA extraction and 5 ng of total DNA was used for qPCR. Mitochondrially encoded NADH dehydrogenase 1 (ND1) was used to measure mitochondrial copy number and was normalized to nuclear β-actin. Primer sequences were as follows: ND1: sense 5′-TAGAACGCAAAATCTTAGGG-3′, antisense 5′-TGCTAGTGTGAGTGATAGGG-3′; β-actin: sense 5′- GGGATGTTTGCTCCAACCAA-3′,antisense 5′-GCGCTTTTGACTCAAGGATTTAA-3′.

2.6. Statistical analysis

Data are presented as means ± SEM and were subjected to one-way analysis of variance (ANOVA). Multiple means were compared using Student–Newman–Keuls test with $p < 0.05$ considered to be a statistically significant difference between means.

3. Results and discussion

3.1. FA induces AKI with rapid functional recovery

FA decreased glomerular function as serum creatinine levels increased ~3-fold vs. controls 1–2 d after FA injection and returned to control levels at 4 d (Fig. 1A). BUN concentrations increased ~8-fold vs. controls 1–2 d after FA injection, and did not return to control levels until 10 d (Fig. 1B). Over 90% of mice developed AKI, defined as a doubling of serum creatinine or BUN levels at 2 d. As an indicator of tubular function we measured urinary glucose concentrations, which increased 2-fold over controls 1–2 d after FA injection (Fig. 1C). Urinary glucose concentrations returned to

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