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Research Paper

Meclizine Preconditioning Protects the Kidney Against Ischemia–Reperfusion Injury

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A B S T R A C T

Global or local ischemia contributes to the pathogenesis of acute kidney injury (AKI). Currently there are no specific therapies to prevent AKI. Potentiation of glycolytic metabolism and attenuation of mitochondrial respiration may decrease cell injury and reduce reactive oxygen species generation from the mitochondria. Meclizine, an over-the-counter anti-nausea and -dizziness drug, was identified in a 'nutrient-sensitized' chemical screen. Pretreatment with 100 mg/kg of meclizine, 17 h prior to ischemia protected mice from IRI. Serum creatinine levels at 24 h after IRI were 0.13 ± 0.06 mg/dl (sham, n = 3), 1.59 ± 0.10 mg/dl (vehicle, n = 8) and 0.89 ± 0.11 mg/dl (meclizine, n = 8). Kidney injury was significantly decreased in meclizine treated mice compared with vehicle group (p < 0.001). Protection was also seen when meclizine was administered 24 h prior to ischemia. Meclizine reduced inflammation, mitochondrial oxygen consumption, oxidative stress, mitochondrial fragmentation, and tubular injury. Meclizine preconditioned kidney tubular epithelial cells, exposed to blockade of glycolytic and oxidative metabolism with 2-deoxyglucose and NaCN, had reduced LDH and cytochrome c release. Meclizine upregulated glycolysis in glucose-containing media and reduced cellular ATP levels in galactose-containing media. Meclizine inhibited the Kennedy pathway and caused rapid accumulation of phosphoethanolamine. Phosphoethanolamine recapitulated meclizine-induced protection both in vitro and in vivo.

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1. Introduction

Acute kidney injury (AKI) is a common clinical problem associated with an increasing prevalence, high morbidity, mortality, and prolonged length of hospitalization (Hsu et al., 2007; Lameire et al., 2005; Xue et al., 2006). AKI is also a risk factor for progression to chronic kidney disease (CKD) (Siew and Deger, 2012; Lo et al., 2009; Wald et al., 2009; Ferenbach and Bonventre, 2015; Canaud and Bonventre, 2015). Global or local ischemia contributes to the pathogenesis of AKI which complicates various clinical conditions. Ischemia–reperfusion injury (IRI) is also a risk factor for delayed graft function and chronic allograft nephropathy (Wirthensohn and Guder, 1986; Brezis et al., 1984; Fletcher et al., 2009). As the options to prevent AKI are few and its prognosis is poor, novel interventional strategies are needed. Episodes of nonlethal ischemia can precondition the kidney protecting it against subsequent ischemia (Park et al., 2001; Joo et al., 2006; Ali et al., 2007; Bonventre, 2002). It would be highly desirable to mimic ischemic preconditioning with pharmaceutical intervention. While there are studies reporting that agents, such as high mobility group box 1 (HMGB1) (Wu et al., 2014), isoflurane (Su et al., 2014), inhibitors of hypoxia inducible transcription factor (HIF) or carbon monoxide (Bernhardt et al., 2006) given to animals prior to the ischemic event are protective, in most studies the agents are administered close to the time of IRI. Furthermore the effects are not keep in sustained if given more than a few hours prior to the IRI, nor are the mechanisms understood.

Ischemia plays a central role in the initiation and establishment of AKI because the nephron has a high energy demand and intrarenal oxygen tensions in the outer medulla are low and further reduced by hypoperfusion (Brezis and Rosen, 1995). The kidney proximal tubular epithelial cells are particularly sensitive to IRI because they have minimal glycolytic capacity and rely on mitochondrial metabolism for ATP synthesis (Wirthensohn and Guder, 1986; Klein et al., 1981; Uchida and Endou, 1988). In addition, in the setting of tubular cell injury, mitochondrial respiration can result in the generation of oxidants. Therefore, shifting energy metabolism from mitochondrial respiration to glycolysis could be a viable therapeutic strategy to minimize cell injury.

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2. Material & Methods

2.1. Animal Experiments

All mouse studies followed the fundamental guidelines for Animal Care and Use in Research and Education and were performed in accordance with the animal use protocol approved by the Institutional Animal Care and Use Committee of Harvard Medical School. Experiments were performed in 8–10 wk old male C57BL/6 mice purchased from Charles River Laboratories. Animals were anesthetized with sodium pentobarbital (60 mg/kg body weight intraperitoneally) prior to surgery. Kidneys were exposed through flank incisions and rendered ischemic by clamping the renal pedicles with nontraumatic vascular clamps (Roboz, Rockville, MD). After 27 min at 36.5–37 °C the clamps were removed. Male mice were used because they are more susceptible to ischemia and have a more consistent response to ischemia (Park et al., 2004). Successful renal ischemia and reperfusion was documented by visual inspection of the kidney. Blood pressure was not monitored in this study. Two hours after surgery, 1 ml of NaCl 0.9% was administered intraperitoneally. Some animals were subjected to sham surgery. In the toxin AKI models mice received a one-time intraperitoneal injection of cisplatin (25 mg/kg body weight, Sigma-Aldrich, St. Louis, MO, USA) or aristolochic acid (10 mg/kg body weight, Sigma-Aldrich) in NaCl 0.9%. The control group was administered NaCl 0.9% only. Meclizine or vehicle (10% Kolliphor® EL in PBS) was administered intraperitoneally at different doses and different time-points.

For the dose–response experiment, mice received 10, 30, 60 or 100 mg/kg body weight of meclizine 17 h and 3 h before IRI. For the time-course experiment, one injection of meclizine (100 mg/kg) was given 8, 17 or 24 h before IRI. For preconditioning experiments, 100 mg/kg of meclizine was injected 17 h before IRI. Some mice received two injections of meclizine (100 mg/kg) 0 (after removing clamps) and 8 h after IRI. Other animals were injected with ethanolamine (150 mg/kg body weight of ethanolamine, Sigma-Aldrich), pH 7.4 in phosphate buffered saline (PBS) or vehicle (PBS) administered intraperitoneally 2 h before, immediately after and 24 h after IRI. Mice were sacrificed 24 or 48 h after release of the pedicle clamps, 3 days after cisplatin injection or 5 days after aristolochic acid, and 48 h after ethanolamine injection for tissue analysis. Mice were randomly divided into experimental groups. Serum creatinine was measured in all mice. Before sacrifice, some animals were placed in metabolic cages for 3 h to collect urine for kidney injury molecule-1 (KIM-1) measurement.

2.2. Renal Function

Serum creatinine was measured by the picric-acid method using the Beckman Creatinine Analyzer II (Beckman, Brea, CA). Serum blood urea nitrogen (BUN) was measured using the Infinity Urea Kit (Thermo Scientific, West Sussex, UK).

2.3. KIM-1 Measurement

Urine KIM-1 concentration was measured using the Luminex xMAP technology (Vaidya et al., 2011). Briefly, 30 μl of urine sample was incubated with ~6000 anti-mouse KIM-1-coupled beads/well for 1 h followed by 3 washes with PBS-Tween 20 (PBST). Beads were then incubated with biotinylated KIM-1 detection antibody for 45 min and washed again 3 times with PBST. Quantification was achieved by incubating samples with picorhodin-coupled streptavidin (Innogenit) and exciting at 532 nm. The signal from this fluorochrome was detected using the Bio-Plex 200 system (BioRad) and is directly proportional to the amount of antigen bound to the microbead surface. Data were interpreted using a 13-point standard five parametric logistic regression model. All samples were analyzed in triplicate and the intra-assay variability was less than 5%.

2.4. Histology

Kidneys were fixed in 10% formalin overnight and then placed into 70% ethanol. Paraffin sections of embedded kidneys were stained with hematoxylin and eosin (H&E) and scored in a blinded fashion. The acute tubular necrosis score was determined by quantitating detachment of epithelial cells, loss of brush border, cast formation, inflammatory cell infiltrate and scored from 0 to 4 based on the % of the area that presented these alterations. 0, no lesion; 1, <25% of parenchyma affected by the lesion; 2, 25–50% of parenchyma affected by the lesion; 3, 50–75% of parenchyma affected by the lesion and 4, >75% of parenchyma affected by the lesion.

2.5. Immunofluorescence Staining

Kidneys were fixed in 4% PLP (4% paraformaldehyde, 75 mM l-lysine, 10 mM sodium periodate) for 2 h at 4 °C, and then placed in 30% sucrose overnight. Tissues were snap frozen in optimal cutting temperature compound (OCT, Sakura FineTek, Torrance, CA) and cryosections of 7 μm were mounted on microscope slides. Sections were incubated overnight with primary antibodies as indicated: anti-F4/80 (hybridoma supernatant) and anti-GR1+ (eBioscence), or anti-Kidney-specific cadherin (Ksp-cadherin) (Morizane et al., 2013). Slides were then incubated with Cy3 or FITC labeled secondary antibodies (Jackson ImmunoResearch). Sections were mounted in Vectashield containing DAPI to stain the nuclei (VectorLabs, Burlingame, CA). Neutrophils were expressed as the mean number of GR1+ cells per 400× magnification field and macrophages as the mean per unit area of F4/80+ cells in 400× magnification fields using ImageJ. Ksp-cadherin positive area was also expressed as the mean of KSP stain positive area in 200× magnification fields. Ten randomly selected images per mouse were quantified using ImageJ software (http://rsbweb.nih.gov/ij/) (Schrimpf et al., 2012; Grigc et al., 2012). All images were obtained by standard or confocal microscopy (Eclipse 90i, C1 Eclipse, respectively; both from Nikon).

2.6. Quantitative RT-PCR

Total RNA was isolated from cells or kidneys using the TRIzol reagent (Sigma) according to the standard protocol. First-strand cDNA was synthesized using the MML-V reverse transcriptase (Promega, USA). Real-time PCR was performed using the iQ5 SYBR Green Supermix (BioRad) and the iQ5 Multicolor Real-Time PCR Detection System (BioRad) for

Meclizine was identified by using a small molecule screening strategy to identify clinically useful drugs that are capable of shifting cellular energy metabolism from respiration to glycolysis (Gohil et al., 2010). Meclizine is an “over-the-counter” FDA approved histamine receptor blocker used for the treatment of nausea, vomiting, and dizziness associated with motion sickness and has been used for many decades. Meclizine shifts cellular energy metabolism from mitochondrial respiration to glycolysis (Wirthensohn and Guder, 1986), via direct targeting of cytosolic phosphoethanolamine metabolism (Gohil et al., 2013). We found that meclizine reduced inflammation, mitochondrial oxygen consumption, oxidative stress, mitochondrial fragmentation and tubular injury after kidney IRI. Meclizine pretreatment of HK-2 cells reduced NaCN-induced LDH release, cytochrome c release and mitochondrial-dependent ATP production and increased lactate production. In addition, meclizine inhibited the Kennedy pathway and led to rapid accumulation of phosphoethanolamine. Exogenous addition of ethanolamine in vivo and in vitro was protective, suggesting that meclizine mediated protection occurs via cytosolic phosphoethanolamine metabolism. Thus, our study not only offers a clinically used drug as a potential therapeutic agent but also identifies a previously unidentified pathway that can be targeted for kidney IRI.
mRNA detection. Rn18s and Actb were used as housekeeping genes. The mRNA expression was normalized to housekeeping genes, and relative mRNA levels are expressed as fold change compared with the DMSO treated cells or sham animals. Primers used were as follows: Rn18s forward: ATGGCCGTCTTATGTTG, reverse: GAAGCCCACTTGTCCTCTA; Havcr1 forward: AAAACAGGATACCCACCC, reverse: GTGCTGGGTCTT CCGTCTG; Tnf forward: CCCTGAGGGGCTGACTCA, reverse: ACCT GCGCGAATCCGGCAA; Ilb forward: CTCTCAGATGGAAGCATGA, reverse: AAGCTCACAAACAGCAGGT; Ilf6 forward: TACTTCCCTTCCA AATTTC, reverse: TTGTTCTCTGACACCTCTC; Clcl2 forward: TGCATCGTGTTGTTGG; Nos2 forward: ACCCTCCTCGTTCAGCTCACCTTC, reverse: TCGCTCCAAGATTCCC TGCCCTAAGGTCTTC, reverse: AAGTGCTTGAGGTGGTTGTGG; Slc2a1 forward: ACCCTCCTCGTTCAGCTCACCTTC, reverse: TCGCTCCAAGATTCCC TGCCCTAAGGTCTTC, reverse: AAGTGCTTGAGGTGGTTGTGG; Slc2a1 forward: ACCCTCCTCGTTCAGCTCACCTTC, reverse: TCGCTCCAAGATTCCC TGCCCTAAGGTCTTC, reverse: AAGTGCTTGAGGTGGTTGTGG; Slc2a1 forward: ACCCTCCTCGTTCAGCTCACCTTC, reverse: TCGCTCCAAGATTCCC TGCCCTAAGGTCTTC, reverse: AAGTGCTTGAGGTGGTTGTGG; Slc2a1 forward: ACCCTCCTCGTTCAGCTCACCTTC, reverse: TCGCTCCAAGATTCCC TGCCCTAAGGTCTTC, reverse: AAGTGCTTGAGGTGGTTGTGG.

2.7. Assays of Mitochondrial Physiology

C57BL/6 mice were treated with two intraperitoneal injections of 100 mg/kg meclizine at 17 and 3 h before sacrificing. Mitochondria were isolated from kidneys by differential centrifugation and resuspended in experimental buffer containing glutamate and malate as respiratory substrates (125 mM) to a final concentration of 0.5 mg/ml (Gohil et al., 2010). Coupled and uncoupled respiration was measured following addition of 0.1 mM ADP and 5 μM carbonyl cyanide m-fluorophenyl hydrazone, respectively. O2 consumption was monitored at 25 °C with a Fiber Optic Oxygen Sensor Probe (Ocean Optics).

2.8. Electron Microscopy

Pieces of mouse kidney tissue were fixed in 4% paraformaldehyde, post-fixed in 1% osmium tetroxide, dehydrated in graded alcohols, and embedded in Epon. A tissue block of approximately 1 mm3 was collected from each kidney, including a portion of renal cortex and outer medulla for standard processing. Semithin sections of each block were stained with toluidine blue stain and examined by light microscopy to select for ultrathin sectioning. Ultrathin sections were cut, placed on nickel grids, and examined using a digital electron microscope (JEOL USA JEM-1010). Mitochondrial area was measured by using Imagej software (Birk et al., 2013).

2.9. Cell Culture

The HK-2 (human kidney-2; human proximal epithelial cell) and LLC-PK1 cells were purchased from the American Type Culture Collection. Cells were cultured in DMEM or DMEM/F12 containing 10% fetal bovine serum, in a humidified atmosphere with 5% CO2 at 37 °C.

2.10. Cell Injury Models

Cells were treated with sodium cyanide (NaCN) (1.5 mM) and 2-deoxyglucose (2-DG) (10 mM) to induce cellular stress and injury by chemical anoxia. In this study, cells were seeded in 12-well plates at 0.5 × 105 cells/well or in 6 cm dishes at 2.5 × 105 cells/well, incubated overnight, and pretreated with different concentrations of meclizine (0, 10, 25 μM) or ethanolamine (0, 10, 100 μM) for 17 h. After 17 h preconditioning with meclizine or ethanolamine, the cells were treated with NaCN and 2-DG for 2 h.

2.11. Measurement of Lactate Production

Increased lactate production was used as a marker of upregulation of glycolysis. Briefly, HK-2 cells were subcultured 1:4 from a confluent culture plate into a 10 cm dish. Once confluence was reached, cells were treated with 25 μM of meclizine or vehicle for 17 h. After incubation cells were washed and incubated in 1 ml PBS at 37 °C for 1 h, and then incubated in 1 ml of PBS containing 1 mM glucose at 37 °C for 1 h. Samples were collected and 50 μl of 1.6 M perchloric acid was added to 1 ml of PBS containing 1 mM glucose to stop metabolism. Lactate production was measured at a wavelength of 340 nm after incubation of 100 μl of each sample with a 1 ml reaction buffer (0.1 M Tris, 0.4 M hydrazine, 0.4 mM EDTA, 10 mM MgSO4, 80 mg/ml NAD, LDH 5 mg/ml, pH 8.5) for 1 h at room temperature. Results were normalized to the protein content of the sample.

2.12. LDH Assay

Cell viability after various treatments was evaluated by LDH microplate titer assay as previously described (Chen et al., 1990). At the end of various treatments, 100 μl of culture medium was collected to measure media LDH levels. Then total LDH levels were determined by addition of Triton X-100 (final concentration 0.1%) to the cells at 37 °C for 30 min to release all LDH. The percentage of LDH release was calculated by dividing the media LDH after a treatment by total LDH.

2.13. MTT Assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay was performed as previously described (Alley et al., 1988).

2.14. ATP Measurement

Cells were seeded into a 12 well plate (0.5 × 105 cells/well) and allowed to grow overnight. Growth medium (DMEM or DMEM/F12 containing 10% fetal bovine serum) was then replaced with 25 mM glucose or 10 mM galactose medium and cells cultured for 24 h under 21% or 5% O2 for 24 h. For the meclizine or ethanolamine pre-conditioning experiment, after incubation with 25 μM of meclizine, 10 μM ethanolamine or DMSO for 17 h growth medium was replaced with 10 mM galactose medium and cells were cultured for another 24 h. ATP content was assessed using the ATP Bioluminescent Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) and normalized to total cellular protein.

2.15. Determination of the Intracellular Concentration of Phosphoethanolamine

Intracellular concentration of phosphoethanolamine in meclizine-treated HK-2 cells was determined as follows: HK-2 cells were seeded into a 6 cm plate (0.25 × 105 cells/dish). After 20 h of growth, cells were treated with 25 μM meclizine or DMSO for approximately 17 h. Cells were scraped, collected in methanol extraction solution (80% methanol, 20% H2O), and phosphoethanolamine levels were quantified by liquid chromatography–mass spectrometry (LC–MS) (Gohil et al., 2013).

2.16. Analysis of the Release of cytochrome c

To determine the cytochrome c released from mitochondria during chemical hypoxia with or without meclizine pretreatment, cells were permeabilized with 0.05% (wt/vol) digitonin in an isotonic sucrose buffer for 2–4 min (Brooks et al., 2009). The cytosolic fraction released by digitonin was collected for western blot analysis using specific antibodies to cytochrome c.
2.17. Western Blot Analysis

HIF1α stabilization and the release of cytochrome c were analyzed by western blot analysis. Extracts (20 μg/lane protein) from cells pretreated with either 0.1% DMSO, 25 μM meclizine or 500 μM CoCl2 (Sigma) or digitonin lysate, were separated by SDS-PAGE, transferred onto a PVDF membrane, and subjected to Western blotting using anti-HIF-1α (Novus Biologicals, Littleton, CO), anti-cytochrome c (BD Pharmingen, San Diego, CA), or anti-β-actin (Cell signaling) antibodies. β-actin was used as a loading control. Proteins were visualized using HRP-conjugated secondary antibodies (Dako, Glostrup, Denmark) and ECL detection reagents (GE Healthcare, Milwaukee, WI). The ECL film

Fig. 1. Pretreatment with meclizine protects the kidney against IRI. (A) Scheme illustrating the strategy for the dose–response experiments. (B) Serum creatinine levels at 24 h after IRI, in animals pretreated 17 and 3 h before IRI with vehicle or various doses of meclizine. (C) Scheme illustrating the strategy comparing effectiveness of treatment at various times prior to IRI. (D) Serum creatinine levels at 24 h after IRI, in animals pretreated with a one-time injection of meclizine at different time-points before IRI. (E) BUN levels at 24 h after IRI in mice pretreated 17 h before IRI with a one-time injection of 100 mg/kg of meclizine or vehicle. Sham (n = 3), IRI + Veh (n = 8) and IRI + meclizine (n = 8). (F) Real-time PCR analysis of KIM-1 mRNA (Havcr1) in sham, vehicle and meclizine pretreated mice at 48 h after IRI. Mice were pretreated 17 h before IRI with a one-time injection of 100 mg/kg of meclizine or vehicle. Sham (n = 3), IRI + Veh (n = 4) and IRI + meclizine (n = 4). (G) Representative images after hematoxylin and eosin (H & E) and Periodic acid-Schiff (PAS) staining of tissue taken 48 h after IRI. Original magnification 200 ×, scale bar = 100 μm. (H) Tubular necrosis was semi-quantified by scoring H&E stained slides. Sham (n = 3), IRI + Veh (n = 8) and IRI + meclizine (n = 8). ***p < 0.001; **p < 0.01 and *p < 0.05. Statistical significance was determined using one-way ANOVA followed by Tukey’s post-hoc test. The columns and error bars are the mean ± SEM.
was scanned using a commercial office scanner (Epson Expression 1680 Scanner) and evaluated in ImageJ.

2.18. Statistical Analysis

Statistical analysis was performed using Prism 6.0 (GraphPad Software Inc.). Evaluation of the data was carried out using the unpaired two-tailed t test when two groups were compared or one-way Analysis of Variance (ANOVA) followed by Tukey’s post-test when multiple groups were compared. A p value lower than 0.05 was considered to be significant. Statistical power analyses were performed to evaluate sample numbers necessary for the main group comparisons reflected in Fig. 1B and D. Animal number in each group was chosen to have a statistical power higher than 0.80 (Cohen, 1992; Faul et al., 2007, 2009).

When not specifically stated results are presented as means of at least three independent experiments and error bars indicate ± SEM.

3. Results

3.1. Pretreatment With Meclizine Protects the Kidney Against IRI

To evaluate whether meclizine pretreatment protects the kidney against IRI, we treated mice twice, 17 and 3 h before 27 min of ischemia, with different doses of meclizine and analyzed kidney function 24 h after surgery (Fig. 1A). A flow diagram for the meclizine dose–response experiments is presented in Supplementary Fig. 1. Serum creatinine levels were increased in mice subjected to IRI, reflecting kidney dysfunction (Fig. 1B). There was a dose-dependent protection afforded by meclizine which was statistically significant at the 100 mg/kg dose level. At this dose of meclizine serum creatinine levels were 0.90 ± 0.10 (mean ± SEM) mg/dl in the meclizine-treated group vs 1.40 ± 0.20 mg/dl in the vehicle group (p < 0.01) (Fig. 1B). In a second set of animals meclizine (100 mg/kg) was administered at different time points before IRI (Fig. 1C). A flow diagram for the meclizine time course experiments is presented in Supplementary Fig. 2. Kidney function was analyzed 24 h after surgery. KIM-1 mRNA (Havcr1) levels and histology were evaluated 48 h after surgery. When mice were treated with only one dose of meclizine (100 mg/kg) 17 or 24 h before IRI, there was a significant decrease in creatinine levels 24 h after reperfusion compared to the respective vehicle-treated group (17 h pretreatment: 0.89 ± 0.11 vs 1.59 ± 0.10 mg/dl, p < 0.001; 24 h pretreatment: 0.60 ± 0.17 vs 1.50 ± 0.25 mg/dl, p < 0.05) (Fig. 1D). There was no difference in the amount of body weight loss between the vehicle and meclizine (100 mg/kg 17 h before IRI) treated mice 24 h after IRI (vehicle 24.35 ± 0.4 to 22.45 ± 1.03 g, meclizine: 24.90 ± 0.74 to 22.13 ± 0.69 g) or in weights 48 h after vehicle or meclizine administration at 17 and 3 h prior to IRI (23.60 ± 0.40 g, vehicle; 23.88 ± 0.38 g, meclizine). Pretreatment for 8 h before ischemia resulted in decreased serum creatinine levels 24 h after reperfusion but this difference failed to meet statistical significance when compared to the vehicle-treated group (Fig. 1D). Blood urea nitrogen (BUN) levels were also decreased in the group treated 17 h before injury with 100 mg/kg meclizine in comparison to the vehicle group (90 ± 10 vs 122 ± 7 mg/dl, p < 0.05) (Fig. 1E). In addition to serum creatinine and BUN, there was less upregulation of Havcr1 mRNA in the 48 h post-IRI kidney from mice pretreated 17 h prior to ischemia with meclizine (123 ± 14 fold increase) when compared to vehicle-treated mice (311 ± 22 fold increase; p < 0.001) (Fig. 1F). Pretreatment with meclizine (100 mg/kg) 17 h before IRI reduced tubular necrosis score when compared to the vehicle-treated IRI group (2.5 ± 0.1 vs. 3.8 ± 0.1 p < 0.001) (Fig. 1G, H).

3.2. Inflammation After IRI Is Reduced With Meclizine (100mg/kg) Pretreatment 17 h prior to IRI

Because inflammation contributes to IRI, we analyzed inflammatory cell infiltration and cytokine production 48 h after IRI in animals pretreated with meclizine or vehicle. As expected, there was an increase in GR-1+ neutrophils and F4/80+ macrophage infiltration in the kidney subjected to IRI, as well as an increase in tissue mRNA levels of inflammatory mediators: Il1b, Tnf, Il6 and Ccl2 when compared to vehicle-treated sham non-ischemic animal kidneys (Fig. 2). In meclizine pre-treated animals there were fewer infiltrating neutrophils 48 h after ischemia (1.5 ± 0.1 vs 3.6 ± 0.8 cells per high power field, p < 0.05) (Fig. 2A and B). There was also a trend toward reduced numbers of infiltrating F4/80+ macrophages (Fig. 2C). Meclizine pretreatment resulted in reduced fold-increases in mRNA expression of inflammatory cytokines Il1b (4.38 ± 0.96 vs 7.80 ± 0.83 (vehicle-treated) p < 0.05), Tnf (1.89 ± 0.65 vs 8.47 ± 2.00, p < 0.01), Il6 (40 ± 16 vs 164 ± 39, p < 0.05) and the chemokine Ccl2 (5.6 ± 1.0 vs 12.8 ± 2.2, p < 0.05) (Fig. 2D–G). Thus the protection afforded by pretreatment with meclizine reduced inflammation after IRI.

3.3. Meclizine Inhibits Mitochondrial Respiration and Reduces Kidney Injury After IRI

We tested meclizine as a potential therapeutic agent for kidney IRI based on its previously reported activity as mitochondrial respiration attenuating agent. While earlier work (Gohil et al., 2010, 2013) has clearly shown that meclizine attenuates mitochondrial respiration in an in vitro cell culture system, it is not known whether meclizine would attenuate respiration when administered to a whole organism. Therefore, to test the effect of meclizine on kidney respiration, mitochondria were isolated from the kidneys of mice pretreated with meclizine. Mice were treated with two doses of meclizine (100 mg/kg), 17 h and 3 h before sacrifice. Kidney mitochondria isolated from mice that received meclizine, had decreased O2 consumption after ADP addition and a further decrease after exposure to the uncoupling agent carbonyl cyanide 3-chlorophenylhydrazone (CCCP), when compared with kidney mitochondria isolated from vehicle-treated mice (Fig. 3A).

Heme-oxygenase-1 (Hmox1) and inducible nitric oxide synthase (Nos2) are up-regulated after kidney injury, related to IR-induced oxidative stress (Aragno et al., 2003; Sze et al., 2011). As expected, the kidney mRNA expression levels were up-regulated 24 h after IRI in both meclizine and vehicle-treated animals. Meclizine-pretreated mice subjected to IRI showed lower fold-increases of Hmox1 (7.09 ± 2.01 fold) when compared with the vehicle-treated group (13.3 ± 1.87 fold) (p < 0.05) and lower increases in Nos2 (1.17 ± 0.35, in the meclizine group vs 2.94 ± 0.74 in the vehicle-treated control group, p < 0.05), indicating a reduced oxidative stress (Fig. 3B and C). To check the number of viable tubular cells after IRI, we evaluated Kidney-specific cadherin (Ksp-cadherin) expression (Thomson et al., 1995). When tubular cells are damaged, the number of Ksp-cadherin positive tubular cells is decreased (Morizane et al., 2014). Ischemia led to a decrease in the normalized number of Ksp-cadherin positive tubular cells, but meclizine pretreatment partially mitigated this decreased expression (0.32 ± 0.05 in the vehicle group vs 0.74 ± 0.12 in the meclizine-treated group, when normalized to sham-treated mice, p < 0.05) (Fig. 3D and E). Electron microscopy revealed loss of brush borders of proximal tubule cells with extensive damage to the mitochondria which is reflected by round and fragmented mitochondria after IRI in vehicle-treated mice (Fig. 3F and G) (Brooks et al., 2009). In contrast, the representative images from a meclizine-pretreated kidney (Fig. 3H and I) showed intact brush borders and many elongated mitochondria on the basal side of the tubular cells. Mean mitochondrial area is 2.73 ± 0.43 times greater in the vehicle vs meclizine pretreated kidney (p < 0.01) (Fig. 3J). These data revealed that meclizine reduced tubular damage–induced oxidative stress and inhibited IRI-induced mitochondrial structural changes.
3.4. Meclizine Is Not Protective If Given After IRI or in Two Toxicity Models of AKI

To evaluate whether meclizine would also be protective when given after the injury had been already established, we treated mice with 100 mg/kg of meclizine twice, one injection right after reperfusion and a second injection 8 h after IRI. No significant differences were measured in serum creatinine levels, tubular necrosis score or kidney KIM-1 mRNA (Havcr1) expression between the vehicle and meclizine-treated mice at 48 h after IRI. Sham (n = 3), IRI + Veh and IRI + Meclizine (n = 4 to 6). ***p < 0.001; **p < 0.01 and *p < 0.05. Statistical significance was determined using one-way ANOVA followed by Tukey’s post-hoc test. The columns and error bars are the mean ± SEM.

We also tested whether preconditioning with meclizine was effective in two toxicity models of AKI. Mice were treated with meclizine 17 h or 1 h before the injection of aristolochic acid or cisplatin. Serum creatinine and urinary KIM-1 were measured at the peak of the toxic injury, 5 days after aristolochic acid injection or 3 days after cisplatin injection. Mice injected with aristolochic acid had increases in serum creatinine levels and urinary KIM-1 compared to sham control mice. In contrast to the protection observed with meclizine preconditioning in the IRI model, pretreatment with meclizine either 17 h or 1 h before aristolochic acid injection had no effect on creatinine and urinary KIM-1 levels (Fig. 4F, G).

Fig. 2. Inflammation after IRI is reduced with meclizine (100mg/kg) pretreatment 17 h prior to ischemia. (A) Representative images of GR1+ (neutrophils) and F4/80 (macrophages) stained kidneys from sham, vehicle and meclizine pretreated mice at 48 h after IRI. Original magnification 400 ×, scale bar = 20 μm. (B and C) Quantification of GR1+ positive cells and F4/80 positive area. (D–G) Real-time PCR analysis of Il1b, Ccl2, Tnf, Il6 and Il18 mRNA in sham, vehicle and meclizine pretreated mice at 48 h after IRI. Sham (n = 3), IRI + Veh and IRI + Meclizine (n = 4 to 6). ***p < 0.001; **p < 0.01 and *p < 0.05. Statistical significance was determined using one-way ANOVA followed by Tukey’s post-hoc test. The columns and error bars are the mean ± SEM.
3.5. Meclizine Attenuates LDH and cytochrome c Release During 2-DG and NaCN Treatment of Tubular Epithelial Cells

To evaluate whether meclizine protected kidney tubular epithelial cells in vitro, cells were pretreated with meclizine 17 h prior to chemical anoxia induced by 1.5 mM of NaCN and 10 mM of 2-DG. A significant decrease of % LDH release in LLC-PK1 and HK-2 cells was observed in cells pretreated with 25 μM of meclizine when compared with cells pretreated with DMSO only (LLC-PK1 cells, 11.6 ± 3.2% vs 25.9 ± 1.5, p < 0.01, Fig. 5A; HK-2 cells, 27.0 ± 5.9 vs 47.6 ± 6.3%, p < 0.05, Fig. 5B, respectively). Furthermore meclizine pretreatment blocked the release of injury-associated cytochrome c from HK-2 cells exposed to chemical anoxia (Fig. 5C, D).

3.6. Meclizine Up-regulates Glycolysis in Glucose Containing Media and Reduces Cellular ATP Levels in Galactose Media

Culturing cells in galactose as a sugar source forces mammalian cells to rely on mitochondrial oxidative phosphorylation (OXPHOS) (Aguer et al., 2011; Gohil et al., 2010). LDH release was increased and cell viability was reduced in galactose media when oxygen was decreased from 21 to 5%. A significant increase in % LDH release and decrease in cell viability were observed in cells cultured in 5% O2 with 10 mM galactose when compared with cells cultured in 5% O2 with 25 mM glucose (% LDH release: 33.0 ± 3.89 vs 15.7 ± 0.24%, p < 0.05, Fig. 6A; cell viability: 12.0 ± 4.23 vs 65.1 ± 2.83%, p < 0.01, Fig. 6B). Cellular ATP levels also decreased when cells were cultured in 5% O2 with 10 mM galactose vs...
5% O2 with 25mM glucose (5.58 ± 0.46 vs 9.98 ± 0.31 nmol/mg protein, p < 0.05, Fig. 6C). Thus culturing cells with galactose as an energy source forces kidney tubular epithelial cells to rely on mitochondrial oxidative respiration rather than glycolysis.

To evaluate whether pretreatment with meclizine affected energy metabolism in vitro, lactate production of HK-2 cells was analyzed. Meclizine pretreatment for 17 h in 21% O2 increased lactate production (51.61 ± 8.05 vs 25.87 ± 3.67 nmol/h/mg, p < 0.05) in glucose containing media (Fig. 6D). Meclizine pretreatment increased the mRNA levels of representative enzymes of glycolysis, hexokinase 2 (Hk2), phosphoglycerate kinase 1 (Pgk-1) and lactate dehydrogenase-A (Ldha) (Fig. 6E). Meclizine pretreatment also decreased ATP levels in HK-2 cells grown in galactose containing media for 24 h (2.3 ± 0.12 vs 3.1 ± 0.14 nmol/mg protein, p < 0.05) (Fig. 6F). We did not observe HIF1-α stabilization following meclizine pretreatment. As a positive control, CoCl2 pretreatment induced HIF1-α stabilization (Fig. 6G and H). Taken together, these findings suggest that meclizine is capable of shifting reliance to glycolysis relative to mitochondrial respiration in a HIF1-α independent manner on HK-2 cells. Despite this decrease in cellular ATP levels, the meclizine-pretreated cells were protected against injury (Fig. 5A–D).

3.7. Cellular Phosphoethanolamine Is Increased by Meclizine and Recapitulates Meclizine-induced Protection

It has been shown that meclizine attenuated mitochondrial respiration by targeting cytosolic phosphoethanolamine metabolism (Gohil
Treatment with meclizine significantly decreased ATP levels when cells were cultured in galactose-containing medium (3.0 ± 0.48 vs 5.2 ± 0.54 nmol/mg protein, p < 0.05) and was associated with increased lactate production (37.57 ± 12.71 vs 24.50 ± 3.18 nmol/h/mg, although not statistically significant p = 0.3352) (Fig. 7B, C). Ethanolamine pretreatment also inhibited % LDH release in response to 1.5 mM of NaN₃ and 10 mM of 2-DG-induced chemical hypoxia in HK-2 cells (10 μM: 23.9 ± 4.6 vs 49.0 ± 4.2%, p < 0.01 and 100 μM: 28.9 ± 5.2 vs 49.0 ± 4.2%, p < 0.05) and LLC-PK1 cells (10 μM: 39.1 ± 1.84 vs 65.0 ± 4.39%, p < 0.05) (Fig. 7D, E). When ethanolamine was given 2 h before ischemia, immediately after and 24 h after reperfusion, there was a significant decrease in BUN levels in mice exposed to IR when compared to the vehicle group (24 h: 88.23 ± 12.23 vs 133.6 ± 7.15 mg/dl, p < 0.05; 48 h: 84.13 ± 19.62 vs 150.8 ± 12.26 mg/dl, p < 0.05) (Fig. 7F, G). Compared with vehicle-injected mice, the renal morphology of ethanolamine-injected mice showed less kidney injury (Fig. 7H). These data revealed that phosphoethanolamine recapitulated meclizine-induced protection both in vitro and in vivo.

4. Discussion

Meclizine is an attractive potential therapeutic agent for IRI, since it is well established to be safe in humans and it has an unusual mechanism of protection. Meclizine inhibits mitochondrial respiration. Meclizine decreases post-ischemic serum creatinine levels when given at least 17 h before injury. The fact that prolonged pretreatment is necessary would make this safe drug appropriate for situations where there is a predictable increase in the probability of developing AKI, such as cardiac surgery, ICU stay or perhaps during allograft preservation prior to transplantation.

The shift in cellular energy production from mitochondrial respiration, which consumes oxygen, to anaerobic glycolysis, is a natural adaptation to reduced oxygen availability (Ramirez et al., 2007). Redirecting energy metabolism toward anaerobic glycolysis can reduce ischemia-induced ROS production, oxidative damage and suppress apoptosis (Vaughn and Deshmukh, 2008; Jeong et al., 2004). We have shown that meclizine attenuates mitochondrial respiration likely through increase in cellular phosphoethanolamine and increases mRNA levels of glycolytic enzymes and lactate production. Significantly lowered expression of post-ischemic HO-1 and iNOS in meclizine-treated mice reflect decreased oxidative stress caused by IRI (Birk et al., 2013; Jeong et al., 2004). This protective effect is brought about by a HIF-independent mechanism (Gohil et al., 2010, 2013). Thus FDA-approved meclizine shifts energy metabolism (Gohil et al., 2010) and may be a useful candidate for chemical ischemic preconditioning in kidney. While the doses used here are high, more effective agents may be developed which can be used at lower concentrations with potentially fewer off-target effects.

Reducing inflammation has been shown to be important for a better outcome in IRI-related organ damage (Mauriz et al., 2001; Meng et al., 2001). Meclizine pretreatment of mice attenuates IRI-induced kidney tubular damage and is associated with a reduction of inflammation including a reduction in granulocytes and expression of a number of cytokine genes, all of which are well established to contribute to the post-ischemic inflammatory milieu (Szeto et al., 2011) (Kieler et al., 2005) (Kreisel et al., 2011).

Postischemic structural and functional changes in mitochondria are closely linked (Kasik et al., 2007). IRI in kidney induces fragmentation of mitochondria which leads to sustained energetic deficits, release of cytochrome c, and activation of cell death pathways in proximal tubule epithelial cells (Brooks et al., 2009; Barsoum et al., 2006). We have shown that meclizine attenuates mitochondrial structural changes and release of cytochrome c.
Meclizine does not protect the kidney when administered after the initial injury. Acute exposure to meclizine did not decrease O$_2$ consumption on kidney mitochondria isolated from mice (Gohil et al., 2010). By contrast when mice received meclizine 17 h before IRI there was a decrease in isolated mitochondrial O$_2$ consumption. Thus meclizine must be present prior to an insult to be effective for the reduction of mitochondrial respiration. Meclizine inhibits phosphate cytidylyltransferase 2 (PCYT2) and causes an increase in cytosolic phosphoethanolamine, an ethanolamine derivative that is a central precursor in the biosynthesis of membrane phospholipids (Gohil et al., 2013). High levels of intracellular phosphoethanolamine inhibit mitochondrial respiration (Gohil et al., 2013; Modica-Napolitano and Renshaw, 2004). Ethanolamine, a precursor of phosphoethanolamine, also inhibits mitochondrial respiratory activity (Modica-Napolitano and Renshaw, 2004; Gohil et al., 2013). In this study we show that meclizine pretreatment increases intracellular phosphoethanolamine in HK-2 cells and ethanolamine has protective effects both in vitro in renal epithelial cells and in vivo in the kidney. Thus a renoprotective effect of meclizine may be mediated by accumulation of intracellular phosphoethanolamine (Fig. 7I).

While the dose used in these studies is higher than the dose used clinically in humans there was no meclizine induced weight loss or other evidence for toxicity in mice at these doses (Gohil et al., 2010). At the dose used in this study mice were protected against acetaminophen-induced liver toxicity (Huang et al., 2004). Although meclizine is classified as a histamine (H1) antagonist and a muscarinic acetylcholine receptor antagonist, the other 64 annotated H1 receptor antagonists and 33 annotated muscarinic antagonists in the original chemical library screen had no effect on oxygen consumption (Gohil et al., 2010). It is likely that the renal protective effect is independent of histaminergic or muscarinic signaling or HIF-stabilization. This study justifies the further development of meclizine-like agents that can be selected for their mitochondrial effects and given to humans to affect mitochondrial respiration while minimizing anti-histaminergic and anticholinergic effects and maintaining efficacy in protecting against kidney injury.

We did not measure blood pressure. Although we closely monitored and tightly controlled the body temperature between 36.5 and 37 °C and the technical success of ischemia–reperfusion by checking the kidney color after clamping and after removing the clips (Park et al., 2004; Wei and Dong, 2012), we cannot completely exclude the possibility that altered hemodynamics may contribute to a modification of kidney injury after ischemia-reperfusion.

In conclusion, we have shown that pretreatment with 100 mg/kg of meclizine 17 or 24 h prior to ischemia protected mice from IRI. Meclizine reduced mitochondrial oxygen consumption, and attenuated oxidative stress and mitochondrial fragmentation after IRI. Meclizine induced intracellular phosphoethanolamine accumulation which inhibits mitochondrial respiration. These findings suggest that pretreatment with meclizine, or a derivative, may reduce kidney injury induced by shock, sepsis, cardiovascular surgery and early allograft dysfunction. Further studies of efficacy are required to rigorously determine optimal dosing.
of this widely used drug with an excellent safety profile or a related drug in humans.

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Author Contributions

S.K., V.M.G., V.K.M. and J.V.B. designed the experiments. S.K., G.C., V.M.G. and P.B. performed the experiments with the assistance of C.B., R.M., V.S. and T.I.

S.K., V.M.G., C.B., R.M., V.S., T.I., V.K.M. and J.V.B. analyzed the data. S.K. and J.V.B. wrote the manuscript.

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Conflicts of Interest

J.V.B. and T.I. are co-inventors on KIM-1 patents, which have been assigned to Partners Healthcare and licensed to a number of companies.
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