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Altered Mitochondrial Functioning Induced by Preoperative Fasting May Underlie Protection Against Renal Ischemia/Reperfusion Injury

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ABSTRACT

We reported previously that the robust protection against renal ischemia/reperfusion (I/R) injury in mice by fasting was largely initiated before the induction of renal I/R. In addition, we found that preoperative fasting downregulated the gene expression levels of complexes I, IV, and V of the mitochondrial oxidative phosphorylation (OXPHOS) system, while it did not change those of complexes II and III. Hence, we now investigated the effect of 3 days of fasting on the functioning of renal mitochondria in order to better understand our previous findings. Fasting did not affect mitochondrial density. Surprisingly, fasting significantly *increased* the protein expression of complex II of the mitochondrial OXPHOS system by 19%. Complex II-driven state 3 respiratory activity was significantly reduced by fasting (46%), which could be partially attributed to the significant decrease in the enzyme activity of complex II (16%). Fasting significantly inhibited Ca^{2+}-dependent mitochondrial permeability transition pore opening that is directly linked to protection against renal I/R injury. The inhibition of the mitochondrial permeability transition pore did not involve the expression of the voltage-dependent anion channel by fasting. In conclusion, 3 days of fasting clearly induces the inhibition of complex II-driven mitochondrial respiration state 3 in part by decreasing the amount of functional complex II, and inhibits mitochondrial permeability transition pore opening. This might be a relevant sequence of events that could contribute to the protection of the kidney against I/R injury. J. Cell. Biochem. 114: 230–237, 2013.

KEY WORDS: FASTING; KIDNEY; MICE; MITOCHONDRIAL RESPIRATION; MITOCHONDRIAL PERMEABILITY TRANSITION PORE

Renal ischemia/reperfusion (I/R) injury is a major clinical problem. In the context of renal transplantation, renal I/R injury is an important risk factor for delayed graft function [Sola et al., 2004] and primary graft non-function [Asaka et al., 2008]. Indirectly, it may play a role in acute rejection [Troppmann et al., 1995] and chronic allograft nephropathy [Fletcher et al., 2009] as well. Graft dysfunction following renal transplantation is associated with decreased graft and patient survival [McLaren et al., 1999]. To date, treatment of renal I/R injury is still unsatisfactory and the development of new therapeutic interventions to reduce or prevent renal I/R injury remains warranted.

Renal I/R injury involves a complex cascade of events, including excessive generation of reactive oxygen species during reperfusion to levels above the normal scavenging capacity of the antioxidant defense systems in the kidney [Paller et al., 1984; Plotnikov et al., 2005]. Excessive ROS generation during reperfusion contributes to renal dysfunction by damaging local macromolecules such as lipids, and proteins [Erdogan et al., 2006].

Mitochondria are considered the main intracellular source for ROS generation, especially by complexes I and III of the mitochondrial oxidative phosphorylation (OXPHOS) system [Ide et al., 1999; Guzy et al., 2005]. Prolonged periods of warm ischemia alter the enzymatic activity of the mitochondrial OXPHOS system [Rouslin, 1983; Petrosillo et al., 2003; Paradies et al., 2004], which can result in increased mitochondrial ROS generation upon reperfusion. In addition, excessive mitochondrial ROS generation,

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as well as mitochondrial Ca^{2+} overload, can induce a mitochondrial permeability transition pore (MPTP) in the inner mitochondrial membrane [Kowaltowski et al., 1996; Kim et al., 2006]. Opening of the MPTP results in ATP depletion by mitochondrial uncoupling of the OXPHOS system, and promotes the influx of small molecules with a size smaller than 1,500 Da. The influx of small molecules induces mitochondrial matrix swelling, which can cause rupture of the outer mitochondrial membrane, followed by the release of pro-apoptotic cytokines such as cytochrome *c* and, finally, cell death [Halestrap, 2009]. It has been reported that apoptosis induced by ischemia significantly contributes to renal I/R injury, and that inhibition of the apoptotic process prevents both inflammation and subsequent kidney injury following renal I/R [Daemen et al., 1999].

We have shown previously that preoperative fasting offers robust protection against renal I/R injury in mice, and causes among others the downregulation of a number of genes of the mitochondrial OXPHOS system [Mitchell et al., 2010]. Moreover, we have demonstrated that these changes were largely initiated before the induction of renal I/R, as postoperative fasting did not induce similar protection. In the present study, we set out to investigate whether the protection induced by fasting is associated with functional changes of renal mitochondria as well. Here, we report that 3 days of fasting induces the inhibition of complex II-driven mitochondrial respiration state 3 partly by decreasing the amount of functional complex II, and prevents MPTP opening, which may be relevant to fasting-induced protection against renal I/R injury.

MATERIALS AND METHODS

ANIMALS

Male C57BL/6 mice, weighing approximately 25 grams, were obtained from Harlan (Horst, The Netherlands). Upon arrival, mice were housed at random under standard conditions in individually ventilated cages (n = 4 mice per cage) with free access to tap water (pH 2.4–2.7) and food (Special Diet Services, Witham, United Kingdom). At the start of the experiment, all mice were transferred to clean cages at 5:00 pm. Ad libitum fed mice (n = 12) were allowed free access to food and water for 3 consecutive days. Three-day fasted mice (n = 12) had free access to water, but were withheld from food for 3 days. No deaths occurred during this fasting regimen. Following 3 days of fasting or ad libitum feeding, mice were anesthetized with a 5% isoflurane/O_2/N_2 gas mixture. Immediately after induction of the anesthetic, the kidneys were removed through a midline abdominal incision and processed for analysis. The experimental protocol was approved by the Animal Experiments Committee under the Dutch National Experiments on Animals Act and complied with the 1986 directive 86/609/EC of the Council of Europe.

MITOCHONDRIAL ISOLATION

Mitochondria were isolated from kidneys of ad libitum fed mice (n = 12) and 3-day fasted mice (n = 12) as described [McFalls et al., 2006]. Briefly, the left kidneys were placed in ice cold mitochondrial isolation buffer (MIB), pH 7.15, containing 50 mM sucrose, 200 mM mannitol, 1 mM EGTA, 5 mM KH_2PO_4, 5 mM MOPS, 0.1% fatty acid-free BSA, and protease and phosphatase inhibitors. All chemicals were obtained from Roche Diagnostics Nederland B.V. (Almere, The Netherlands).

After addition of fresh MIB, a 5% kidney homogenate was made in a glass homogenizer with a Teflon pestle. One hundred microliters of the kidney homogenate was snap frozen in liquid nitrogen, and four samples per group were used for Western Blotting, qRT-PCR and to determine OXPHOS and citrate synthase (CS) activity. Homogenates were centrifuged one time at 750*g* for 10 min at 4°C and two times at 8,000*g* for 10 min at 4°C. The mitochondria were collected for mitochondrial respiration analysis (n = 12 per group) and detection of mitochondrial permeability transition pore opening (n = 4 per group). The protein concentration was determined with the Bio-Rad DC protein assay (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands).

OXPHOS AND CITRATE SYNTHASE ACTIVITY

OXPHOS activity was measured at 37°C in kidney homogenates from ad libitum fed mice (n = 4) and 3-day fasted mice (n = 4) by spectrophotometric methods as described before [Trounce et al., 1996; Birch-Machin and Turnbull, 2001] with some essential modifications. The spectrophotometric methods were scaled down by the use of submicrocell quartz cuvettes (Hellma GmbH, Müllheim, Germany) of 100 µl, by which the concentration of the homogenate could be increased without requiring large amounts of kidney tissue. Complex I activity was determined as the rotenone-sensitive oxidation of NADH with ubiquinone$_1$ as electron acceptor (100 µM final concentration) at 340 nm, with 380 nm as the reference wavelength [de Wit and Sluiter, 2009]. To assess complex II activity, the extinction difference between 600 and 520 nm was used to follow the ubiquinone$_2$-coupled TTFA-sensitive reduction of 2,6-dichlorophenolindophenol, with succinate as the substrate. Complex III was measured as the change in extinction difference between 550 and 540 nm for the antimycin-A-sensitive reduction of cytochrome *c* by ubiquinol$_2$. Complex IV was determined by following the oxidation of reduced cytochrome *c* at 550 nm, with 540 nm as the reference wavelength in the presence of *n*-dodecyl-β-D-maltoside. Complex V was measured as oligomycin-sensitive Mg-ATPase activity immediately after brief sonication of the homogenate according to Rustin et al. [1994]. All inhibitors and substrates were obtained from Sigma–Aldrich Chemie B.V. (Zwijndrecht, The Netherlands). The activity of citrate synthase (CS) was measured according to Srere [1969]. All enzyme activity was normalized for the amount of protein in the homogenate (Bio-Rad DC protein assay, Bio-Rad Laboratories B.V.) and expressed as U/g or mU/g protein. Each sample was tested at least in duplicate.

MITOCHONDRIAL RESPIRATION

The respiration rate (expressed in nmol O_2 per minute and per mg mitochondrial protein) of isolated mitochondria (n = 12 per group) resuspended in buffer containing 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, and 1 g/L BSA, pH 7.1 were assessed at 37°C by high-resolution respirometry (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria). Because the anesthetic isoflurane is known to inhibit complex I activity [Hanley et al., 2002], complex I-driven mitochondrial respiration was not determined. For complex

II-driven mitochondrial respiration, 5 μM rotenone was added to completely inhibit complex I-dependent respiration, followed by 10 mM succinate to induce complex II-driven state 2 respiration, and 250 μM ADP to induce complex II-driven state 3 respiration. Then, 2 μg/ml oligomycin was added to achieve the pseudo-resting respiration state (state 4) by inhibition of complex V, and finally 1 μM of the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was added to obtain the maximal respiratory rate. All chemicals, inhibitors and substrates were obtained from Sigma–Aldrich Chemie B.V. The respiratory control ratio (RCR), which reflects the efficiency of coupling of the mitochondrial OXPHOS system, was calculated by division of the ADP-activated flux (state 3) by the flux in the controlled state without phosphorylation of ADP (state 4).

MITOCHONDRIAL PERMEABILITY TRANSITION PORE OPENING

Mitochondrial permeability transition pore opening by Ca^{2+}-induced swelling of isolated kidney mitochondria from ad libitum fed mice (n = 4) and 3-day fasted mice (n = 4) was measured as described [Wang et al., 2005]. Briefly, kidney mitochondria were resuspended in swelling buffer containing 120 mM KCl, 10 mM Tris–HCl (pH 7.4), 20 mM MOPS, and 5 mM KH_2PO_4 to a final protein concentration of 0.25 mg/ml. MPTP opening was induced by 2 μM $CaCl_2$ in the absence and presence of 30 nM of the MPTP inhibitor cyclosporine A (CsA). All chemicals were obtained from Roche Diagnostics Nederland B.V. Opening of the MPTP was measured spectrophotometrically by the reduction rate in absorbance at 520 nm wavelength during 5 min after the addition of calcium. Mitochondrial swelling dependent on MPTP opening was calculated as follows: total swelling in the absence of CsA minus remaining swelling in the presence of CsA, and expressed as the mean rate of the decrease in absorbance at 520 nm wavelength per minute during the first 5 min after the addition of calcium.

WESTERN BLOTTING

The protein expression levels of the mitochondrial complexes I to V, the voltage-dependent anion channel (VDAC) and prohibitin were determined in kidney homogenates from ad libitum fed mice (n = 4) and 3-day fasted mice (n = 4). Twenty micrograms of protein was supplemented with Laemmli buffer [Laemmli, 1970] and 100 mM dithiothreitol (DTT), boiled for 5 min at 95°C, and loaded onto 12% SDS–PAGE with protein size standards (Precision Plus Protein™ Standards, Bio-Rad Laboratories B.V.). After electrophoresis (100 V, ~1.5–2.5 h), the proteins were transferred to Immobilon-FL PVDF membranes (Millipore B.V., Amsterdam, The Netherlands) during 1 h at 250 mA. The membranes were blocked by incubation with 5% non-fat dry milk/Tris buffered saline (TBS) for 1 h, followed by three washes of 5 min each with 1× TBS/Tween 0.1%. Primary antibody incubations against the mitochondrial complexes I–V (MitoProfile® Total OXPHOS Rodent WB Antibody Cocktail, dilution 1:250, MitoSciences Inc., Eugene, OR) and VDAC (dilution 1:1,000, Cell Signaling Technology Inc., Danvers, MA) or prohibitin (dilution 1:1,000, Abcam, Cambridge, United Kingdom) were performed simultaneously overnight at 4°C. After three washes of 5 min each with 1× TBS/Tween 0.1%, the membranes were incubated for 1 h with the IRDye® 800CW conjugated goat anti-mouse IgG (H + L) and the IRDye® 700DX conjugated goat (polyclonal) anti-mouse IgG (H + L) antibodies (dilutions 1:5,000, both from LI-COR Biosciences GmbH, Bad Homburg, Germany). Antibody binding was detected using the LI-COR Odyssey infrared imaging system (LI-COR Biosciences GmbH) and analyzed using the LI-COR Odyssey infrared imaging system application software (v2.1). Each sample was tested at least in duplicate.

QUANTITATIVE RT-PCR

With the Trizol reagent (Invitrogen, Breda, The Netherlands), total RNA was extracted from frozen kidney tissues obtained from ad libitum fed mice (n = 4) and 3-day fasted mice (n = 4). Total RNA was purified by a DNase treatment (RQ1 RNase-free DNase; Promega Benelux B.V., Leiden, The Netherlands), and reverse transcribed to cDNA using random hexamer primers, and Superscript II RT (both from Invitrogen) according to manufactures instructions. Quantitative RT-PCR was performed using a MyiQ Single-color Real-Time PCR Detection System with SYBR Green incorporation (both from Bio-Rad Laboratories B.V.). Primer sequences are available upon request. Relative expression was calculated using the equation $2^{-(\Delta C_t \text{sample} - \Delta C_t \text{control})}$. Each sample was tested at least in triplicate.

STATISTICAL ANALYSIS

Data were expressed as the mean ± SEM. Differences between the groups were compared by Mann–Whitney *U* tests using SPSS (version 15). Differences were considered significant at $P < 0.05$.

RESULTS

THE NUMBER OF MITOCHONDRIA WAS NOT AFFECTED BY 3 DAYS OF FASTING

We showed previously that preoperative fasting offers robust protection against renal I/R injury in mice, and causes among others the preoperative downregulation of genes of the mitochondrial OXPHOS system [Mitchell et al., 2010]. Here, we hypothesized that the decreased expression of these genes by fasting was caused by a reduction in the number of mitochondria, and investigated this in kidney tissue homogenates of ad libitum fed mice and 3-day fasted mice. The quantitative marker citrate synthase (CS), an enzyme of the tricarboxylic acid cycle that resides in the mitochondrial matrix, was used as surrogate measure. First, the enzymatic activity of citrate synthase was determined with an in vitro enzyme assay.

As shown in Figure 1A, there was no difference in the number of mitochondria in kidneys from 3-day fasted mice (0.18 ± 0.01 U CS/g of protein) as compared with ad libitum fed mice (0.22 ± 0.03 U CS/g of protein, P = ns). In accordance, CS mRNA expression levels were not significantly lower in kidneys from 3-day fasted mice (0.93 ± 0.06 vs. 1.21 ± 0.19 in kidneys from ad libitum fed mice; Fig. 1B).

THREE DAYS OF FASTING INCREASED THE PROTEIN EXPRESSION OF MITOCHONDRIAL COMPLEXES II AND IV

Because the downregulation of genes encoding for complexes I, IV, and V of the mitochondrial OXPHOS system [Mitchell et al., 2010] was not reflected by a reduction in mitochondrial numbers, we further investigated these genes at the protein level. In addition, we studied the protein expression of the mitochondrial complexes II

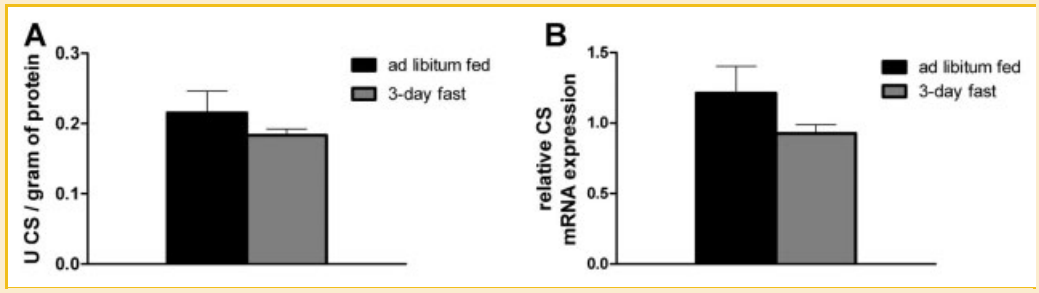

Fig. 1. Mitochondrial numbers. Mitochondrial numbers as reflected by (A) citrate synthase (CS) enzyme activity and (B) CS mRNA expression levels did not differ significantly between the kidneys from 3-day fasted mice and ad libitum fed mice. Data are expressed as the mean ± SEM (n = 4 per group).

and III. Three days of fasting significantly increased the protein expression of complex II (21.6 ± 0.6 vs. 18.2 ± 0.7 arbitrary units [AU] in ad libitum fed mice, $P = 0.021$) by 19% and complex IV (0.5 ± 0.1 vs. 0.2 ± 0.1 AU in ad libitum fed mice, $P = 0.021$) by 150%, while there was no change in the expression of complexes I, III, and V (Fig. 2A,B). Since we were only able to detect the total protein expression levels of complexes I to V with the MitoProfile® Total OXPHOS Rodent WB Antibody Cocktail, thus without assessing protein activity, we performed an additional experiment in which we measured the enzyme activity of these complexes spectrophotometrically. As shown in Figure 2C, 3 days of fasting only significantly reduced the enzyme activity of complex II by 16% (0.27 ± 0.02 vs. 0.32 ± 0.01 in ad libitum fed mice, $P = 0.043$).

FASTING REDUCED COMPLEX II-DRIVEN MITOCHONDRIAL RESPIRATION

Next, we investigated the functional OXPHOS activity by assaying the mitochondrial oxygen consumption rate in isolated kidney mitochondria from 3-day fasted mice and ad libitum fed mice. In the presence of the complex II-linked substrate succinate and ADP, a

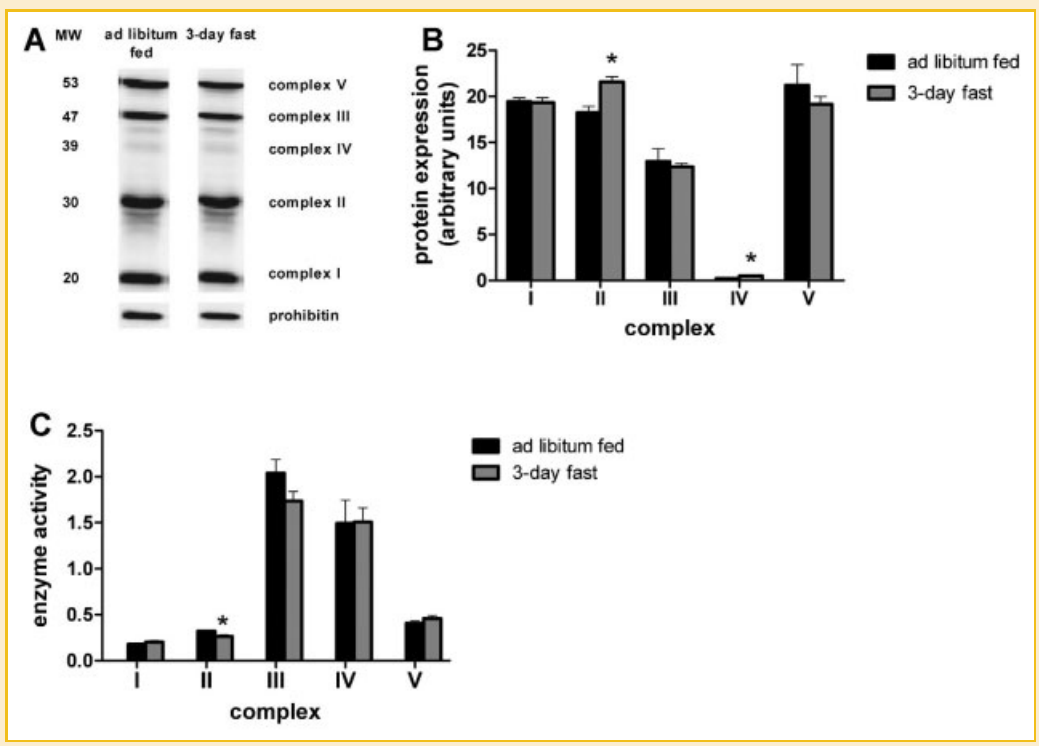

Fig. 2. Mitochondrial oxidative phosphorylation (OXPHOS) system. A: Representative Western blot showing the protein expression levels of the cornerstone subunits of complexes I (20 kDa), II (30 kDa), III (47 kDa), IV (39 kDa), and V (53 kDa), and the mitochondrial marker prohibitin (32 kDa) in kidney homogenates from 3-day fasted mice and ad libitum fed mice. B: Protein expression levels of the mitochondrial complexes I–V. The protein expression levels of complexes II and IV were significantly increased by 3 days of fasting, while the expression of complexes I, III, and V did not change. The protein expression levels of complexes I–V as detected by Western blotting were normalized for the prohibitin expression levels of the same samples. All samples were tested at least in duplicate. Data are expressed as the mean ± SEM (n = 4 per group). $^*P < 0.05$ versus ad libitum fed mice. C: Enzymatic activity of mitochondrial complexes I–V under optimal in vitro conditions. Three days of fasting only reduced the enzymatic activity of complex II significantly. Data are expressed as the mean ± SEM (n = 4 per group). $^*P < 0.05$ versus ad libitum fed mice.

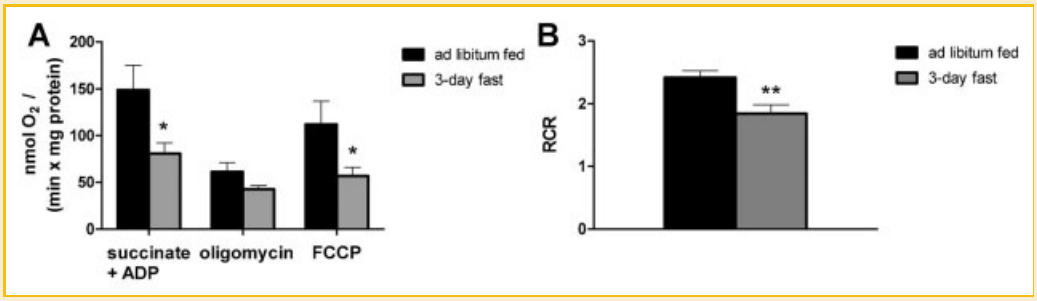

Fig. 3. Mitochondrial respiration. A: Complex II-dependent mitochondrial respiration. State 3 (succinate + ADP) oxygen consumption was significantly lower in isolated kidney mitochondria from 3-day fasted mice versus mitochondria from ad libitum fed mice. When complex V was inhibited by oligomycin, no significant difference was detected in state 4 respiration. The maximal respiration rate (FCCP) in mitochondria from fasted mice was significantly lower than that from ad libitum fed mice. Data are expressed as the mean ± SEM (n = 12 per group). $^{*}P < 0.05$, $^{**}P < 0.01$ versus ad libitum fed mice. B: The respiratory control ratio (RCR). The RCR was significantly reduced in isolated kidney mitochondria from 3-day fasted mice versus mitochondria from ad libitum fed mice. Data are expressed as the mean ± SEM (n = 12 per group). $^{*}P < 0.05$, $^{**}P < 0.01$ versus ad libitum fed mice.

46% reduction in state 3 oxygen consumption was detected in mitochondria from 3-day fasted mice (81 ± 12 vs. 149 ± 26 nmol O_2/min/mg protein in ad libitum fed mice, $P = 0.018$; Fig. 3A). No significant difference in state 4 oxygen consumption was found in the mitochondria from both groups (43 ± 4 vs. 61 ± 10 nmol O_2/min/mg protein in kidney mitochondria from ad libitum fed mice; Fig. 3A). Consequently, there was a significant decrease in the respiratory control ratio (RCR) of mitochondria from 3-day fasted mice (1.8 ± 0.1 vs. 2.4 ± 0.1 in ad libitum fed mice, $P = 0.006$; Fig. 3B). Treatment of the isolated mitochondria with the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) causes collapse of the mitochondrial membrane potential, and results in a maximal increase in mitochondrial oxygen consumption in the absence of ATP synthesis. After addition of FCCP, the mitochondrial maximal respiratory rate was significantly lower in kidney mitochondria from 3-day fasted mice (57 ± 9 vs. 112 ± 25 nmol O_2/min/mg protein in ad libitum fed mice, $P = 0.043$; Fig. 3A).

MITOCHONDRIAL PERMEABILITY TRANSITION PORE OPENING WAS INHIBITED BY FASTING

Necrotic cell death after I/R is the consequence of MPTP opening [Devalaraja-Narashimha et al., 2009]. We have shown previously that, after induction of renal I/R injury, there was less acute tubular necrosis in kidneys from preoperatively 3-day fasted mice as compared with preoperatively ad libitum fed mice [Mitchell et al., 2010], suggesting that fasting before the ischemic insult inhibits MPTP opening. Hence, we investigated the effect of 3 days of fasting on the opening of the MPTP in isolated kidney mitochondria by assessing mitochondrial swelling. In the presence of 2 μM $CaCl_2$, mitochondrial swelling was significantly decreased in mitochondria from 3-day fasted mice as compared with ad libitum fed mice (0.00 ± 0.00 vs. 0.26 ± 0.09 AU in mitochondria from ad libitum fed mice, $P = 0.047$; Fig. 4).

THREE DAYS OF FASTING DID NOT AFFECT VDAC PROTEIN EXPRESSION

VDAC is a voltage-dependent anion channel that is located on the outer mitochondrial membrane and an element of the MPTP [Abou-Sleiman et al., 2006]. We investigated if a reduction in the protein expression of VDAC caused the inhibition of MPTP opening by fasting. To account for any differences in mitochondrial density the protein expression of VDAC was normalized with the protein expression of prohibitin, an evolutionarily conserved protein located in the inner membrane of mitochondria [Merkwirth and Langer, 2009]. As shown in Figure 5, the VDAC protein expression level did not change with fasting (2.1 ± 0.1 vs. 2.6 ± 0.5 AU in kidney homogenates from ad libitum fed mice).

DISCUSSION

We have shown previously that preoperative fasting offers robust protection against renal I/R injury in mice, among others through downregulation of the gene expression levels of complexes I, IV, and V of the mitochondrial oxidative phosphorylation (OXPHOS) system, improved insulin sensitivity, increased mRNA expression levels of antioxidant and cytoprotective genes, and reduced mRNA expression levels of inflammatory genes [Mitchell et al., 2010]. In addition, when the kinetics of fasting was studied, we found that the beneficial effects of fasting were not only largely initiated before the induction of renal I/R, but also persisted for several days beyond

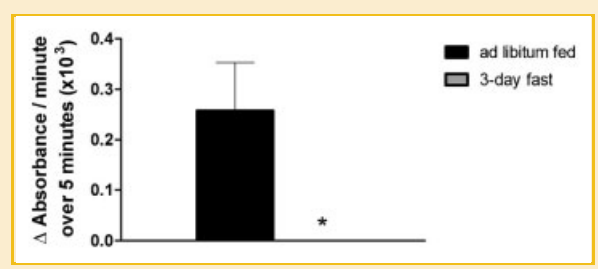

Fig. 4. Mitochondrial swelling. Mitochondrial swelling was assessed by the rate of decrease in absorbance ($\Delta Abs \times 10^3$/min) during 5 min after the addition of 2 μM calcium. The difference in mitochondrial swelling in isolated kidney mitochondria from 3-day fasted and ad libitum fed mice was statistically significant. Data are expressed as the mean ± SEM (n = 4 per group). $^{*}P < 0.05$ versus ad libitum fed mice.

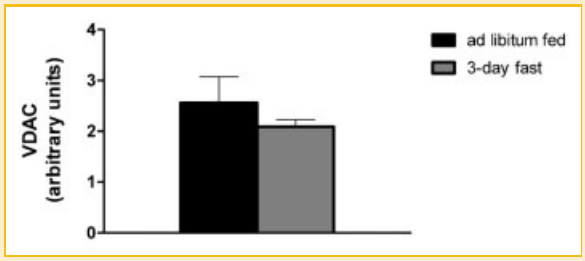

Fig. 5. Voltage-dependent anion channel (VDAC) protein expression. The protein expression of VDAC did not differ significantly between the kidneys from 3-day fasted mice and ad libitum fed mice. VDAC and prohibitin expression were normalized for complex II expression of the same samples, and subsequently VDAC was normalized for prohibitin of the same samples. Data are expressed as the mean ± SEM (n = 4 per group).

the fasting period. Analogous to ischemic preconditioning we have called this preoperative dietary intervention "nutritional preconditioning."

Because the mechanism underlying nutritional preconditioning has not yet been fully clarified, we investigated if 3 days of preoperative fasting was associated with functional changes of kidney mitochondria. Here, we report that fasting did not affect mitochondrial density, significantly *increased* the protein expression levels of complex II of the mitochondrial OXPHOS system by 19%, significantly *decreased* the enzyme activity of complex II by 16%, significantly *decreased* complex II-driven mitochondrial respiration by 46%, and significantly inhibited Ca^{2+}-dependent mitochondrial permeability transition pore (MPTP) opening as compared to mitochondria of ad libitum fed mice.

Mitochondria have multiple functions to preserve cell homeostasis, including ATP production by the OXPHOS system, regulation of calcium homeostasis, and mediation in apoptotic cell death by the release of pro-apoptotic signals such as cytochrome *c*. I/R injury impairs mitochondrial function [Baumann et al., 1989; Petrosillo et al., 2003; Paradies et al., 2004; Brooks et al., 2009]. Moreover, the imbalance between ROS generation and mitochondrial antioxidant enzyme activity during renal I/R injury is considered to be a major contributor to renal dysfunction [Baker et al., 1985; Kim et al., 2009]. Several animal experimental studies have demonstrated that pharmacologically induced increases in mitochondrial antioxidant enzyme activity at baseline [Yin et al., 2001; Rahman et al., 2009], and following renal I/R [Baker et al., 1985; Kim et al., 2009] ameliorate ROS-mediated oxidative renal injury. We have shown previously that 3 days of fasting increased the renal gene expression of the antioxidant glutathione reductase and the stress response gene heme oxygenase-1 before induction of renal I/R injury [Mitchell et al., 2010]. Increasing the expression of the antioxidant defense system and enhancing cellular stress response by preoperative fasting before the ischemic injury may thus promote protection against renal I/R injury.

When the mitochondrial genes encoding for complexes I–V were investigated at the protein level, we found that fasting significantly increased the expression of the mitochondrial complex II by 19% and that of complex IV by 150%, while it did not change the expression of the other three complexes. These findings were contrary to our previous results that demonstrated that fasting reduced the gene expression levels of complexes I, IV, and V, and did not affect those of complexes II and III [Mitchell et al., 2010]. Although the relative change in complex IV protein expression appeared impressive, the actual expression levels were very low and therefore any change should be considered in that perspective.

The picture became even more complex when we determined the *functional* OXPHOS enzyme activities. Fasting induced a 16% *decrease* in the activity of complex II, while the enzyme activity of the other complexes remained unaltered. Further investigation showed the impact of fasting on the *integrated* respiration driven by electron flow from complex II to complex IV (and coupled proton flow to complex V), that is, a decrease by 46% in state 3, no change in state 4, and a decrease by 49% in uncoupled respiration. Clearly, the results of the present study indicate the complexity of the effects of fasting on the electron transport chain. If restricted to complex II, we found discordance with respect to gene expression (unchanged), protein content (increased), enzyme activity (decreased), and complex II-driven respiration (decreased).

Do all these findings reflect the real complex situation evoked by fasting in vivo? Some reserve may be appropriate here. In the first place, although in opposite directions, the effects of fasting on protein content and enzyme activity of complex II were relatively small and its gene expression was even unaltered. Better than our broad approach of using an antibody cocktail for Western blotting, a two-dimensional blue native gel electrophoresis or liquid chromatography–mass spectrometry should be used instead to firmly assess any changes of the OXPHOS complexes at the protein level. Furthermore, the small decrease in complex II activity (as found by our in vitro spectrophotometric analysis reflecting the functional, maximal enzyme activity) may not be the actual enzyme activity in vivo, because enzyme activity can be regulated by substrate availability, stimulators and inhibitors that might be generated during fasting, and possibly by phosphorylation/dephosphorylation. While that latter aspect could be resolved with the Pro-Q Diamond phosphoprotein gel staining [Murray et al., 2004], we determined the *actual* OXPHOS activity by assaying the mitochondrial oxygen consumption rate, and found that fasting significantly decreased the complex II-driven state three respiratory activity by 46%. Since the modest decrease in the enzyme activity of complex II could only partially explain the large reduction in complex II-driven state three respiratory activity, these findings suggest that the occurrence of a complex II inhibitor might cause the extra reduction in respiratory activity.

Ischemic preconditioning (IPC) is an endogenous protective mechanism in which brief non-lethal periods of I/R can protect organs such as the kidneys [Joo et al., 2006] and the heart [Murry et al., 1986] from subsequent prolonged periods of I/R. In the context of cardiac I/R injury, mitochondria are associated with the endogenous cardioprotective mechanism of ischemic preconditioning [Vuorinen et al., 1995; Takeo and Nasa, 1999]. Recently, it has been demonstrated that, under conditions mimicking IPC in rat cardiac mitochondria, malonate, the inhibitor of complex II, is generated [Wojtovich and Brookes, 2008]. Malonate has a similar structure as succinate, the natural substrate of complex II, and functions as a classical competitive inhibitor by increasing the K_M

value of the enzyme complex. Interestingly, malonate not only inhibits complex II activity, but also induces the opening of the mitochondrial ATP-sensitive potassium (mK_{ATP}) channel in the inner mitochondrial membrane [Wojtovich and Brookes, 2008]. Opening of the mK_{ATP} channel by pharmacological mK_{ATP} channel agonists has been shown to confer protection against I/R injury of the brain [Gaspar et al., 2008], heart [Escande and Cavero, 1992] and kidney [Rahgozar et al., 2003], and is associated with MPTP inhibition [Costa et al., 2006]. Unfortunately, we could not detect any malonate in kidney mitochondria from fasted animals by the method described by Fedotcheva et al. [2006] and Wojtovich and Brookes [2008], but unexpectedly neither in those from ad libitum fed controls treated with hydrogen peroxide in vitro (results not shown). Additional studies are required to reveal if malonate is still involved in the inhibition of complex II and indirectly with MPTP inhibition during fasting.

Mitochondria can sequester large amounts of intracellular Ca^{2+} in the mitochondrial matrix through a Ca^{2+} uniporter, a specific Ca^{2+} channel which becomes activated in response to the electrical difference across the mitochondrial membrane [Szabadkai and Duchen, 2008]. Uncontrolled mitochondrial Ca^{2+} uptake, as seen after I/R, results in mitochondrial Ca^{2+} overload, followed by the opening of a MPTP, mitochondrial swelling, and cell death [Kowaltowski et al., 1996; Halestrap, 2009]. We observed that 3 days of fasting inhibited MPTP opening, and demonstrated that this effect was not caused by a reduction in VDAC protein expression. This suggests that the inhibition of the complex II-driven mitochondrial respiration by fasting and the subsequent reduction in mitochondrial membrane potential slows down the Ca^{2+} transport into mitochondrial matrix. This might by itself, or together with malonate for example, prevent MPTP opening.

Recently, we have reported that the protection against renal I/R injury by preoperative fasting is not mediated by an increase in the appetite-stimulating hormone ghrelin [van Ginhoven et al., 2010a] or the stress responding steroid hormone corticosterone [Van Ginhoven et al., 2010b]. Here we show that the protection might, at least in part, be mediated by the reduction in complex II-driven mitochondrial respiration and the inhibition of MPTP opening. However, the contribution of other protective processes, such as starvation-induced autophagy should also be considered.

In conclusion, the results of the present study indicate that at the physiological level 3 days of fasting induces inhibition of complex II-dependent state three respiration and MPTP opening. This might be a relevant sequence of events in the protection of the kidney against I/R and offer new perspectives for intervention.

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