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ORIGINAL ARTICLE

Preoperative fasting induces protection against renal ischemia/reperfusion injury by a corticosterone-independent mechanism

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Summary

Three days of fasting protects mice against lethal renal ischemia–reperfusion (I/R) injury. We hypothesize that the protection imposed by fasting is mediated by increased levels of corticosterone, induced by the stress of food deprivation. C57Bl/6 mice were fasted for 3 days after which serum corticosterone levels were determined. Mice underwent a bilateral adrenalectomy (ADX). Ten days later, they were either fasted or given a corticosterone receptor antagonist while fasting. Bilateral renal I/R injury was induced by clamping the artery and vein of the left and right kidney simultaneously for 37 min. Survival and kidney function were determined. Fasting significantly increased corticosterone levels. Only 8% of the ADX mice which were fasted prior to I/R injury survived, whereas all sham-ADX operated mice survived I/R injury after fasting. After ADX and fasting, 70% of the mice subjected to sham I/R succumbed to the surgical procedure. After fasting with concomitant blockade of the glucocorticoid receptor all animals survived renal I/R. Three days of fasting protects against I/R injury and increases serum corticosterone levels. ADX renders mice incapable of withstanding subsequent abdominal surgery. Glucocorticoid receptor blockade does not interfere with the protective effects of fasting. Thus, the protection against renal I/R injury induced by preoperative fasting is mediated by corticosterone-independent mechanisms.

Introduction

Renal transplantation is considered the treatment of choice for people with end-stage renal disease. One of the factors negatively influencing the outcome after kidney transplantation is ischemia–reperfusion (I/R) injury [1,2]. Delayed graft function is primarily a consequence of I/R injury and contributes to the loss of kidney grafts [3]. We have previously shown that dietary restriction protects against I/R injury [4]. Both 3 days of fasting and 2 weeks of reduced (30%) caloric intake prior to renal I/R resulted in protection against I/R injury in mice. Dietary restriction increased baseline levels of cytoprotective and antioxidant genes and resulted in a more expeditious and pronounced response of these genes to I/R injury [4,5]. The mechanism by which dietary restriction induces this protection remains elusive.

During short-term stress responses, activation of the hypothalamic–pituitary–adrenal axis stimulates the release of glucocorticoids from the adrenal gland. Glucocorticoids are one of the main mediators in these stress response pathways [6] and are essential in limiting and resolving inflammation [7]. I/R injury induces inflammation, which is responsible for its detrimental consequences

[8]. Prolonged fasting acts as an acute stressor and increases levels of corticosterone in rodents [9]. We hypothesized that the protection against I/R injury imposed by fasting is mediated by increased systemic levels of corticosterone. We quantified serum corticosterone levels after 3 days of fasting and subjected mice to a bilateral adrenalectomy (ADX) and treatment with the glucocorticoid receptor antagonist Mifepristone during fasting. The effect of glucocorticoid receptor blockade on the increased expression of cytoprotective and antioxidant genes induced by fasting was determined to investigate the relationship between fasting, corticosterone, and the expression profile of these genes.

Materials and methods

Animals

Male C57Bl/6 mice with an average weight of 25 g were purchased from Harlan (Horst, The Netherlands). All mice were maintained under standard conditions with a 12 h light/dark cycle and were allowed food and water *ad libitum*. 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.

Fasting protocol

Mice in the fed group were allowed unrestricted access to food. Mice in the fasting groups were transferred to a clean cage at 17:00 hours and withheld food for 3 days. All animals were given continuous access to water or 0.9% NaCl (discussed next).

Bilateral adrenalectomy

Mice were anesthetized by isoflurane inhalation (5% isoflurane initially and then 2% with oxygen for maintenance). Body temperature was maintained by placing the animals on heating pads until recovery from anesthesia. A small incision (0.5 cm) was made in the left and right flanks after which the adrenal glands were identified. Diathermy coagulation was performed to remove the adrenal glands from the surrounding tissue. Wounds were closed in two layers using 5/0 Safil sutures (B.Braun Medical B.V., Oss, The Netherlands). Sham animals underwent the same procedure without removal of the adrenal glands. After surgery, 0.5 ml phosphate-buffered saline (PBS) was administered subcutaneously for maintenance of the fluid balance. Postoperatively, all animals were given access to 0.9% NaCl to ensure adequate salt balance. The experiments were resumed following a recovery period of 10 days. Corticosterone levels were determined as described below to confirm complete removal of the glands.

Bilateral renal I/R injury

All surgical procedures were conducted between 9:00 and 12:00 hours. Mice were anesthetized by isoflurane inhalation (5% isoflurane initially and then 2% isoflurane with a 1:1 air:oxygen mixture for maintenance of anesthesia). Body temperature was maintained by placing the animals on heating pads until recovery from anesthesia. Following a midline abdominal incision, the renal artery and vein of both the left and right kidney were occluded simultaneously, by using atraumatic microvascular clamps, for 37 min. In a previous study, we showed that this ischemic time induces a mortality rate of 40% [4]. After macroscopic confirmation of ischemia (purple color), the incision was covered with PBS-soaked gauze and the animal was covered with an aluminum foil blanket to maintain body temperature. Following release of the vascular clamp, restoration of blood-flow was confirmed by the kidney returning to normal color. The abdominal wound was closed in two layers using 5/0 Safil sutures. Directly after closing the abdomen 0.5 ml of PBS at body temperature was injected subcutaneously for maintenance of fluid balance.

Glucocorticoid receptor blockage

Mifepristone is a potent glucocorticoid type II receptor antagonist that also blocks the progesterone receptor, albeit to a much lesser extent. Mifepristone (RU-38486; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) to a final concentration of 500 mg/ml. This stock solution was diluted 850- or 85-fold with PBS before intraperitoneal injection yielding a final DMSO concentration of 0.12% or 1.16%, respectively, to minimize the effect of DMSO on I/R injury [10]. As these treatments differ in final DMSO concentration, we used two vehicle solutions containing either 0.12% or 1.16% DMSO to correct for this difference.

Serum measurements

Blood samples were obtained under anesthesia by retro-orbital venous plexus puncture (during the experiments) or heart puncture (at the end of the experiment). Serum urea levels were determined using a QuantiChrom assay kit, DIUR-500 (Gentaur, Brussels, Belgium). Serum corticosterone was determined using a corticosterone ELISA kit (Sigma Aldrich) according to the manufacturer's

protocol. Corticosterone serum levels were determined from blood samples obtained between 9:00 and 10:00 hours.

Influence of fasting and ADX on corticosterone levels

Animals were fed *ad libitum* or fasted for 1, 2, or 3 days ($n = 6$/group), after which they were scarified by exsanguination under anesthesia. Furthermore, blood samples were obtained from ADX and sham (ADX)-operated mice ($n = 6$/group) after a 3-day fast. Corticosterone levels were determined to confirm complete removal of the adrenal glands (Fig. 1a).

Survival following renal I/R injury after ADX and subsequent fasting

ADX mice ($n = 13$) or sham (ADX)-operated mice ($n = 8$) underwent 3 days of fasting and subsequent renal I/R. Animals were observed twice a day for 1 week to monitor survival. In addition, survival was assessed in fasted ADX mice that had been subjected to a sham I/R procedure ($n = 8$) (Fig. 1b).

Survival following renal I/R injury after mifepristone treatment

To asses the effect of glucocorticoid receptor blockade on renal I/R injury after a 3-day fast several experiments were performed. First, either the vehicle (PBS containing 0.12% DMSO, $n = 6$) or mifepristone (10 mg/kg, $n = 6$) was injected intraperitoneally (i.p.) 30 min prior to I/R after 3 days of fasting. Next, vehicle ($n = 6$) or mifepristone ($n = 6$) were administered once daily at 17:00 hours during the 3-day fast before renal I/R was applied. Finally, either vehicle (PBS containing 1.16% DMSO) ($n = 8$) or mifepristone in a ten times higher dose (100 mg/kg, $n = 8$) was injected daily i.p. during the 3-day fast before renal I/R was applied. To investigate the effects of mifepristone on renal I/R without preoperative fasting, vehicle ($n = 6$) or mifepristone (100 mg/kg, $n = 6$) was administered daily i.p. to *ad libitum* fed control mice (no ADX) during 3 days preceding I/R injury (Fig. 1c).

Mifepristone was administered in dosages that have been reported to effectively block all glucocorticoid receptors [11,12]. Serum corticosterone levels increase after

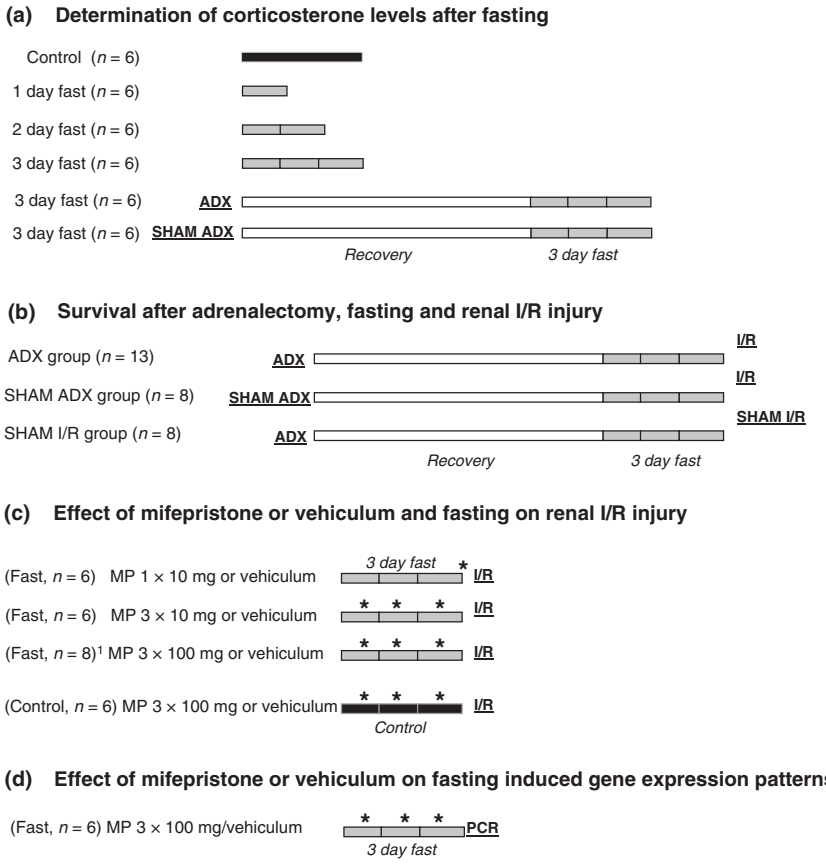

Figure 1 Overview of experimental design. (a) Corticosterone levels were determined in control animals and in animals after 1, 2, or 3 days of fasting. (b) Animals were subjected to either a bilateral adrenalectomy or a sham procedure. After a recovery period of 10 days, animals in all groups were fasted for 3 days followed by either bilateral renal ischemia and reperfusion injury or a sham procedure. Survival was monitored following this second operation. (c) All animals were subjected to 3 days of fasting while mifepristone or the vehiculum was administered, except for the last group which was fed *ad libitum*. *Administration of mifepristone or the vehiculum. Next, all groups were subjected to renal I/R injury and survival was monitored. [1]Renal function was measured in this group. MP, Mifepristone.

administration of mifepristone [11] as a result of feedback inhibition of the pituitary gland. Therefore, increased corticosterone levels were used to indirectly assess blockade of the glucocorticoid receptors by mifepristone.

Quantitative real-time PCR

During the 3-day fast, either vehicle (PBS containing 1.16% DMSO, $n = 6$) or mifepristone 100 mg/kg i.p. ($n = 6$) was injected daily i.p. (Fig. 1d). As the most robust upregulation of cytoprotective and antioxidant genes upon fasting was observed in the liver; we investigated the effect of mifepristone treatment on the expression of these genes in the liver. Livers were harvested and snap frozen in liquid nitrogen after the 3-day fast. For gene expression analysis, total RNA was extracted from frozen liver tissue using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. To prevent contamination by genomic DNA, the isolated RNA was purified by a DNase treatment (RQ1 Rnase-Free Dnase; Promega, Madison, WI, USA). Two μg of total RNA was reverse transcribed to cDNA using random hexamer primers (Invitrogen), and Superscript II RT (Invitrogen) according to the manufacturer's instructions. Quantitative real-time PCR was performed using a MyiQ Single-color Real-Time PCR Detection System (Bio-Rad Laboratories, Herculus, CA, USA) in combination with SYBR Green as DNA probe (Bio-Rad Laboratories). The following primers were used: B2m, forward 5′-TCACTGACCGGCCTGTATGC-3′, reverse 5′-GAGGCGGGTGGAACTGTGTT-3′, Hsp32/HO-1, forward 5′-GAAGGCTTTAAGCTGGTGATGG-3′, reverse 5′-CTTCGGTGCAGCTCCTCAGG-3′, SOD2, forward 5′-TCTGGCGGGAGATGTACAA-3′, reverse 5′-GGGCTCAGGTTTGTCCAGAAAAT-3′, GSR, forward 5′-CCGCCTGAACACCATCTAT-3′, reverse 5′-TTCCCATTGACTTCCACCG-3′. Relative mRNA expressions were calculated using the equation $2\text{^}-(\Delta C_t \text{ sample} - \Delta C_t \text{ control})$. Each sample was assayed in duplicate.

Statistical analysis

Categorical data are presented as number (percentage) and continuous variables as mean ± SEM (normal distribution, assessed visually and by means of Shapiro–Wilks test) or median ± interquartile distance (no normal distribution). Means between two groups were compared using either the nonparametric Mann–Whitney *U*-test or the *t*-test for parametric data. Survival curves were compared using a log-rank (Mantel–Cox) test. A value of $P < 0.05$ was considered significant. All analyses were performed using Statistical Package for the Social Sciences 15.0 (SPSS, Chicago, IL, USA).

Results

Fasting induces increased levels of corticosterone

Baseline corticosterone levels were 298 ± 40 nmol/l. One, 2, and 3 days of fasting significantly increased corticosterone levels compared with baseline to 1135 ± 163 nmol/l ($P = 0.0022$), 1253 ± 234 nmol/l ($P = 0.0022$), and 1287 ± 167 nmol/l ($P = 0.0022$), respectively (Fig. 2a). ADX in combination with 3 days of fasting led to significantly reduced corticosterone values of 2.6 ± 0.3 nmol/l, when compared with the sham (ADX)-operated group, who had corticosterone levels of 1186 ± 150 nmol/l ($P = 0.0022$) after a 3-day fasting period (Fig. 2b).

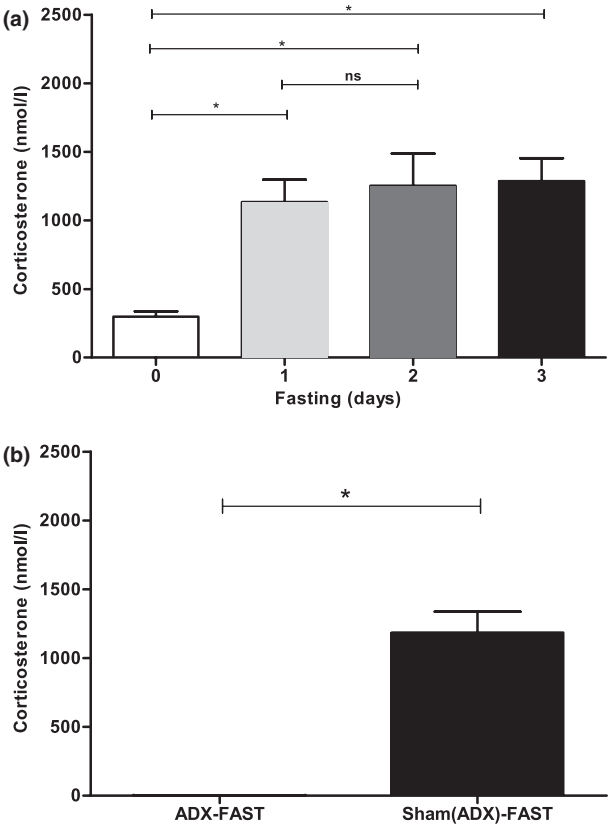

Figure 2 (a) Corticosterone levels after fasting. Animals were fasted for 0, 1, 2, or 3 days after which the serum corticosterone levels were determined. Data are presented as mean ± SEM. An asterisk (*) designates a statistically significant difference between the indicated groups ($P = 0.0022$ for all comparisons). NS, not statistically different. (b) Corticosterone levels after adrenalectomy. ADX-FAST animals underwent an adrenalectomy 10 days prior to fasting and subsequent I/R. Sham (ADX)-FAST animals served as a control group. Animals in this group underwent a sham adrenalectomy 10 days prior to fasting. Data are presented as mean ± SEM. An asterisk (*) designates a statistically significant difference between the indicated groups ($P = 0.0022$).

ADX abolishes the protective effect of fasting on renal I/R injury

Mice recovered rapidly from the ADX as reflected by their return to preoperative weight on postoperative day 2. When ADX mice were subjected to a 3-day fast followed by renal I/R injury only 8% of the animals survived (Fig. 3a). By contrast, survival of sham (ADX)-operated mice after fasting and subsequent I/R was 100% ($P < 0.0001$). To determine whether the high mortality rate was resulting from I/R injury or the absence of adrenal glands, the survival of ADX mice subjected to a sham I/R procedure after 3 days of fasting was assessed (Fig. 3b). The 7-day survival in this group was 30%, similar to the ADX mice that had undergone renal I/R ($P = 0.501$), indicating that mice are not able to withstand abdominal surgery after bilateral adrenalectomy.

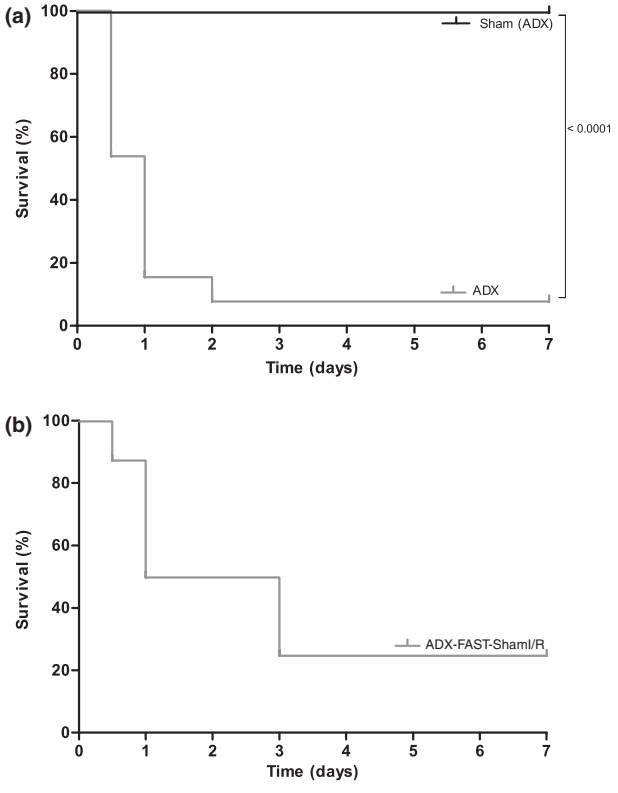

Figure 3 (a) Survival of adrenalectomized (ADX) mice versus sham (ADX) mice after a 3-day fast and subsequent renal I/R injury. Survival in the sham-operated group is 100% vs. 8% in the adrenalectomized group ($P < 0.0001$). (b) Survival of adrenalectomized mice after a 3-day fast and subsequent sham I/R injury. Survival in the sham-operated group is 30%. This is not statistically different from the survival of the ADX group in (Fig. 2a) ($P = 0.5010$).

Glucocorticoid receptor blockade does not affect the benefits of fasting on renal I/R injury

To asses the effect of glucocorticoid receptor blockade on renal I/R injury after and during a 3-day fast several experiments were performed. In the first experiment, mice received either 10 mg/kg mifepristone or vehicle after 3 days of fasting and 30 min prior to renal I/R injury. In both groups survival was 100%. Subsequently, we increased the frequency of mifepristone administration to once daily during the 3-day fast preceding I/R injury. All animals survived the experiment. When a tenfold higher mifepristone dosage (100 mg/kg) was given, again all animals in the control and mifepristone groups survived I/R after the 3-day fast. Following the high dose of mifepristone, kidney function assessed by serum urea concentrations before and 24 and 48 h after I/R showed no differences between the two groups (Fig. 4). To rule out that mifepristone or the vehicle interfered with the renal I/R injury model, the 3-day treatment as described above was applied to *ad libitum* fed control mice (no ADX, only I/R injury). The survival of mifepristone and vehicle-treated mice (Fig. 5a) was similar to the survival of untreated (no mifepristone or vehicle), *ad libitum* fed mice [4]. To confirm effective glucocorticoid receptor blockade by mifepristone serum levels of corticosterone were measured. Corticosterone levels were significantly increased ($P < 0.05$) in mifepristone-treated animals, confirming adequate blockade of the glucocorticoid receptors during fasting and subsequent I/R (Fig. 5b).

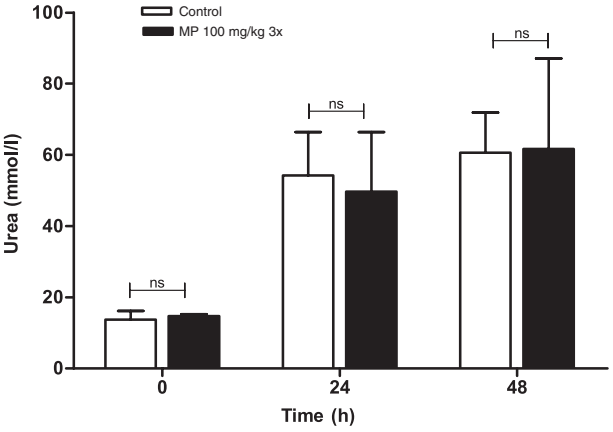

Figure 4 Kidney function after renal I/R injury as indicated by serum urea values. Mifepristone treatment was given once daily (100 mg/kg) during the 3-day fast preceding I/R. The control group received a vehicle. There were no statistically significant differences between both groups.

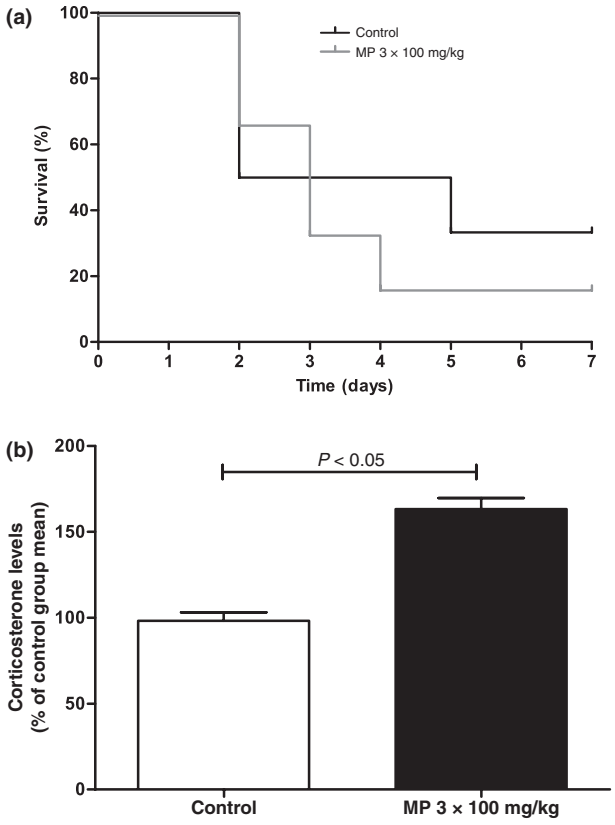

Figure 5 (a) Survival of mifepristone-treated and vehicle-treated control animals after renal I/R injury. Mifepristone (100 mg/kg) was administered once daily, starting 3 days prior to I/R. Vehicle (PBS containing 1.16% DMSO) was administered to the control group. There was no significant difference in survival. Survival was similar to that observed in control mice without treatment [4] (data not shown). (b) Corticosterone levels of mifepristone-treated and control animals after a 3-day fast. Mifepristone (MP) (100 mg/kg) was administered once daily during the 3-day fast ($n = 4$). Vehicle (PBS containing 1.16% DMSO) was administered to the control group, during the 3-day fast ($n = 4$). After the 3-day fast, corticosterone levels were measured and expressed as a percentage of the control group. Corticosterone levels were significantly ($P = 0.0268$) higher in the mifepristone-treated group, when compared with the control group. This indicates total blockade of the glucocorticoid receptor.

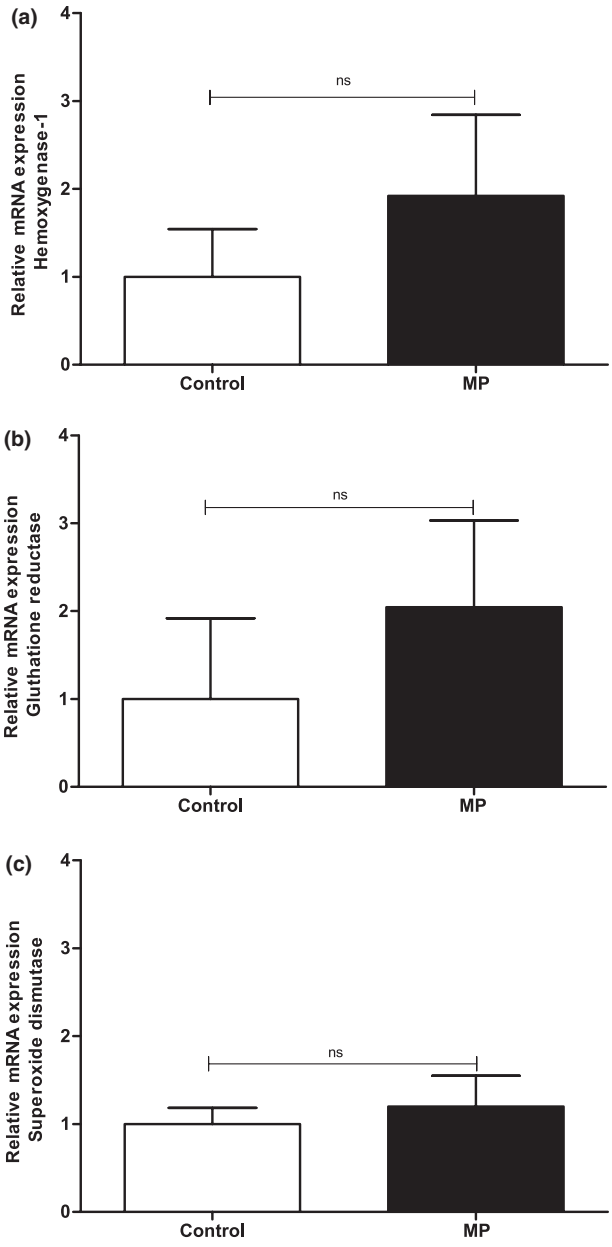

Figure 6 Hepatic mRNA expression levels of hemoxygenase-1 (a), glutathione reductase (b), and superoxide dismutase-2 (c). Mifepristone treatment was given once daily (100 mg/kg) during a 3-day fast after which the livers were harvested ($n = 6$). The control group ($n = 6$) received a vehicle. There were no statistically significant differences in mRNA expression between both groups.

The effect of mifepristone on fasting-induced upregulation of cytoprotective genes

We have previously shown that 3 days of fasting led to significantly higher baseline expression levels of antioxidant defense genes in the liver [4]. Here, we determined mRNA expression levels of hepatic tissue after 3 days of fasting with or without mifepristone treatment (3 days, 100 mg/kg/day). No significant differences were observed in mRNA expression levels of hemoxygenase-1, glutathione reductase, and superoxide dismutase, suggesting that corticosterone receptor inhibitor treatment did not interfere with the induction of cytoprotective and antioxidant genes by fasting (Fig. 6).

Discussion

Renal ischemia and reperfusion injury negatively influences the outcome of kidney transplantation. Strategies to

reduce I/R injury are important to improve patient survival as well as graft function and survival, as I/R is one of the main factors contributing to graft loss [3]. We have recently reported that fasting is able to protect both kidney and liver against I/R injury [4]. Current experiments were designed to investigate whether the protection afforded by fasting against I/R injury is mediated by increased levels of corticosterone. Fasting led to significantly higher levels of corticosterone when compared with *ad libitum* feeding [9]. Bilateral ADX was performed to investigate the effect of corticosterone on renal I/R injury. After ADX, mice exhibited higher mortality rates after I/R compared with control mice. However, survival after laparotomy in ADX mice without I/R injury resulted in similar mortality rates. These experiments did not address our hypothesis that the protection afforded by fasting is attributed to increased corticosterone levels.

Mifepristone, a glucocorticoid receptor antagonist, blocks the downstream signaling of the glucocorticoid receptor. The use of mifepristone therefore enables controlled studies on the effects of corticosterone on renal I/R injury without bilateral ADX. Glucocorticoid receptor blockade 30 min prior to I/R injury did not abolish the protective effects of fasting on renal I/R injury. This suggests that either glucocorticoid receptor blockade does not interfere with the protective effects of fasting or that fasting induces its protection during the 3-day fast. The latter is supported by elevated levels of corticosterone already after 1 day of fasting. Therefore, mifepristone was administered daily during the 3-day fast. However, this regime did not affect the protective effect of fasting on renal I/R injury. Finally, a higher dosage of mifepristone was used based on the previous studies [13]. Moreover, this regime did not abolish the protection afforded by fasting on renal I/R injury. Survival rates and kidney function were similar in both the treatment and the control group. We therefore conclude that fasting-induced protection against renal I/R injury is mediated by corticosterone/glucocorticoid receptor-independent pathways. This is partially in line with earlier reports indicating that mice subjected to social stress [13] or high physiological titers of endogenous glucocorticoids [14] exhibited exacerbated ischemic injury. By contrast, a study in rats concluded that bilateral ADX prevents renal I/R injury [15]. However, this protection is probably induced by the depletion of mineralocorticoid hormones only, as these rats were supplemented with dexamethason, a potent exogenous glucocorticoid agonist. Administration of exogenous glucocorticoids is known to protect against cerebral [16], cardiac [17,18], and renal I/R injury [19]. In addition, clinical studies have shown that donor pretreatment with steroids significantly decreased tissue (liver) and serum expression of proinflammatory cytokines [20] after I/R injury. A recent prospective randomized study investigated the effects of donor pretreatment with methylprednisolone on organ function and outcome after liver transplantation. The use of steroids significantly reduced I/R injury and inflammation and improved graft function [21]. We did not administer exogenous glucocorticoids in our model because they are already known to improve I/R injury and because our hypothesis predicted the involvement of endogenous steroids.

If increased levels of endogenous corticosteroids do not mediate the protective effects of fasting, the question remains which mechanisms do contribute to the induced protection. In the previous experiments, we have shown that 3 days of fasting lead to significantly higher expression levels of cytoprotective and antioxidant defense genes in the kidney and liver [4,5]. The strongest response to fasting was observed in the liver; therefore we investigated the effect of mifepristone treatment on the expression of these cytoprotective and anti-oxidant genes in the liver. This study demonstrated that mifepristone treatment did not interfere with the upregulation of antioxidant defense systems. It would be interesting to investigate whether exact mimicking of corticosterone induction by fasting, by corticosteroid administration, would be able to increase the expression of cytoprotective genes as well. However, as it is difficult, if not impossible, to duplicate the physiological response to fasting, we have not performed these additional experiments. Together, these data support a hypothesis that the up-regulated expression of these genes was instrumental in the protection afforded by fasting against I/R injury, but that these changes are independent of corticosterone. Future experiments are warranted to investigate the relation between these fasting-induced changes in gene expression patterns and I/R injury.

In conclusion, our data demonstrate that fasting increases serum corticosterone levels. However, the protective effect of fasting on I/R injury is induced independently of corticosterone levels and glucocorticoid receptor availability. The upregulation of antioxidant genes is independent of the availability of the glucocorticoid receptor. The latter may represent an important clue to elucidate the mechanisms by which fasting affords protection against I/R injury.

Authorship

TMG: designed the research, performed most of the experiments, conducted the data analysis and wrote the first drafts of the manuscript. JWB: participated in performance of the experiments and writing of the manuscript. WAD: participated in the research design and writing of the manuscript. JNMI: contributed to the interpretation

of the data, and involved in revising and improving the manuscript. RWFB: contributed to conception and design and interpretation of data, and involved in revising and improving the manuscript.

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