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# Renal ischemia and reperfusion activates the eIF2 alpha kinase PERK

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**Abstract**

Inhibition of protein synthesis occurs in the post-ischemic reperfused kidney but the molecular mechanism of renal translation arrest is unknown. Several pathways have been identified whereby cell stress inhibits translation initiation via phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF2α, phospho-form eIF2α(P)]. Here, we report a 20-fold increase in eIF2α(P) in kidney homogenates following 10 min of cardiac arrest-induced ischemia and 10 min reperfusion. Using immunohistochemistry, we observed eIF2α(P) in tubular epithelial cells in both cortex and medulla, where the greatest eIF2α(P) staining was found in epithelial cells of the so-called watershed area at the corticomedullary junction. We further show that increased eIF2α(P) is accompanied by activation of the PKR-like endoplasmic reticulum eIF2α kinase (PERK). These observations indicate that renal ischemia and reperfusion induce stress to the endoplasmic reticulum and activate the unfolded protein response in renal epithelial cells. As the unfolded protein response can result alternatively in a pro-survival or pro-apoptotic outcome, the present study demonstrates an new additional mechanism involved in cell damage and/or repair in ischemic and reperfused kidney.


*Keywords:* Renal ischemia and reperfusion; eIF2α(P); Endoplasmic reticulum stress; PERK; Protein synthesis inhibition; Unfolded protein response

## 1. Introduction

Renal ischemia and reperfusion (I/R) result in significant mortality and morbidity in a variety of clinical contexts [1,2]. However, in spite of a number of identified damage mechanisms, including deleterious inflammatory responses [3,4], endothelial dysfunction [5], nitric oxide dysregulation and oxidative stress [6–8], and caspase activation [9], the understanding of renal injury following I/R is still incomplete.

Depression of protein synthesis is characteristic of reperfused kidney [10]. Plestina and Gamulin [11] showed that renal I/R results in polysome disaggregation, which is indicative of inhibited translation initiation [12]. However, the mechanism of translation initiation inhibition in post-ischemic kidney is unknown. A variety of stresses cause translation initiation inhibition, including heme depletion [13], viral infection [14], stress to the endoplasmic reticulum [15], and amino acid depletion [16]. Each of these stresses activates specific regulatory pathways leading to the phosphorylation of the alpha subunit of eukaryotic initiation factor 2 [eIF2; alpha subunit, eIF2α; alpha subunit phospho-form, eIF2α(P)].

Under conditions of active protein synthesis, the function of eIF2 is to deliver the first amino acid, which is always a methionine in eukaryotes, to the 40S ribosomal subunit during the process of translation initiation [12]. To do so, eIF2 requires binding of GTP. At the end of translation

*Abbreviations:* ER, endoplasmic reticulum; eIF2α, alpha subunit of eukaryotic initiation factor-2; ischemia and reperfusion, I/R; PERK, PKR-like ER eIF2α kinase; tg, thapsigargin; UPR, unfolded protein response

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initiation, the GTP bound to eIF2 is hydrolyzed to GDP and the eIF2–GDP complex is functionally inactive. Therefore, a second initiation factor, eIF2B, exchanges the GDP bound to eIF2 with GTP, thereby recycling eIF2 for another round of translation initiation. When the alpha subunit of eIF2 is phosphorylated, this increases the binding affinity between eIF2B and eIF2(αP) by 150-fold [17]. Thus, eIF2(αP) effectively removes eIF2B from the catalytic cycle, and allows the accumulation of inactive eIF2–GDP, which halts translation initiation and leads to polysome disaggregation. Further, the typical stoichiometry of eIF2 to eIF2B is 5:1 [18], so that phosphorylation of only 20% of total eIF2 can sequester essentially all eIF2B, leading to complete translation arrest.

A well-studied example of clinically relevant protein synthesis inhibition is that which occurs in the brain following I/R. Here, it has been shown that protein synthesis inhibition is also due to inhibited translation initiation which is caused by a massive and rapid increase in phosphorylation of eIF2α at the onset of reperfusion ([19], reviewed in [20]). Further, eIF2α phosphorylation in reperfused brain is due to activation of the endoplasmic reticulum (ER) eIF2α kinase PERK [25]. PERK is activated as part of the unfolded protein response (UPR), an endoplasmic reticulum (ER) stress system (reviewed in [21]).

We reasoned that the cellular stress response would be conserved between brain and kidney following I/R and so we undertook studies of eIF2α phosphorylation and PERK activation in ischemic and reperfused kidney. We show here that this response is indeed conserved and report an approximately 20-fold increase in eIF2α(P) in reperfused kidney, concurrent with PERK activation. Further, immunohistochemical mapping localized eIF2α(P) to tubular epithelial cells, indicating that these cells experience ER stress during renal I/R. Our observations provide further insight into the cellular response of renal tubular epithelium to ischemic injury.

## 2. Materials and methods

Polyclonal anti-PERK antiserum was a gift from David Ron (New York University, NY) and has been previously described [22]. Phospho-specific PERK antisera [PERK(P)] was purchased from Cell Signaling Technology, Inc. (Beverly, MA, U.S.A.) and detects PERK phosphorylation at Thr980. Antibodies specific for total eIF2α or specific for the serine-51 phosphorylated form of eIF2α [eIF2α(P)] were purchased from Biosource International (Cammarillo, CA, U.S.A.). All other chemicals were reagent grade.

### 2.1. Ischemia protocol

All animal experiments were approved by the Wayne State University Animal Investigation Committee and were conducted following the *Guide for the Care and Use of Laboratory Animals* (National Research Council, revised 1996).

The thoracic compression method of cardiac arrest was used to induce whole-body ischemia in male Long Evans rats (250–300 g) (Harlan, Indianapolis, IN) by methods we have previously described [23,24]. Briefly, male Long Evans rats weighing 250–300 g were anesthetized with intraperitoneal ketamine (45 mg/kg) and xylazine (5 mg/kg). Femoral vascular access was established for arterial pressure monitoring and intravenous fluid and drug administration. All animals were maintained normothermic (37±0.5 °C) during both the ischemic and reperfusion periods by means of a homeostatic blanket control unit (Harvard Apparatus, Holliston, MA). The electrocardiogram was monitored by limb leads. A tracheostomy was performed on the rats with a 14-gauge angiocath for positive pressure mechanical ventilation. Immediately after instrumentation, cardiac arrest was initiated by thoracic compression, causing circulatory arrest within 5 s. Following a 10-min duration of cardiac arrest, mechanical ventilation began, and manual chest compressions was delivered at a rate of 200–300 per minute, until establishment of return of spontaneous circulation (average time 94±21 s). Reperfusion was defined as a sustained mean arterial pressure>60 mm Hg. Mechanical ventilation was with 100% $O_2$ at 70 breaths per minute, a tidal volume of 1.0 ml/100 g body weight and positive end expiratory pressure of 3 cm $H_2O$. Mechanical ventilation was provided for the entire reperfusion duration. Experimental groups ($n$=3 per group) were: nonischemic controls (NIC), 5 min ischemia only (5I), 10 min ischemia only (10I), 5 min ischemia and 10 min reperfusion (5I/10R), 10 min ischemia and 10 min reperfusion (10I/10R), and 10 min ischemia and 90 min reperfusion (10I/90R).

### 2.2. Renal tissue preparation and immunoprecipitation

At the indicated ischemia or reperfusion times, animals were decapitated, placed on ice, and kidneys were immediately dissected bilaterally. For some experiments, renal cortex was dissected from the renal medulla. Tissue was kept on ice, minced and then homogenized in a ground glass mechanical homogenizer until liquified. Homogenization buffer consisted of 20 mM HEPES pH 7.5, 10% glycerol, 1 mM EDTA, 10 mM tetrasodium pyrophosphate, 100 mM NaF, 17.5 mM β-glycerophosphate, 1:42 dilution of Sigma protease inhibitor cocktail (P8340), 5 μM okadaic acid, and 0.04% trypsin inhibitor. Unfractionated homogenate protein concentrations were determined by the Folin phenol reagent method, and homogenates were stored at −80 °C until used.

Total PERK or PERK(P) were detected using antisera for total PERK or PERK(P), respectively, by first immunoprecipitating and then detecting immunoprecipitates by Western blot. The immunoprecipitation (IP) protocol was as previously described [25]. For both PERK and PERK(P) immunoprecipitations, 30 mg homogenate protein was used per IP reaction.

For eIF2α(P) Western blots, 125 μg protein from unfractionated homogenates was run per lane on SDS-PAGE, and electroblot transferred to nitrocellulose as previously described [23]. For Western blotting, primary antisera dilutions were: eIF2α(P), 1:750; PERK(P), 1:2000; PERK, 1:10,000. Quantitation of Western blots was by densitometric analysis (Bio Image Intelligent Quantifier, version 3.1). Groups were compared by analysis of variance and LSD post hoc with significance set at $P<0.05$.

### 2.3. Immunocytochemistry and light microscopy

Localization of eIF2α(P) in rat kidney using immunohistochemistry was done on kidneys fixed by retrograde perfusion via the aorta with 2% paraformaldehyde in 0.1 M phosphate buffered saline, pH 7.4. Kidneys were dehydrated using an alcohol series and embedded in paraffin. Sagittal sections (4 μm) of whole kidney were stained with hematoxylin. For immunoperoxidase labeling, endogenous peroxidase was blocked by 3% $H_2O_2$ in absolute methanol for 10 min at room temperature. To reveal antigens, section were put through a 1-mM sodium citrate solution at pH 6.0 at 95 °C for 20 min. Nonspecific binding of immunoglobulin was prevented by blocking in PBS supplemented with 1% horse serum for 10 min. Sections were incubated overnight for 18 h at 4 °C with primary antibody to eIF2α(P) diluted (1:100) in PBS. Sections were washed three times with PBS, then incubated with a biotinylated secondary antibody for 10 min (anti rabbit Ig Vectastain kit [Vector, Inc.]), followed by horseradish peroxidase-conjugated streptavidin for 10 min. Sections were incubated with aminoethyl carbazole (AGC) chromogen and development of immunostaining was monitored microscopically. The microscopy was carried out using the Axioplan 2 Imaging System (Carl Zeiss, Oberkochen, Germany). Digital images were acquired with the AxioCam MRm high resolution digital camera.

### 2.4. Creatinine assessment

Trunk blood was obtained from each rat at the time of sacrifice and placed into heparinized tubes. Plasma creatinine was measured in duplicate using a creatinine assay kit based on a modification of the Jaffe reaction (Cayman Chemicals, Ann Arbor, MI).

## 3. Results

In homogenates of unfractionated whole kidney, eIF2α(P) levels increased during reperfusion in proportion to the ischemic duration. Five minutes of ischemia followed by 10 min reperfusion lead to a 7-fold increase in whole kidney eIF2α(P) (ANOVA $P=0.048$), whereas 10 min ischemia and 10 min reperfusion resulted in a 20-fold increase in eIF2α(P) (ANOVA $P<0.001$). Total levels of eIF2α were unchanged (ANOVA $P=0.43$) (Fig. 1). There was no increase in eIF2α(P) (ANOVA $P=0.28$) nor change in the level of eIF2α (ANOVA $P=0.37$) in kidneys after either 5 or 10 min ischemia only (Fig. 2). To determine if the eIF2α(P) increase was selective for renal cortex or medulla, these were dissected and assessed separately for eIF2α and

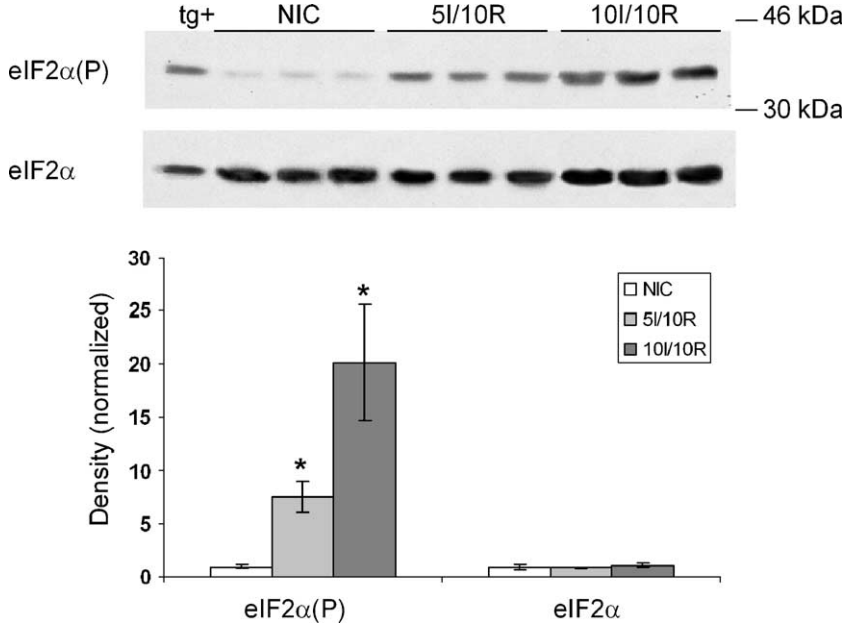


Fig. 1. Levels and phosphorylation of eIF2α as assessed by Western blot of 125 μg homogenate protein per lane in unfractionated homogenates of whole kidney in nonischemic controls (NIC), following 5 min ischemia and 10 min reperfusion (5I/10R), and following 10 min ischemia and 10 min reperfusion (10I/10R). At a constant reperfusion time of 10 min, phosphorylation of eIF2α increased as a function of ischemic duration. Thapsigargin treated (tg+) NB104 cells provides a positive mobility control for eIF2α(P). Bar graph shows mean ± standard deviation. Values are normalized such that NIC average is 100%. *$P<0.05$ compared to NIC group.

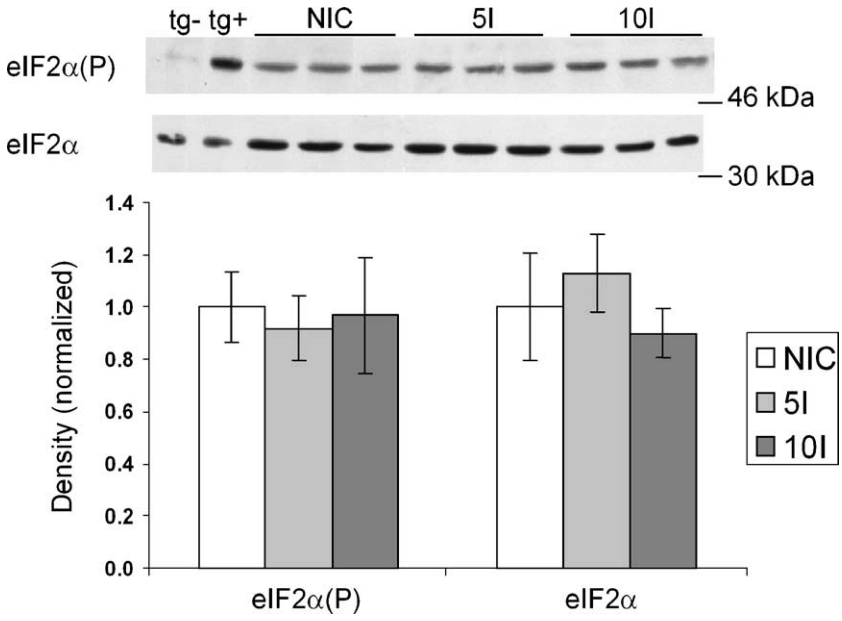


Fig. 2. Levels and phosphorylation of eIF2α as assessed by Western blot of 125 μg homogenate protein per lane in unfractionated homogenates of whole kidney in nonischemic controls (NIC), or following 5 min (5I) or 10 min (10I) of cardiac arrest-induced ischemia. There was no change in eIF2α levels or eIF2α(P) phosphorylation in the ischemic period. Thapsigargin treated (tg+) NB104 cells provides a positive mobility control for eIF2α(P). Bar graph shows mean±standard deviation of scanning densitometry. Values are normalized such that NIC average is 100%.

eIF2α(P) (Fig. 3). At 10 min ischemia and 10 min reperfusion, eIF2α(P) increased approximately 8-fold in both cortex and medulla (Fig. 3, ANOVA $P<0.05$). The 16-fold increase in eIF2α(P) that results from adding cortical and medullary eIF2α(P) was statistically indistinguishable from the 20-fold eIF2α(P) increase obtained in whole kidney (two-tailed $t$-test, $P=0.12$). At 90-min reperfusion following 10 min ischemia, eIF2α(P) returned to control levels in both cortex and medulla. There was no change in total eIF2α in cortex (ANOVA $P=0.33$) or medulla (ANOVA $P=0.28$) (Fig. 3). We note that endogenous eIF2α(P) levels in NIC kidney were higher than in the untreated NB104 cells. Even compared to NIC brain samples, endogenous renal eIF2α(P) levels were higher

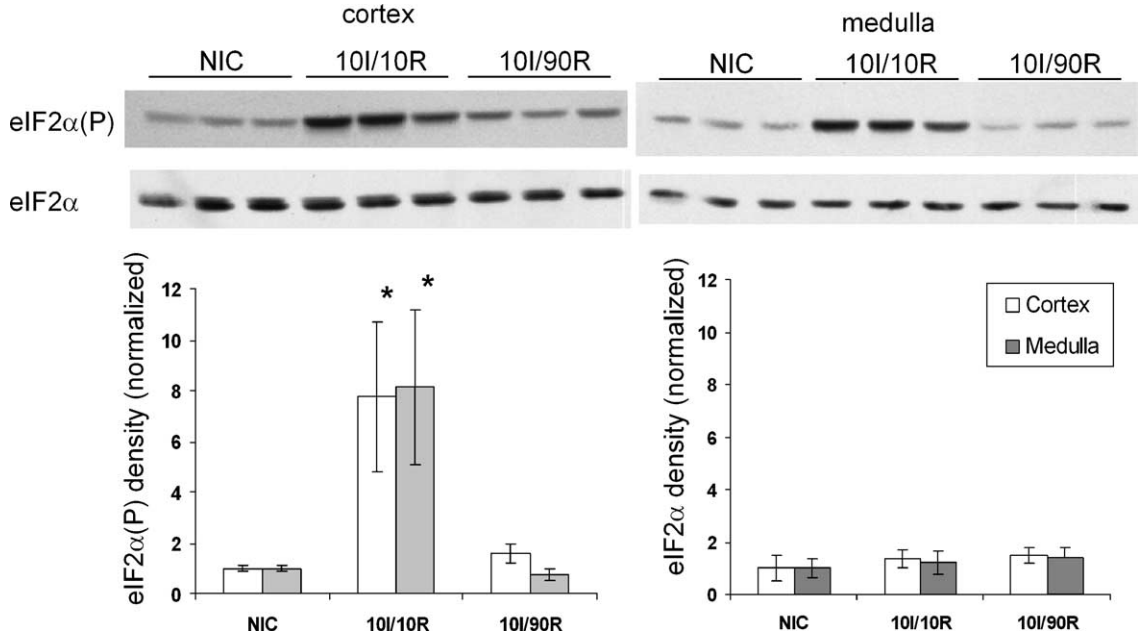


Fig. 3. Levels and phosphorylation of eIF2α in cortical and medullary homogenates. Following 10 min ischemia and 10 min reperfusion (10I/10R), eIF2α(P) levels in cortex and medulla were equal and were ~8-fold those of nonischemic controls (NIC). By 90 min reperfusion following 10 min ischemia (10I/90R), eIF2α(P) levels had returned to control levels. There was no change in total eIF2α. Bar graph shows mean±standard deviation. Values are normalized such that NIC average is 100%. *$P<0.05$ compared to NIC group.

(data not shown). We also note that apparent disparities in the NIC levels of eIF2α(P) among Figs. 1–3 are due to different film exposure conditions. Because the increase in eIF2α(P) is so high in reperfused samples, Western blots containing reperfused samples required specific exposure conditions to be able to visualize all three groups adequately on the same film development while maintaining linearity for densitometric analysis.

To determine the cause of eIF2α phosphorylation, the activation status of PERK was assessed following immunoprecipitation and detection by Western blot. Activation of PERK is accompanied by its autophosphorylation, causing PERK to migrate slower on SDS-PAGE gels [22,25]. Immunoprecipitation with an antisera that recognizes both the phosphorylated and unphosphorylated forms of PERK showed increased PERK(P) following ischemia and reperfusion (Fig. 4). In nonischemic control (NIC) kidneys, 27% of PERK was in the phosphorylated, active form (Table 1). At 5I/10R, 65% of PERK was in the active, phosphorylated form, and this decreased slightly to about 60% in the 10I/10R and 10I/90R groups (Table 1). Thus, following reperfusion, the majority of PERK is in the active state. We note that inactive renal PERK migrated slightly below the inactive PERK band from the NB104 cells (Fig. 4), and this is consistent with a previous study showing that inactive renal PERK migrated below inactive PERK from other tissues [26]. Total PERK [PERK+PERK(P)] was observed to be significantly decreased at 90 min reperfusion to roughly 10% of control levels. To further confirm PERK activation, immunoprecipitation was also performed with an antisera specific only for PERK(P) (Fig. 5). In the 10I/10R group, multiple bands, corresponding to the different phospho-forms of PERK were detect. As PERK has been shown to contain 11 authentic phosphorylation sites [27], the presence of multiple PERK(P) bands in not unexpected. The 10I/10R and 10I/90R groups showed 10-fold and 2-fold increases in PERK(P), respectively, over the other experimental groups (Fig. 4C, ANOVA $P<0.05$).

We next ascertained which renal cells contained eIF2α(P) by immunohistochemistry. As a control for eIF2α(P)

Table 1
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| | NIC | 5I/10R | 10I/10R | 10I/90R |
|---|---|---|---|---|
| PERK(P) | 27±11% | 65±13%* | 61±5%* | 60±4%* |
| PERK | 73±11% | 35±13% | 39±5% | 40±4% |
| Relative levels | 1±0.8 | 1±0.9 | 2±1.3 | 0.1±0.07* |

Percentages of inactive PERK and phosphorylated PERK [PERK(P)], and relative levels of total PERK [PERK+PERK(P)] following ischemia and reperfusion determined from scanning densitometry of total PERK IP as shown in Fig. 4. Shown are the percentages of PERK in the inactive and active forms such that the totals add to 100%. The bottom row shows the relative levels of total PERK. Abbreviations as in Figs. 1 and 3.
* Post hoc $P<0.05$ compared to NIC group.

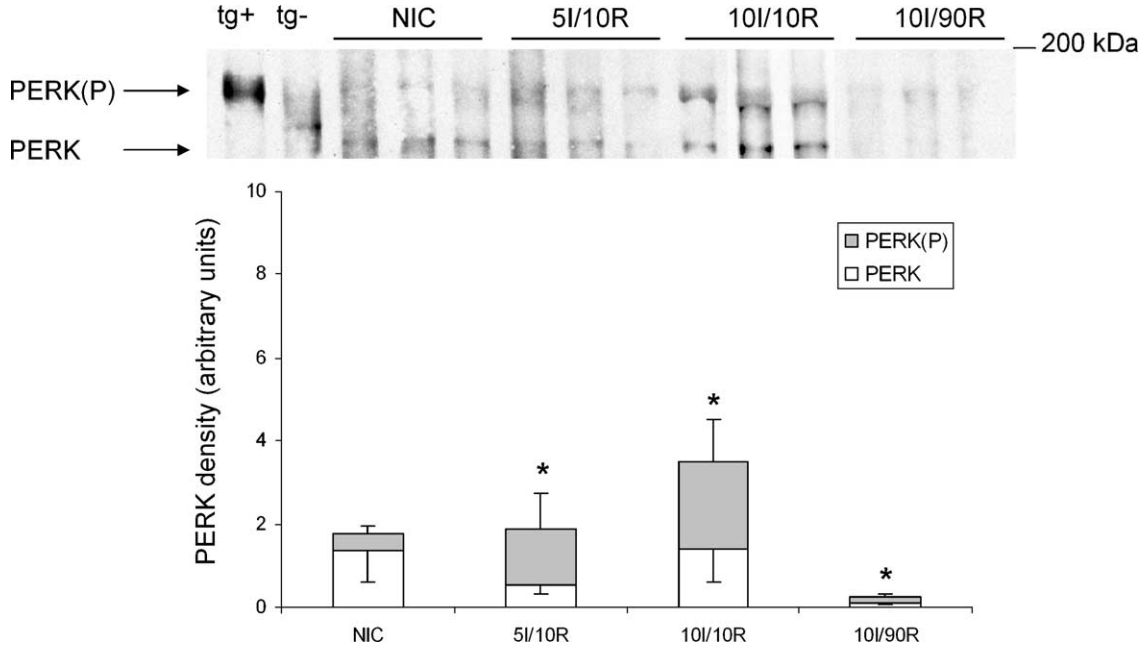



Fig. 4. PERK is activated by renal ischemia and reperfusion. Following immunoprecipitation with PERK antisera and detection by Western blot with the same antisera, active, hyperphosphorylated PERK migrates slower than inactive PERK on SDS-PAGE and is therefore identified by its decreased mobility. Neuroblastoma cells were either treated (tg+) or not treated (tg−) with thapsigargin, an agent that causes stress to the endoplasmic reticulum and PERK activation, providing mobility controls for active [PERK(P)] and inactive PERK. Note the inactive renal PERK migrates slightly below inactive PERK from NB104 cells, as shown in Harding et al. [26]. Scanning densitometry shows a shift from predominantly inactive PERK in the nonischemic (NIC) group to predominantly active PERK after 5 min ischemia and 10 min reperfusion (5I/10R), 10 min ischemia and 10 min reperfusion (10I/10R), and 10 min ischemia and 90 min reperfusion (10I/90R) (* $P<0.05$ compared to NIC group for levels of PERK(P)). There is also an absolute loss of PERK in the 10I/90R group (and $P<0.05$ for total levels of PERK [PERK+PERK(P)]). Bar graph shows mean±standard deviation of densitometry. These data are summarized numerically in Table 1.

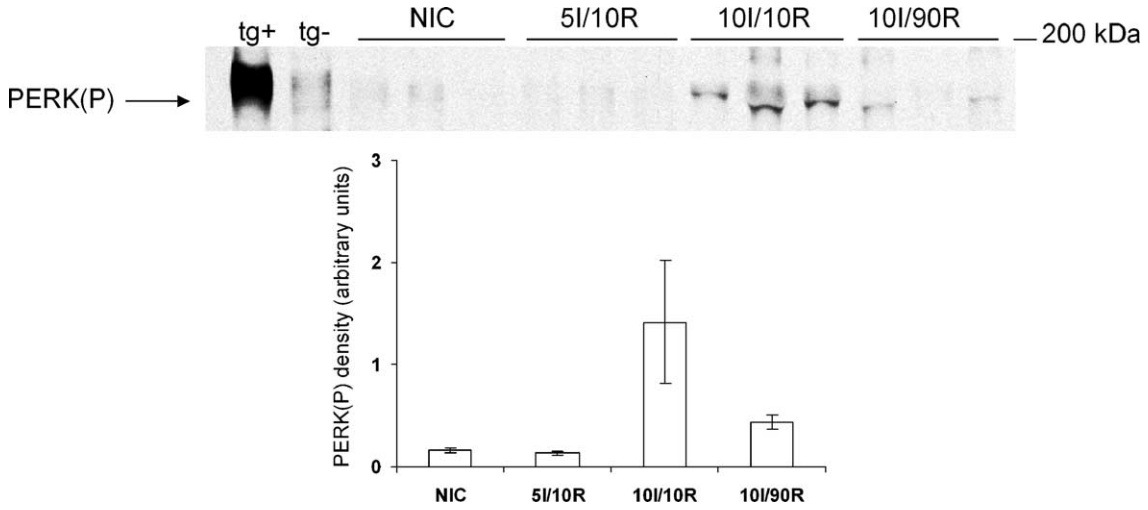



Fig. 5. Immunoprecipitation followed by Western blot with antisera against phosphorylated PERK [PERK(P)] gave the largest signal in the 10I/10R group. At least two different phospho-forms of PERK were detected at 10R and 90R. Scanning densitometry shows a 10-fold increase in PERK(P) in the 10I/10R group and a 2-fold increase in the 10I/90R group, compared to nonischemic controls (NIC) and 5 min ischemia and 10 min reperfusion.

staining, immunohistochemistry was also performed on cerebellum from NIC and 10I/10R animals, in which we previously showed undetectable eIF2α(P) in NICs, and intense eIF2α(P) staining in reperfused cerebellar neurons [28,29]. As shown in Fig. 6A and B, our previous results in cerebellum were confirmed. Further, Western blots for eIF2α(P) in unfractionated kidney homogenates showed only a single band migrating at 35 kDa, indicating the monospecificity of the eIF2α(P) antisera in kidney and its suitability for immunohistochemical analysis.

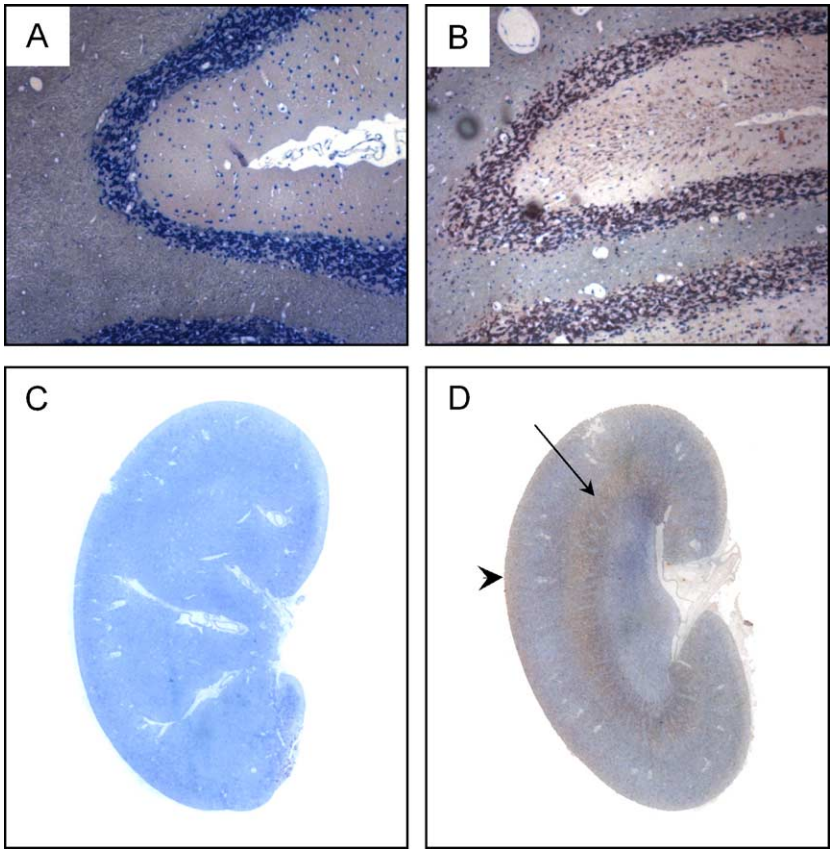
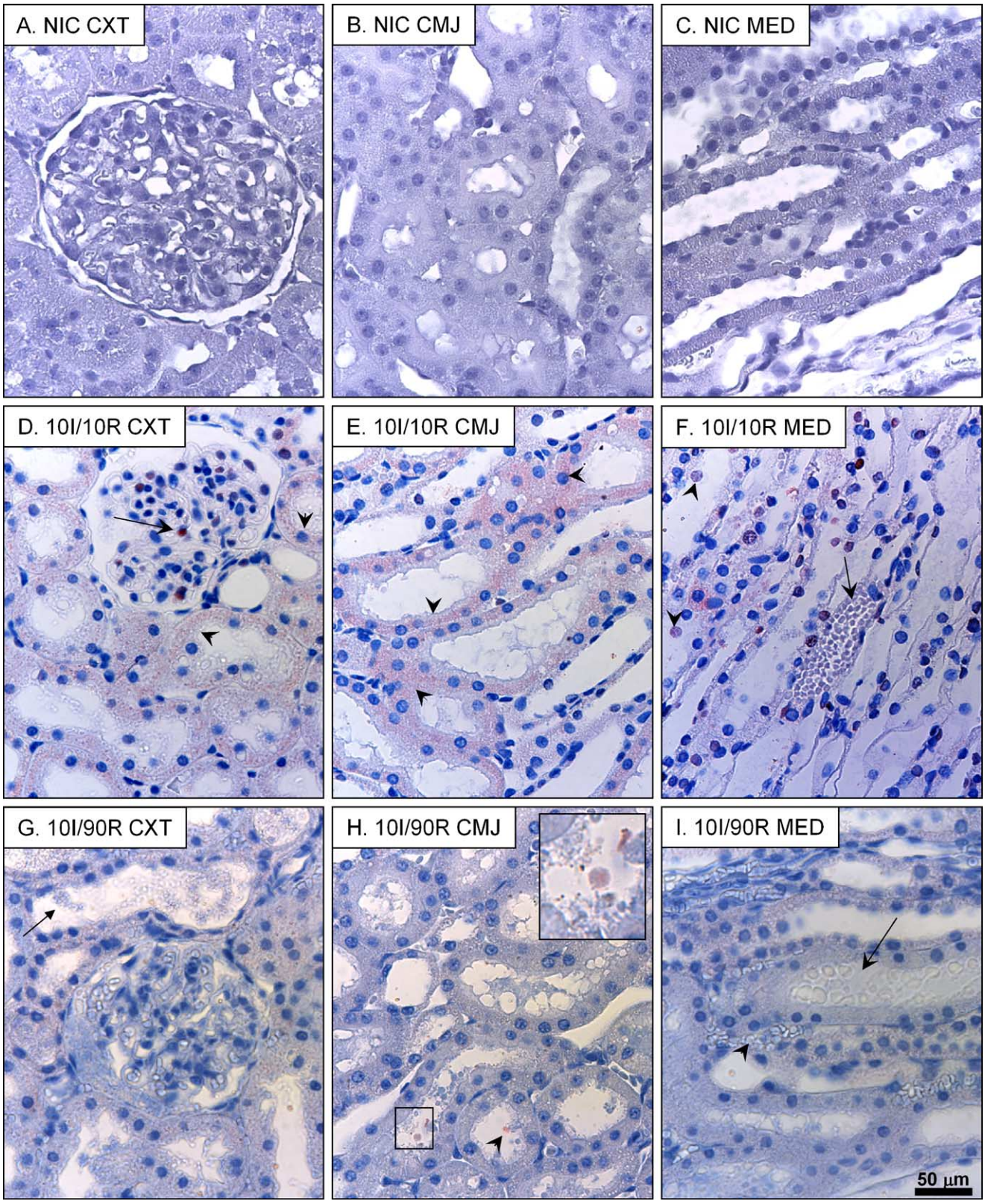



Fig. 6. Immunoperoxidase staining of eIF2α(P) using paraffin-embedded tissues from (A, C) nonischemic rat cerebellum (40×) and kidney (10×) and (B, D) 10 min ischemia/10 min reperfusion (10I/10R) rat cerebellum and kidney. Immunolabeling of eIF2α(P) is associated with Purkinje and granular cells of the cerebellum and prominently at the corticomedullary junction of the kidney (arrow) as well as superficial cortical areas (arrowhead).

Low magnification views of eIF2α(P) staining showed no signal in NICs (Fig. 6C). However, following 10 min ischemia and 10 min reperfusion, a prominent band of eIF2α(P) staining was observed at the corticomedullary junction (Fig. 6D). Staining was also evident along the most superficial cortex. At low magnification, the 10I/90R group resembled controls (data not shown).



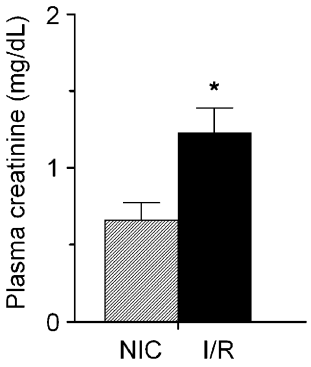



Fig. 8. Plasma creatinine values in nonischemic (NIC) and ischemia+reperfusion (I/R) groups. The I/R group consists of the pooled values from the 10I/10R and 10I/90R groups. Values are mean ±SE ($n=6$ per group, *$P<0.02$ vs. NIC).

At high magnification (×20 objective) no eIF2α(P) was detectable in NIC cortex (CTX), corticomedullary junction (CMJ), or medulla (MED) (Fig. 7A–C). At 10I/10R, most glomerular cells showed no eIF2α(P). However, some cells within the glomerulus, putatively identified as inflammatory cells on the basis of their morphology, showed intense eIF2α(P) staining around the nucleus (Fig. 7D, arrow). The majority of cortical eIF2α(P) staining was observed in tubular epithelial cells, notably the proximal cortical tubule (7D, arrowheads). Faint staining of distal tubule segments was also present. The strongest eIF2α(P) staining was obtained in proximal tubule cells at the corticomedullary junction (Fig. 7E). Dilated and congested capillaries were visible within the medulla (7F, arrow) at 10I/10R, as well as staining of some tubular epithelial cells and inflammatory cells. By 90 min reperfusion, eIF2α(P) staining abated significantly, although faint residual staining was observed in cortex, corticomedullary junction and medullary epithelial cells (Fig. 7G–I). Cells positive for eIF2α(P) were evident within the tubular lumen (Fig. 7H, arrowhead and inset). Capillary congestion (Fig. 7I, arrowhead) and casts (Fig. 7I, arrow) were present within the inner medulla. Thus, eIF2α(P), and by extension, eIF2α kinase activity, is largely confined to the tubular epithelial cells of the nephron, and is greatest in the poorly vascularized "watershed" area at the corticomedullary junction.

Since there were no significant differences in plasma creatinine between the 10I/10R and 10I/90R groups, these values were grouped together. Despite the relatively brief period of whole body ischemia, plasma creatinine was significantly higher in the ischemic group (Fig. 8).

## 4. Discussion

The novel findings of the present study are that, following a clinically relevant 10 min duration of cardiac arrest, eIF2α(P) greatly increased and PERK was activated in renal tubular epithelial cells, providing a cellular mechanism for the inhibition of protein synthesis that occurs in kidney following I/R [10]. Further, these changes co-occurred with morphological and physiological evidence of renal ischemic damage (Figs. 7 and 8). Activation of PERK is a marker of ER stress and activation of the unfolded protein response (UPR) [15]. Although these mechanisms have been thoroughly investigated in brain (as reviewed in 20), demonstrating their occurrence in reperfused kidney is novel for the following reasons. First, our results show conservation of the ER stress response in reperfused kidney and brain. Given the extreme sensitivity of brain to ischemic injury as compared to kidney, it is not an a priori given that both organs would respond similarly to I/R. Second, the present results have implications for the treatment of pathologies associated with renal I/R, such as renal transplantation surgery, shock, or following resuscitation from cardiac arrest. As elaborated below, ER stress and the UPR may play a causal role in renal dysfunction and recovery following I/R.

In the present study, we did not measure protein synthesis as its depression following renal reperfusion is well known (reviewed in 10). Instead, our aim here was to investigate a well-established pathway of translation arrest to provide deeper insight into the renal cellular response to I/R. Plestina and Garmulin [11] showed a 16% decrease in polysomes in mouse kidney following 5 min ischemia alone, and a 36% decrease in polysomes with reperfusion durations out to 6 h. Further, these authors showed that administration of cycloheximide, an elongation inhibitor, prevented ischemic-, but not reperfusion-induced polysome disaggregation. Our results, in conjunction with this previous study, indicate that multiple mechanisms underlie renal translation arrest following I/R.

In kidney after 10 min ischemia induced by renal pedicle ligation, ATP levels fell to roughly 12% of control values and energy charge was 7% of controls [30]. Thus, during ischemia, polysome disaggregation could be due to run off of existing polysomes and decreased rate of translation initiation due to depletion of ATP. This would explain the ability of cycloheximide to prevent polysome disaggregation during ischemia, by "freezing" polysomes and preventing run-off, and is consistent with our demonstration of

Fig. 7. Immunoperoxidase staining of eIF2α(P) in renal cortex (CTX), corticomedullary junction (CMJ) and medulla (MED) at 20× magnification. (A–C) Nonischemic kidney (NIC) showing no detectable immunolabeling for eIF2α(P) at any level. (D–F) After 10 min ischemia and 10 min reperfusion (10I/10R), glomeruli were largely free of immunolabeled eIF2α(P), however, some cells (consistent with inflammatory cells) within the glomerulus showed intense eIF2α(P) staining around the nucleus (D, arrow). Strong immunoreactivity for eIF2α(P) staining in tubular epithelial cells, especially proximal tubules (D, arrowheads). Faint staining of distal tubule segments was also present. The strongest eIF2α(P) staining was obtained in proximal tubule cells at the corticomedullary junction (E). Dilated and congested capillaries were visible within the medulla (F, arrow) as well as staining of tubular epithelial cells lining the lumen or shed into the lumen (arrowheads). (G–I) Following 90 min reperfusion, faint residual staining for eIF2α(P) staining in cortex, corticomedullary junction and medullary epithelial cells. Cellular debris (G, arrow) and epithelial cells positive for eIF2α(P) (H, arrowhead and inset) were evident within the tubular lumen. Capillary congestion (I, arrowhead) and casts within the tubular lumen (I, arrow) were present within the medulla.

lack of eIF2α(P) increase during ischemia only. Upon reperfusion, renal ATP returns [30], and eIF2α(P) rapidly increases, which could account for the cycloheximide-refractory disaggregation of polysomes observed by Plestina and Garmulin [11]. However, we observed eIF2α(P) to return to control levels by 90 min reperfusion. Thus, polysome disaggregation outlasts eIF2α phosphorylation. In fact, this is similar to the situation observed in brain in which protein synthesis inhibition and polysome disaggregation outlast eIF2α phosphorylation [31]. Thus, in both brain and kidney, a mechanisms of translation inhibition other than eIF2α phosphorylation must occur following longer reperfusion durations. What this mechanism might be is currently unknown (reviewed in [20]).

In addition to providing insight into the mechanisms of translation arrest following renal I/R, our observations indicate the cellular context within which eIF2α(P)-mediated translation arrest occurs. The evidence for PERK activation shown above (Figs. 4 and 5) is definitive molecular evidence for UPR activation and hence indicates that renal epithelial cells experience ER stress following I/R.

We note that in the 5I/10R group, following total PERK IP (Fig. 4), we observed 65% of total PERK (Table 1) to be in slower migrating, phosphorylated forms. However, following PERK IP using antisera specific for PERK phosphorylated at threonine 980 [PERK(ThrP980)], we observed no increase at 5I/10R compared to controls (Fig. 5). Generally, multiple forms of PERK(P) were observed in kidney following I/R (Fig. 5, 10I/10R group) which is not unexpected as PERK has been shown to possess 11 authentic phosphorylation sites [27]. The functional significance of these phospho-forms, and particularly their role in PERK activation, remains to be investigated. Therefore, one possibility for our result is that PERK(ThrP980) is not an absolute requirement of PERK activation, and the increase in PERK(P) shown in Fig. 4 is more indicative of PERK activation. A second possibility is that PERK(ThrP980) is an absolute indicator of PERK activation, and PERK was not activated in the 5I/10R group. This possibility would indicate that regulation of eIF2α(P) following renal ischemia is more complex and involves multiple eIF2α kinases. Further studies will be required to distinguish between these two possibilities.

We also observed an absolute loss of PERK at 90 min renal reperfusion, which also occurs following 90 min brain reperfusion [24]. The significance of PERK loss following its reperfusion-induced activation is not currently understood. We note that the inactive form of renal PERK migrated slightly below that from the NB104 cells. The smaller form of inactive renal PERK has been previously reported [26], although, to our knowledge, its significance has not been investigated.

Unlike brain [24], the eIF2α phosphorylation we observed in reperfused kidney is reversible within 90 min following a 10 min ischemic insult (Fig. 3). Although at 90R the remaining PERK was predominantly in the active form, the overall loss of PERK would be expected to lead to a net increase in eIF2α(P) phosphatase activity, and may account for the rapid dephosphorylation of eIF2α(P) by 90R. The present model, based on clinically relevant durations of cardiac arrest and resuscitation, has been used extensively to study brain I/R but precludes longer durations of ischemia (20–30 min) typical of renal ischemia studies. We have shown here proof of principle that renal I/R activates PERK. Although requiring further investigation, our observation of PERK activation implies activation of the UPR following renal I/R. Since eIF2α(P) increased as a function of ischemic duration (Fig. 2), it may be expected that longer durations of ischemia will cause a more intense ER stress and sustained UPR activation.

The present study shows clearly that the translation inhibition function of the UPR is activated in kidney following I/R. Other studies have also provided evidence suggesting UPR activation following in vivo renal ischemia or in cultured tubular cells. Kuznetsov et al. observed upregulation of the mRNAs for ER localized proteins GRP78, GRP94, and ERP72 following 30-min acute renal ischemia [32]. Treatment of Madin–Darby canine kidney cells with the ER stress inducers tunicamycin and A23187 resulted in significant cytoprotection after subsequent ATP depletion, suggesting activation of the UPR [33]. In a culture system of simulated ischemia, proteomic analysis of LLC-PK1 cells showed upregulation of GRP78, along with heat shock proteins [34].

UPR activation may be an additional important mechanism whereby renal I/R injury leads to differential susceptibility of tubular epithelial segments via either a pro-survival or pro-apoptotic phenotype in affected cells. The observed increase in eIF2α(P) immunohistochemical staining of tubular segments at the poorly vascularized corticomedullary junction (Fig. 6D, arrow) is consistent with the increased ischemic susceptibility in this region. This highly localized increase in eIF2α(P) may contribute to the greater cellular damage typically seen in cells of the $S_3$ segment of the proximal tubule and outer medullary thick ascending limb in renal ischemic injury. The eIF2α(P) distribution also explains the relatively equal content of eIF2α(P) in cortical and medullary homogenates (Fig. 3), as our dissection likely split the corticomedullary junction equally between dissected regions.

The significance of UPR-mediated renal apoptosis has been discussed with respect to tunicamycin-treated CHOP knockout mice that undergo acute tubular necrosis [35]. CHOP knockout mice showed attenuated renal apoptosis compared to wild types, and slower recovery of renal function. Zinszner et al. proposed that CHOP-mediated cell death may actually be beneficial by promoting the regeneration of epithelium through removal of damaged tubular cells [35]. Thus, UPR expression in reperfused kidney may serve a dual role. Renal tubular epithelial cells subjected to severe ER stress and more intense activation of UPR due to the duration or magnitude of the ischemic insult, nature of

the microcirculation, and/or intrinsic cellular susceptibility will experience apoptotic cell death and removal. Less intensely affected renal tubular cells may undergo UPR-associated transient inhibition of protein synthesis followed by an increase in the processing capacity of the ER with recovery of cell function. Together with activation of the heat shock proteins [36,37], the UPR may contribute to tubular epithelial cells regaining their cytoskeletal architecture and polarity, while others may enter the cell cycle and mitosis resulting in repopulation of lost renal tubular cells. In contrast to its role in the brain, the pro-apoptotic expression of the UPR may therefore serve to complement its pro-survival role in the reperfused kidney.

In conclusion, the present study demonstrated activation of PERK and phosphorylation of eIF2α, indicating ER stress and activation of the UPR, following renal I/R. These findings open the door to ascertaining the significance of ER stress and UPR expression in post-ischemic reperfused kidney. Future studies are needed to assess the status of IRE1 and ATF6, as well as other downstream UPR components in reperfused kidney. The ultimate aim of such investigations will be to determine the relative contributions of pro-survival or pro-apoptotic UPR pathways to renal dysfunction and recovery following I/R. Given the importance of ER-localized protein synthesis for renal tubular function, such as the absolute requirement for synthesis of transmembrane transport proteins, and the unique exposure of the kidney to a variety of substances with potential to stress the ER, it is expected that the results presented here may be generalizable to other forms of clinically relevant renal damage.

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## References

[1] R.W. Schrier, W. Wang, B. Poole, A. Mitra, Acute renal failure: definitions, diagnosis, pathogenesis and therapy, J. Clin. Invest. 114 (2004) 5–13.
[2] R. Thadhani, M. Pacual, J.V. Bonventre, Acute renal failure, N. Engl. J. Med. 334 (1996) 1448–1460.
[3] J.V. Bonventre, A. Zuk, Ischemic acute renal failure: an inflammatory disease? Kidney Int. 66 (2004) 480–485.
[4] J.J. Friedewald, R. Hamid, Inflammatory cells in ischemic acute renal failure, Kidney Int. 66 (2004) 486–490.
[5] T.A. Sutton, H.E. Mang, S.B. Campos, R.M. Sandoval, M.C. Yoder, B.A. Molitoris, Injury of the renal microvascular endothelium alters barrier function after ischemia, Am. J. Physiol. 285 (2003) F191–F198.
[6] H. Ling, C. Edelstein, P. Gengaro, X. Meng, S. Lucia, M. Knotek, A. Wangsiripaisan, Y. Shi, R.W. Schrier, Attenuation of renal ischemia–reperfusion injury in inducible nitric oxide synthase knockout mice, Am. J. Physiol. Renal. Physiol. 277 (1999) F383–F390.
[7] E. Noiri, A. Nakao, K. Uchida, H. Tsukahara, M. Ohno, T. Fujita, S. Brodsky, M.S. Goligorsky, Oxidative and nitrosative stress in acute renal ischemia, Am. J. Physiol. Renal. Physiol. 281 (2001) F948–F957.
[8] L. Yu, P.E. Gengaro, M. Niederberger, T.J. Burke, R.W. Schrier, Nitric oxide: a mediator in rat tubular hypoxia/reoxygenation injury, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 1691–1695.
[9] Y. Shi, V.Y. Melnikov, R.W. Schrier, C.L. Edelstein, Downregulation of the calpain inhibitor protein calpastatin by caspases during renal ischemia–reperfusion, Am. J. Physiol. Renal. Physiol. 279 (2000) F509–F517.
[10] I.H. Chaudry, M.G. Clemens, A.E. Baue, Alterations in cell function with ischemia and shock and their correction, Arch. Surg. 116 (1988) 1309–1317.
[11] S. Plestina, S. Gamulin, Kidney ischaemia–reperfusion injury and polyribosome structure, Nephron 89 (2001) 201–207.
[12] W.C. Merrick, Mechanism and regulation of eukaryotic protein synthesis, Microbiol. Rev. 56 (1992) 291–315.
[13] J.J. Chen, Heme-regulated eIF2α Kinase, in: N. Sonenberg, J.W.B. Hershey, M.B. Mathews (Eds.), Translational Control of Gene Expression, Cold Spring Harbor Laboratory Press, New York, 2000, pp. 529–546.
[14] R.J. Kaufman, Double-stranded RNA-activated protein kinase PKR, in: N. Sonenberg, J.W.B. Hershey, M.B. Mathews (Eds.), Translational Control of Gene Expression, Cold Spring Harbor Laboratory Press, New York, 2000, pp. 503–528.
[15] D. Ron, H.P. Harding, PERK and translational control by stress in the endoplasmic reticulum, in: N. Sonenberg, J.W.B. Hershey, M.B. Mathews (Eds.), Translational Control of Gene Expression, Cold Spring Harbor Laboratory Press, New York, 2000, pp. 547–560.
[16] S.R. Kimball, L.S. Jefferson, Regulation of translation initiation in mammalian cells by amino acids, in: N. Sonenberg, J.W.B. Hershey, M.B. Mathews (Eds.), Translational Control of Gene Expression, Cold Spring Harbor Laboratory Press, New York, 2000, pp. 561–580.
[17] A.G. Rowlands, R. Panniers, E. Henshaw, The catalytic mechanism of guanine nucleotide exchange factor action and competitive inhibition by phosphorylated eukaryotic initiation factor 2, J. Biol. Chem. 263 (1988) 5526–5533.
[18] S. Oldfield, B.L. Jones, D. Tanton, C.G. Proud, Use of monoclonal antibodies to study the structure and function of eukaryotic protein synthesis initiation factor eIF-2B, Eur. J. Biochem. 221 (1994) 399–410.
[19] J. Burda, M.E. Martin, A. Garcia, A. Alcazar, J.L. Fando, M. Salinas, Phosphorylation of the α subunit of initiation factor 2 correlates with the inhibition of translation following transient cerebral ischemia in the rat, Biochem. J. 302 (1994) 335–338.
[20] D.J. DeGracia, Acute and persistent protein synthesis inhibition following cerebral reperfusion, J. Neurosci. Res. 15 (2004) 771–776.
[21] D.J. DeGracia, R. Kumar, S. Owens, G.S. Krause, B.C. White, Molecular pathways of protein synthesis inhibition during brain reperfusion: implications for neuronal survival or death, J. Cereb. Blood Flow Metab. 22 (2002) 127–141.
[22] H.P. Harding, Y. Zhang, D. Ron, Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase, Nature 397 (1999) 271–274.
[23] D.J. DeGracia, R.W. Neumar, B.C. White, G.S. Krause, Global brain ischemia and reperfusion: modifications in eukaryotic initiation factors are associated with inhibition of translation initiation, J. Neurochem. 67 (1996) 2005–2012.
[24] R. Kumar, G.S. Krause, H. Yoshida, K. Mori, D.J. DeGracia, Dysfunction of the unfolded protein response during global brain ischemia and reperfusion, J. Cereb. Blood Flow Metab. 23 (2003) 462–471.
[25] R. Kumar, S. Azam, J.M. Sullivan, S. Owens, D.R. Cavener, P. Zhang, D. Ron, H.P. Harding, J.J. Chen, A. Han, B.C. White, G.S. Krause, D.J. DeGracia, Brain ischemia and reperfusion activates the eukary-

otic initiation factor 2α kinase, PERK, J. Neurochem. 77 (2001) 1418–1421.
[26] H.P. Harding, H. Zeng, Y. Zhang, R. Jungries, P. Chung, H. Plesken, D.D. Sabatini, D. Ron, Diabetes mellitus and exocrine pancreatic dysfunction in perk−/− mice reveals a role for translational control in secretory cell survival, Mol. Cell 7 (2001) 1153–1163.
[27] Y. Ma, Y. Lu, H. Zeng, D. Ron, W. Mo, T.A. Neubert, Characterization of phosphopeptides from protein digests using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and nanoelectrospray quadrupole time-of-flight mass spectrometry, Rapid Commun. Mass Spectrom. 15 (2001) 1693–1700.
[28] D.J. DeGracia, J.M. Sullivan, R.W. Neumar, S.S. Alousi, K.R. Hikade, J.E. Pittman, B.C. White, J.A. Rafols, G.S. Krause, Effect of brain ischemia and reperfusion on the localization of phosphorylated eukaryotic initiation factor 2α, J. Cereb. Blood Flow Metab. 17 (1997) 1291–1302.
[29] A.B. Page, R. Kumar, B.C. White, J.A. Rafols, D.J. DeGracia, Global cerebral ischemia induces eIF2α phosphorylation throughout the entire brain, Transl. Control Abstr. (2000) 255.
[30] M.J. Eng, D.J. Gmur, R.A. Zager with Technical Assistance of C.E. Bredl, Hyperthermia: effects on renal ischemic/reperfusion injury in the rat, Lab. Invest. 63 (1990) 360–369.
[31] C. Martin de la Vega, J. Burda, M. Nemethova, C. Quevedo, A. Alcazar, M.E. Martin, V. Danielisova, J.L. Fando, M. Salinas, Possible mechanisms involved in the down-regulation of translation during transient global ischaemia in the rat brain, Biochem. J. 357 (2001) 819–826.
[32] G. Kuznetsov, K.T. Bush, P.L. Zhang, S.K. Nigam, Perturbations in maturation of secretory proteins and their association with endoplasmic reticulum chaperones in a cell culture model for epithelial ischemia, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 8584–8589.
[33] K.T. Bush, S.K. George, P.L. Zhang, S.K. Nigam, Pretreatment with inducers of ER molecular chaperones protects epithelial cells subjected to ATP depletion, Am. J. Physiol. Renal. Physiol. 277 (1999) F211–F218.
[34] Y. Kumar, U. Tatu, Stress protein flux during recovery from simulated ischemia: induced heat shock protein 70 confers cytoprotection by suppressing JNK activation and inhibiting apoptotic cell death, Proteomics 3 (2003) 513–526.
[35] H. Zinszner, M. Kuroda, X. Wang, N. Batchvarova, R.T. Lightfoot, H. Remotti, J.L. Stevens, D. Ron, CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum, Genes Dev. 12 (1998) 982–995.
[36] B. Bidmon, M. Endemann, T. Muller, K. Arbeiter, K. Herkner, C. Aufricht, HSP-25 and HSP-90 stabilize Na,K-ATPase in cytoskeletal fractions of ischemic rat renal cortex, Kidney Int. 62 (2002) 1620–1627.
[37] C. Lovis, F. Mach, Y.R. Donati, J.V. Bonventre, B.S. Polla, Heat shock proteins and the kidney, Ren. Fail. 16 (1994) 179–192.