



## PERK is activated differentially in peripheral organs following cardiac arrest and resuscitation<sup>☆</sup>

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### Abstract

Visceral organs display differential sensitivity to ischemia and reperfusion injury, but the cellular mechanisms underlying these differential responses are not completely understood. A significant response to ischemia identified in brain is stress to the endoplasmic reticulum (ER), as indicated by PKR-like endoplasmic reticulum eIF2 $\alpha$  kinase (PERK)-mediated phosphorylation of eIF2 $\alpha$ . To determine the generality of this response, we evaluated the PERK pathway in brain, GI tract, heart, liver, lung, kidney, pancreas and skeletal muscle following a clinically relevant, 10 min cardiac arrest-induced whole body ischemia and either 10 or 90 min reperfusion. The potential role of nitric oxide (NO) on PERK activation was investigated by conducting ischemia and reperfusion in the presence and absence of the NO synthase inhibitor nitro-L-arginine methyl ester (L-NAME). Organ stress could be ranked with respect to the degree of eIF2 $\alpha$  phosphorylation at 10 min reperfusion. Brain, kidney and GI tract were reactive organs, showing 15 to 20-fold increases in eIF2 $\alpha$ (P) compared to controls. Moderately reactive organs included liver and heart, showing <10-fold increases in eIF2 $\alpha$ (P). Pancreas, lung and skeletal muscle were nonreactive. Although treatment of cultured neuroblastoma 104 cells with the NO-donor *S*-nitroso-*N*-acetyl-penicillamine (SNAP) activated PERK, administration of L-NAME had no effect on PERK activation or eIF2 $\alpha$  phosphorylation in organs following ischemia and reperfusion. Thus, PERK is activated differentially in reperfused organs independent of NO. These results suggest that ER stress may play a role in differential responses of viscera to ischemia and reperfusion. ER stress in viscera may contribute to the pathophysiology of resuscitation from cardiac arrest and during organ transplantation procedures. © 2005 Elsevier Ireland Ltd. All rights reserved.

**Keywords:** Cardiac arrest; Ischaemia; Nitric oxide; Reperfusion

### 1. Introduction

The organs of the body display differential sensitivities to ischemia and reperfusion (I/R) insults. For example, specific neuron populations show evidence of death following

as little as 5 min cessation of blood flow [1], whereas skeletal muscle can be ischemic for up to 3 h before showing signs of irreversible damage [2]. Generally, ischemic susceptibility has been linked to the extent to which organs can withstand periods of low or no blood flow [3]. However, given the gradation of organ susceptibility to ischemia, the consequences of ATP depletion are incompletely understood, and this has definite clinical consequences. For peripheral organs such as liver, lung, heart and kidney, guarding against ischemic injury is an important factor in the success of organ transplants. Resuscitation from cardiac arrest is another important clinical setting in which organ sensitivity to ischemia contributes to outcome [3]. Although cerebral pathophysiology is the main contributor to mortality following resuscitation from cardiac arrest, cardiac, renal and other organ dysfunction contribute in incom-

*Abbreviations:* ATF4, activating transcription factor of the leucine zipper family 4; CHOP, cyclic AMP response element binding protein homology protein; ER, endoplasmic reticulum; eIF2 $\alpha$ , alpha subunit of eukaryotic initiation factor-2; I/R, ischemia and reperfusion; L-NAME, nitro-L-arginine methyl ester; NO, nitric oxide; NOS, nitric oxide synthetase; PERK, PKR-like ER eIF2 $\alpha$  kinase; SNAP, *S*-nitroso-*N*-acetyl-penicillamine; tg, thapsigargin

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pletely understood fashions to poor resuscitation outcome [4].

It has been recognized for some time that irreversible inhibition of protein synthesis in brain following I/R likely contributes to the death of specific neuronal populations [5]. Translation arrest in reperfused brain is due to phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF2 $\alpha$ ; phosphorylated form, eIF2 $\alpha$ (P)). Phosphorylation of eIF2 $\alpha$  inhibits protein synthesis by acting as a competitive inhibitor of a second initiation factor eIF2B, thereby preventing delivery of the methionine-containing initiator tRNA to the small ribosomal subunit [6]. Further, following brain I/R, eIF2 $\alpha$  is phosphorylated by the PKR-like endoplasmic reticulum eIF2 $\alpha$  kinase (PERK) (reviewed in [7]). PERK, in turn, is activated under conditions of stress to the endoplasmic reticulum (ER) [8]. The ER is the site of post-translational modification of nascent proteins destined for secretion, membrane insertion, or those proteins and enzymes that reside in the secretory compartments. Stress to the ER disrupts post-translational modifications of nascent proteins and triggers a complex protective response that includes PERK-mediated inhibition of protein synthesis [7]. Kohno et al. [9] showed that I/R-induced ER stress can be inhibited by administration of nitric oxide synthase (NOS) inhibitors, suggesting that nitric oxide (NO) contributes to ER stress following brain ischemia and reperfusion.

Here we hypothesized that peripheral organs would demonstrate evidence of ER stress following I/R, as does brain. We further hypothesized that NO activates PERK following I/R. These hypotheses were tested by measuring PERK activation and eIF2 $\alpha$  phosphorylation, as markers of ER stress [8], in several peripheral organs and brain regions following I/R. These studies were conducted in the presence and absence of the nonspecific nitric oxide synthase inhibitor nitro-L-arginine methyl ester (L-NAME). We also tested whether NO can activate PERK by treating cultured neuroblastoma 104 (NB104) cells with the NO-donor S-nitroso-N-acetyl-penicillamine (SNAP). Although we found that SNAP activated PERK in cultured NB104 cells, administration of L-NAME had no effect on PERK activation or eIF2 $\alpha$  phosphorylation following I/R. Further, along with brain, we observed activation of PERK or eIF2 $\alpha$  phosphorylation in kidney, GI tract, liver and heart following I/R. In the clinical context of resuscitation from cardiac arrest, ER stress of kidney and liver, and possibly GI tract and heart, provide an additional pathophysiological mechanism whereby peripheral organ dysfunction may contribute to poor resuscitation outcome.

## 2. Materials and methods

Polyclonal anti-PERK antiserum was a gift from David Ron (New York University, NY) and has been previously described [8]. Phospho-specific PERK antisera [PERK(P)]

was purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Antisera for eIF2 $\alpha$  antibody specific for serine-51 phosphorylated eIF2 $\alpha$  were purchased from Biosource International (Camarillo, CA). Antisera for CHOP (GADD153) and ATF4 were purchased from Santa Cruz Biotech (Santa Cruz, CA). 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), and standard critical care cardiopulmonary resuscitation was used to reperfuse animals by methods we have previously described [10,11].

Male Long Evans rats weighing 250–300 g were anesthetized with intraperitoneal ketamine (80 mg/kg) and xylazine (10 mg/kg). Femoral vascular access was established for arterial pressure monitoring and intravenous fluid and drug administration. All animals were maintained normothermic ( $37 \pm 0.5$  °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. The mechanism of circulatory arrest is complete interruption of venous return due to the increased intrathoracic pressure. Following a 10 min duration of cardiac arrest, mechanical ventilation began and manual chest compressions were delivered at a rate of 200–300 per minute, until establishment of return of spontaneous circulation (ROSC). The effectiveness of manual chest compression rate was determined by monitoring MAP increase during CPR. Reperfusion was defined as a sustained mean arterial pressure  $>60$  mmHg. Mechanical ventilation was with 100% O<sub>2</sub> 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<sub>2</sub>O. Mechanical ventilation was provided for the entire reperfusion duration. Dopamine infusion at 10  $\mu$ g/(kg min) was used as necessary to maintain MAP  $>60$ .

L-NAME was administered intravenously at a concentration of 0.6 g/ml in sterile saline. Following L-NAME administration, animals were kept for 5 min before inducing ischemia. Experimental groups ( $n = 3$  per group) were: non-ischemic controls (NIC), 10 min ischemia and 10 min reperfusion (10R), 10 min ischemia and 10 min reperfusion plus 200 mg/kg L-NAME (10RN), 10 min ischemia and 90 min reperfusion (90R), and 10 min ischemia and 90 min reperfusion plus 200 mg/kg L-NAME (90RN).

## 2.2. Organ tissue preparation

At the indicated reperfusion times, animals were decapitated, placed on ice, and brain, descending colon, duodenum, heart, kidney, liver, lung, pancreas and skeletal muscle were immediately dissected. Brain was further dissected on ice to give brainstem (all area below the inferior colliculus), midbrain (including the inferior and superior colliculi), cerebellum, thalamus, hippocampus and cerebral cortex. For heart, the entire left ventricle was dissected and used for further studies. For skeletal muscle, 1 g was taken from lateral thigh muscles, including the vastus lateralis and tensor fasciae latae. For descending colon, duodenum, kidney, lung, liver and pancreas, each was excised in its entirety.

Brain regions were homogenized (1:5, wt:vol) in buffer described below by sonication and unfractionated homogenates were used for further studies. Peripheral tissues were minced on ice and then homogenized (1:5, wt:vol) on ice in a ground glass mechanical homogenizer in buffer consisting of 1% Triton-X-100, 50 mM HEPES pH 7.5, 10% glycerol, 150 mM NaCl, 1 mM EDTA, 10 mM tetrasodium pyrophosphate, 100 mM NaF, 17.5 mM  $\beta$ -glycerophosphate, 1:42 dilution of Sigma protease inhibitor cocktail (P8340), 2  $\mu$ M okadaic acid and 0.04% trypsin inhibitor. Following mechanical grinding, peripheral tissue homogenates were sonicated on ice with five brief pulses. Insolubles were removed by centrifugation at 14,000 rpm for 10 min at room temperature and the resulting supernatants were used for further studies. Protein concentrations of all samples were determined by the Folin phenol reagent method. All samples were then stored at  $-80^{\circ}\text{C}$  until used.

## 2.3. Treatment of NB104 cells

Neuroblastoma NB104 cells were cultured using published procedures [12]. Cells were maintained in complete media (CM) containing 1:1 DMEM to Ham's F-12, 14.2 mM  $\text{Na}_2\text{CO}_3$ , 15 mM HEPES, 2.5% fetal calf serum and 5% horse serum. Cells were left undifferentiated, or differentiated for 48 h in CM containing 1 mM dibutyryl-cAMP and 1 mM theophylline. For experimental runs, cells were pre-equilibrated in DMEM for 15 min. Differentiated or undifferentiated NB104 cells were treated with 2  $\mu$ M thapsigargin (tg), an agent that induces ER stress [8], or 1 mM of the NO donor *S*-nitroso-*N*-acetyl-penicillamine in DMEM for 3 h. After treatments, cells were scraped, pelleted by  $500 \times g$  centrifugation at  $4^{\circ}\text{C}$ , washed three times in PBS containing 1 mM EDTA, and then lysed by sonication in the above homogenization buffer. Cell lysate protein concentrations were determined by the Folin phenol reagent method, and lysates were stored at  $-80^{\circ}\text{C}$  until used.

## 2.4. Immunoprecipitation and Western blotting

PERK(P) immunoprecipitation of organ samples, and PERK immunoprecipitation of cell lysates were accom-

plished as previously described [13]. For PERK(P) immunoprecipitation, 30 mg of organ homogenate protein was used per reaction. Western blotting was performed as previously described [11]. For eIF2 $\alpha$ , eIF2 $\alpha$ (P), CHOP and ATF4 Western blots, 125  $\mu$ g of homogenate protein per lane was run on SDS-PAGE, and electroblot transferred to nitrocellulose as previously described [10]. For Western blotting, primary antisera dilutions were: eIF2 $\alpha$ , 1:1000; eIF2 $\alpha$ (P), 1:750; PERK(P), 1:2000; PERK, 1:10,000; ATF4, 1:200; CHOP, 1:400.

## 2.5. Quantitation and statistics

Western blots were quantitated by scanning densitometry (Bio Image Intelligent Quantifier, Version 3.1). Groups were compared by one way analysis of variance and Tukey HSD post hoc with alpha set at  $p < 0.05$ . Power analysis was conducted to detect a 50% difference between the largest and smallest means for each organ set with  $\beta > 0.2$  (Systat 10, Systat Software, Inc., 2002).

## 3. Results

### 3.1. Levels of eIF2 $\alpha$ (P)

Fig. 1 shows eIF2 $\alpha$ (P) immunoblots for all brain regions and peripheral organs in nonischemic controls or following 10 or 90 min reperfusion after a 10 min cardiac arrest, with and without L-NAME. Scanning densitometry results are depicted graphically in Fig. 2. Table 1 summarizes the numerical increases in eIF2 $\alpha$ (P) in each brain region or organ, and provides ANOVA  $p$  values, and power analysis values.

All brain regions showed large increases in eIF2 $\alpha$ (P) at 10R, ranging from 7-fold NIC in brainstem to 30-fold NIC in cerebellum. At 90R there was an average decrease of eIF2 $\alpha$ (P) to 45% of the 10R levels.

Kidney homogenates showed a very large eIF2 $\alpha$ (P) increase of 20-fold control at 10R, which returned to control levels by 90R. GI tract, including duodenum and colon showed the second highest level of eIF2 $\alpha$ (P) at 10 min reperfusion, at 14-fold controls. Of all peripheral organs tested, only duodenum showed a statistically significant 3.8-fold increase over controls at 90 min reperfusion. Heart showed a 5-fold increase at 10R that also decreased back to control levels at 90R. Liver showed an average 10-fold increase in eIF2 $\alpha$ (P), but the large dispersion in liver eIF2 $\alpha$ (P) levels prevented this set from clearing statistically (ANOVA  $p = 0.25$ ). However, in both the 10R and 10RN liver groups, specific samples showed definite eIF2 $\alpha$ (P) increases, and further, PERK(P) increased in 10R liver (see below). Together these data suggested that there is an endogenous variability of PERK activation in ischemic and reperfused liver. In lung, pancreas and skeletal muscle, eIF2 $\alpha$ (P) did not change following ischemia and reperfusion.

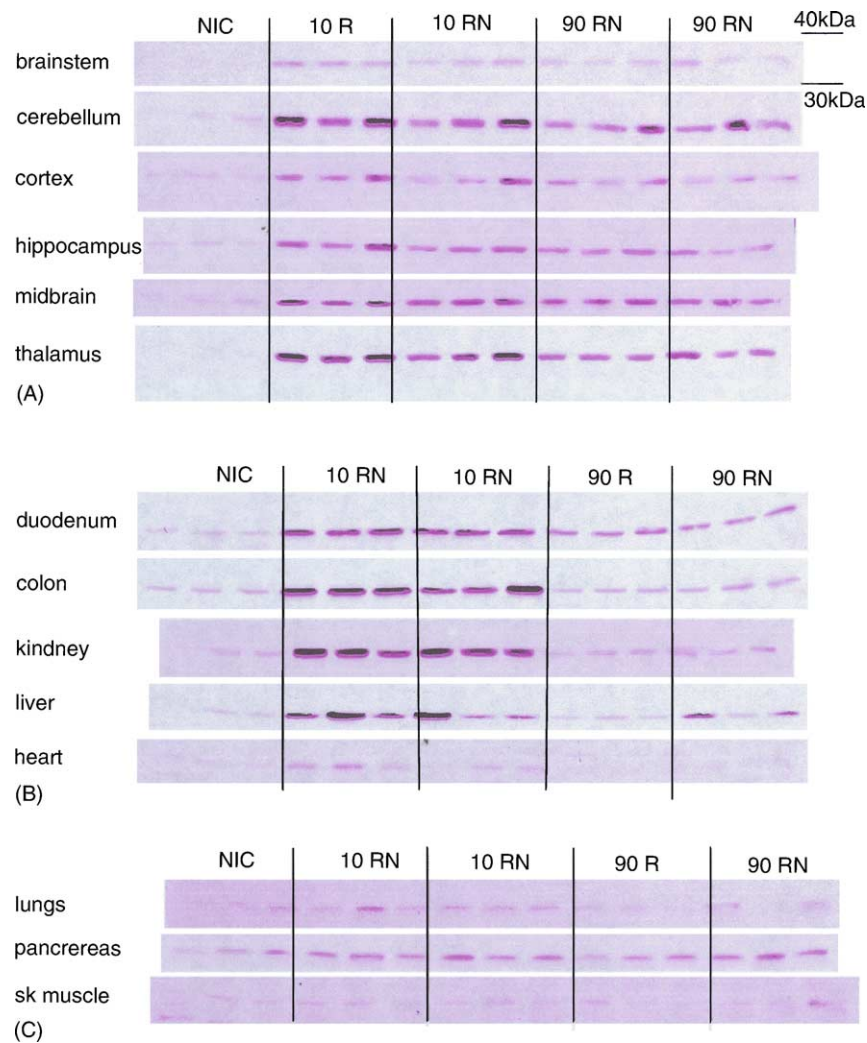


Fig. 1. Western blots of eIF2 $\alpha$ (P) following ischemia and reperfusion, and in the presence or absence of L-NAME. Data is presented in terms of (A) brain regions, (B) peripheral organs that underwent eIF2 $\alpha$  phosphorylation in response to ischemia and reperfusion and (C) peripheral organs that showed no change in eIF2 $\alpha$ (P) following ischemia and reperfusion. For the organs shown in (B), duodenum, colon and kidney were reactive, and liver and heart were moderately reactive with respect to eIF2 $\alpha$  phosphorylation. Abbreviations: NIC, nonischemic control; 10R, 10 min ischemia and 10 min reperfusion; 10RN, 10 min ischemia and 10 min reperfusion plus 200 mg/kg L-NAME; 90R, 10 min ischemia and 90 min reperfusion; 90RN, 10 min ischemia and 90 min reperfusion plus 200 mg/kg L-NAME. Molecular weight marker positions are shown for brainstem in (A) and apply to all blots shown.

The L-NAME treatment protocol used here is based on that of Niiro et al. [37] who showed that L-NAME treatment completely abated NO production in ischemic brain, as measured by an NO sensitive electrode. Before induction of ischemia, animals not administered L-NAME showed mean arterial pressures (MAP) averaging  $95.7 \pm 8.7$  mmHg, whereas those administered L-NAME showed an average MAP of  $150.3 \pm 6.5$  mmHg ( $p$  Student's  $t$ -test =  $3 \times 10^{-10}$ ), indicating physiologic inhibition of NO production in L-NAME-treated animals. During resuscitation, L-NAME had no effect on time to ROSC ( $74.2 \pm 41$  s for untreated animals versus  $45.8 \pm 12$  s for L-NAME treated animals,  $p$  Student's  $t$ -test = 0.08). In every organ in which eIF2 $\alpha$ (P) increased following ischemia and reperfusion, there was no effect of L-NAME treatment on eIF2 $\alpha$ (P) levels at either 10 or 90 min reperfusion (Figs. 1 and 2, and Table 1).

To determine if the amount of eIF2 $\alpha$  in organ homogenates was related to the degree of eIF2 $\alpha$  phosphorylation, quantification of eIF2 $\alpha$  was performed in peripheral organs and normalized to cerebral cortex eIF2 $\alpha$  levels (Fig. 3). Table 2 lists the numerical level of eIF2 $\alpha$  in the various organs relative to cerebral cortex. These ranged from  $\sim 20\%$  of cerebral cortical levels in heart and skeletal muscle to 10-fold cortical levels in pancreas. Indeed, the relative levels of eIF2 $\alpha$  appeared to correlate with the protein synthesis demands of each organ, with liver (3.4 $\times$ ), kidney (4.2 $\times$ ) and pancreas (10 $\times$ ) showing the highest levels of eIF2 $\alpha$ . However, as shown in Fig. 3B, there was no correlation between relative amounts of eIF2 $\alpha$  and the degree of eIF2 $\alpha$ (P) produced in a given organ following ischemia and reperfusion (Pearson's correlation coefficient =  $-0.12$ ).

### 3.2. PERK activation following ischemia and reperfusion

As we showed previously PERK activation in reperfused brain [11,13], here we assessed the amount of phosphorylated PERK [PERK(P)] in peripheral organs following ischemia and reperfusion. PERK becomes activated by autophosphorylation, therefore the standard marker of PERK activation is detection of increased PERK(P) [8]. The PERK(P) results were indistinguishable with or without L-NAME (data not shown), therefore only PERK(P) immunoprecipitations from untreated animals are shown here. Following immunoprecipitation using a phospho-specific PERK anti-

sera, only kidney, liver and pancreas showed evidence of PERK(P) (Fig. 6A). PERK(P) was not detected in duodenum, descending colon, heart, skeletal muscle or lung in any experimental group (Fig. 6A). Fig. 6B shows changes in the levels of PERK(P) in kidney, liver and pancreas. At 10R, kidney showed a 9-fold increase (ANOVA  $p=0.008$ ), and liver a 5-fold increase ( $p=0.03$ ) in PERK(P) compared to NICs. PERK(P) returned to NIC levels in the 90R groups of both liver and kidney. In pancreas, there was no change in PERK(P) levels in any experimental groups (ANOVA  $p=0.17$ ).

Through eIF2 $\alpha$  phosphorylation, PERK activation can lead to a paradoxical increase in translation of two tran-

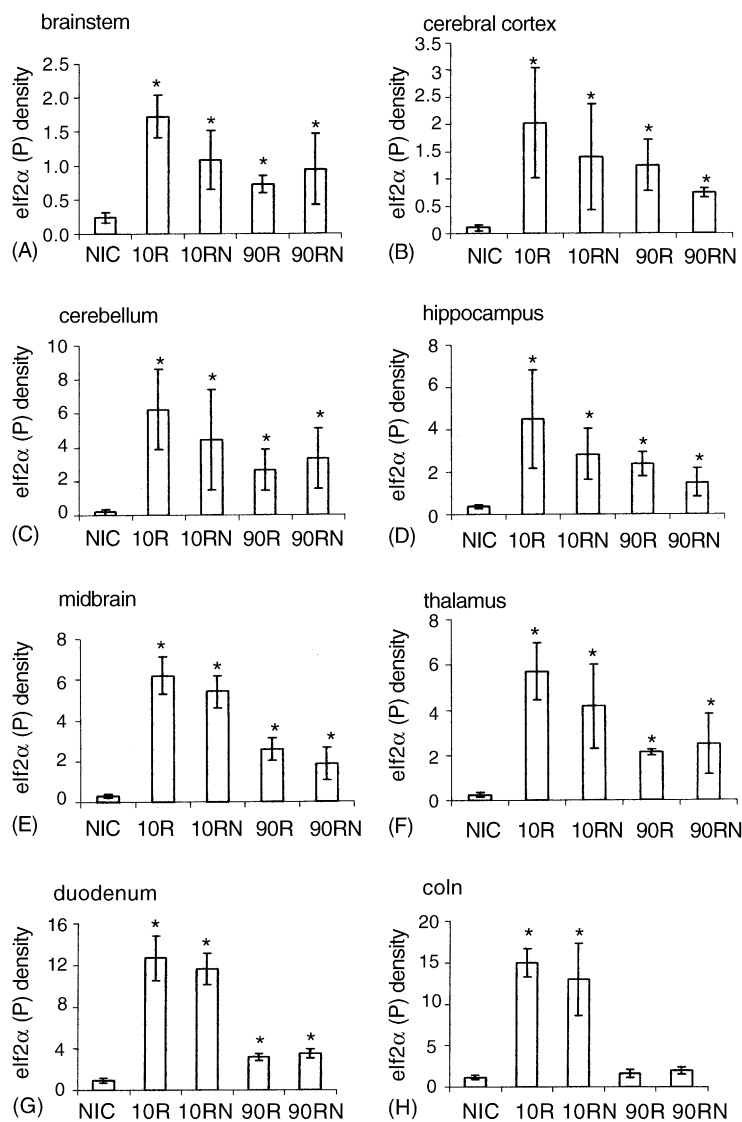


Fig. 2. Scanning densitometry of the eIF2 $\alpha$ (P) blots shown in Fig. 1. All graphs show mean  $\pm$  standard deviation of optical density (in arbitrary units, Y-axis). Asterisk (\*) indicates post hoc  $p < 0.05$  as compared to nonischemic controls (NIC). Note that L-NAME had no effect on eIF2 $\alpha$ (P) levels in any brain region or peripheral organ. Graphs A–F are brain regions. Graphs G–K are peripheral organs that underwent eIF2 $\alpha$  phosphorylation in response to ischemia and reperfusion. Note that for colon, kidney, liver and heart, but not duodenum, eIF2 $\alpha$ (P) levels returned to NIC values by 90 min reperfusion. Graphs L–N are peripheral organs in which eIF2 $\alpha$ (P) levels did not change in response to ischemia and reperfusion. Table 2 provides a summary of the graphs of Fig. 3 along with further statistical analysis. Abbreviations are the same as in Fig. 1.

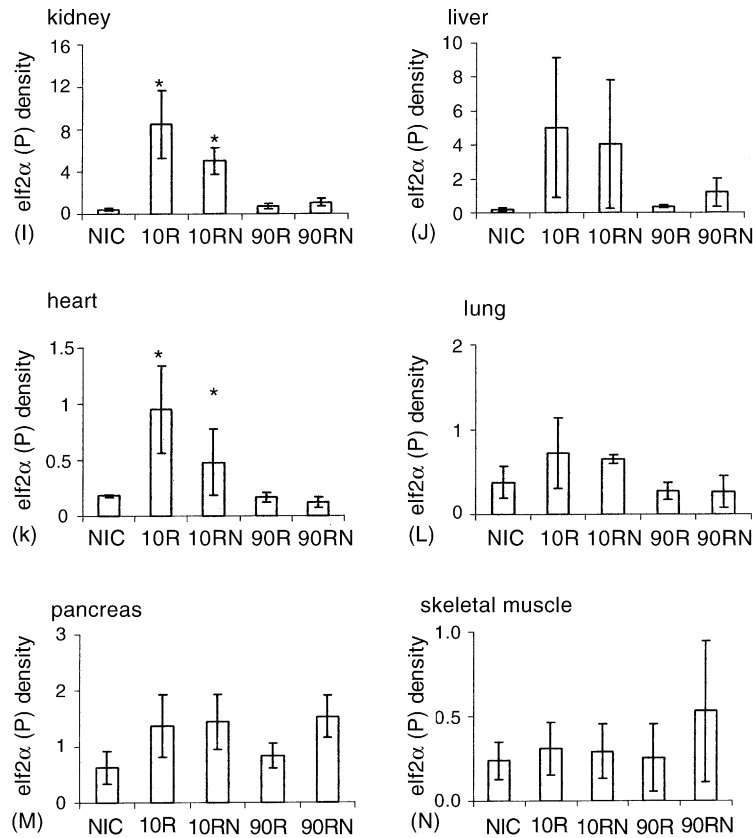


Fig. 2. (Continued).

scription factors, ATF4 and CHOP, via a mechanism termed “by-pass scanning” [14]. When immunoblots were performed for ATF4 and CHOP on organ homogenates, we observed neither ATF4 nor CHOP in any organ of any experimental group (representative immunoblots are shown in Fig. 4).

### 3.3. Effect of SNAP on NB104 cells

When treated with 1 mM SNAP for 3 h, both differentiated and undifferentiated NB104 cells showed increased PERK(P) similar to that seen with thapsigargin treatment (Fig. 5 A and B, respectively). Further, the cells showed

Table 1

Levels of eIF2α(P) following ischemia and reperfusion in various brain regions and peripheral organs

Brain region or organ	10 min eIF2α(P)	90 min eIF2α(P)	Effect of L-NAME	ANOVA <i>p</i>	Power
Brainstem	7×	40% Decrease	None	0.004	0.986
Cerebellum	30×	44% Decrease	None	0.037	0.999
Cerebral cortex	20×	53% Decrease	None	$6.8 \times 10^{-6}$	0.995
Hippocampus	12×	48% Decrease	None	$1.1 \times 10^{-4}$	0.989
Midbrain	21×	32% Decrease	None	$1.3 \times 10^{-9}$	0.996
Thalamus	24×	53% Decrease	None	$1.6 \times 10^{-4}$	0.984
Duodenum	14×	73% Decrease	None	$3.7 \times 10^{-5}$	0.999
Colon	14×	Control level	None	0.0012	0.955
Heart	5×	Control level	None	0.0094	0.922
Kidney	20×	Control level	None	$3.8 \times 10^{-4}$	0.999
Liver	10×	Control level	None	0.25	0.686 (4)
Lung	nc	nc	None	0.475	0.244 (36)
Pancreas	nc	nc	None	0.364	0.364 (7)
Skeletal muscle	nc	nc	None	0.5711	0.244 (22)

Column 2 lists the X-fold increase of eIF2α(P) over nonischemic controls. Column 3 lists the reduction from the 10R level shown in column 2. Column 4 lists the effect of L-NAME on eIF2α(P) levels. Column 5 lists the ANOVA *p* values. For brain regions and duodenum, post hoc testing showed significant *p* values for the 10R and 90 groups, with and without L-NAME, vs. the NIC groups. For colon, heart and kidney, the 10R groups were statistically different from the NIC and 90R groups, the latter were not statistically different. Column 6 lists power values to detect a 50% change between the lowest (NIC) and highest (10R) means. Where power was <0.8, the *n* to achieve power >0.8 is shown in parenthesis. Abbreviations: nc, no change.

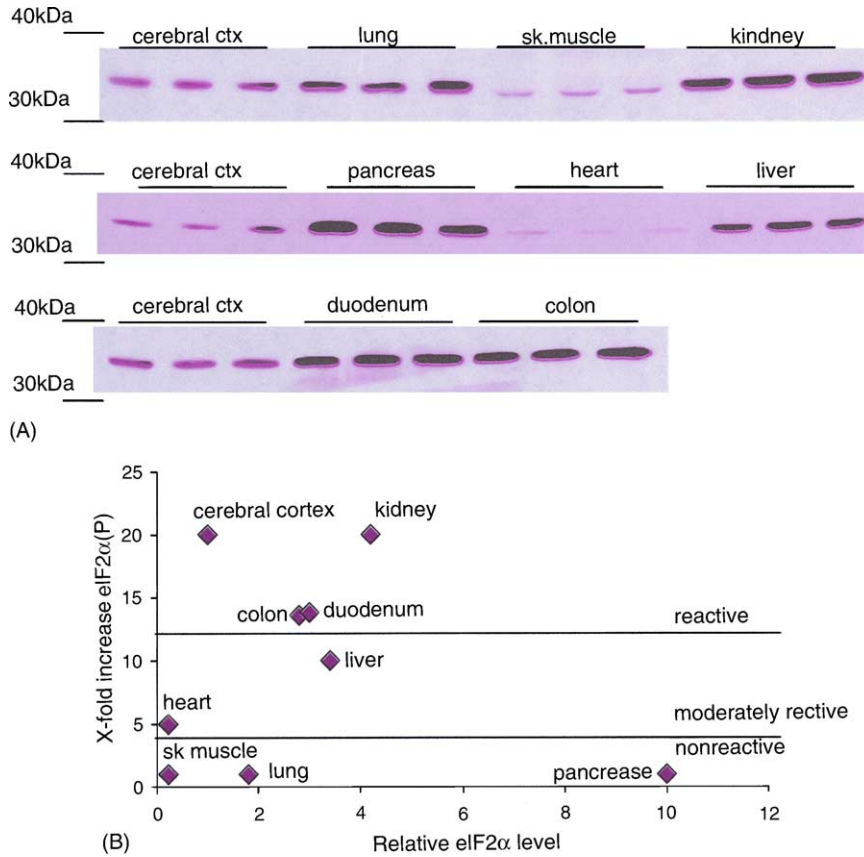


Fig. 3. Relative levels of eIF2α in peripheral organs as compared to cerebral cortex. (A) One hundred and twenty-five micrograms of the respective tissue homogenates were run on SDS-PAGE and immunoblotted for eIF2α. Quantitation of relative levels of eIF2α is given in Table 2. The organs with the greatest protein synthesis requirements (pancreas, liver and kidney) showed the highest relative levels of eIF2α. Molecular weight marker positions are shown at left for all blots. (B) Correlation analysis between the amount of eIF2α in a given organ (expressed such that cerebral cortex is 1.00) and the degree of eIF2α phosphorylation following 10 min ischemia and 10 min reperfusion (expressed as X-fold increase over controls) showed no correlation between these variables (Pearson’s correlation coefficient = -0.12). This graph also illustrates the three categories of organ response: reactive organs showed ~15 to 20-fold increase in eIF2α(P), moderately reactive organs showed <10-fold increase in eIF2α(P), and nonreactive organs showed no increase in eIF2α(P). These patterns of reactivity are independent of the levels of eIF2α.

increased eIF2α(P) that correlated with the appearance of PERK(P), whether induced by SNAP or thapsigargin. Hence, in this cell culture system, NO activated PERK similar to thapsigargin.

#### 4. Discussion

Using PERK activation and eIF2α phosphorylations as markers of ER stress [8], we have evaluated a number of

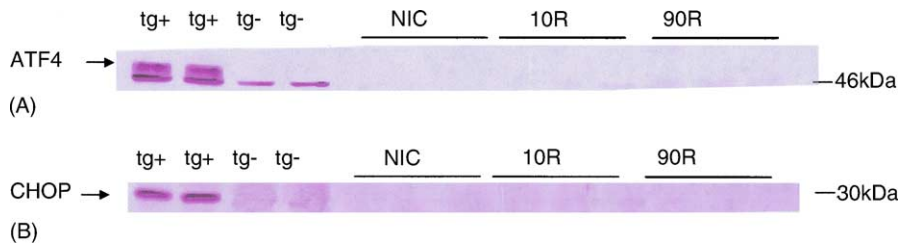


Fig. 4. ATF4 and CHOP were not detected in homogenates of any peripheral organs out to 90 min reperfusion following 10 min complete ischemia. (A) Representative ATF4 Western blot of 125 μg left ventricle homogenates. While ATF4 was readily produced in NB104 cells following thapsigargin treatment (tg+), ATF4 was not detected in untreated NB104 cells (tg-) or in nonischemic control (NIC), or following 10 min reperfusion (10R) or 90 min reperfusion (90R) after 10 min complete ischemia. (B) Representative CHOP Western blot in 125 μg of kidney homogenates. Again, CHOP was readily detected in thapsigargin treated NB104 cells, but not in any of the kidney experimental groups. ATF4 and CHOP were not detected in any organ tested in the study.

Table 2  
Relative eIF2 $\alpha$  levels in various organs compared to cerebral cortex

	Relative eIF2 $\alpha$ level
Cerebral cortex	1.00
Colon	3.00
Duodenum	2.80
Heart	0.23
Kidney	4.20
Liver	3.40
Lung	1.80
Skeletal muscle	0.22
Pancreas	10.00

brain regions and peripheral organs for evidence of ER stress, along with the potential contribution of NO, following cardiac arrest-induced whole body ischemia. Lung, pancreas and skeletal muscle did not show evidence of ER stress with the ischemic durations used in the present study, whereas GI tract, kidney, liver and heart did demonstrate evidence of cell stress. Compared to brain, two salient features emerge from these results: (1) eIF2 $\alpha$  (P) abated in kidney, liver and heart, but not GI tract, by 90 min reperfusion, and (2) the response of kidney at 10 min reperfusion was on the same order as that of brain, GI tract was about 25% less, and liver and heart were over 50% less. These results are of interest for (1) discerning the magnitude of ER stress responses in different tissues to the ischemia and reperfusion conditions used in the present study, and (2) suggesting that cellular stress, as marked by eIF2 $\alpha$  phosphorylation, in GI tract, kidney, liver and heart following cardiopulmonary resuscitation may have consequences for outcome success.

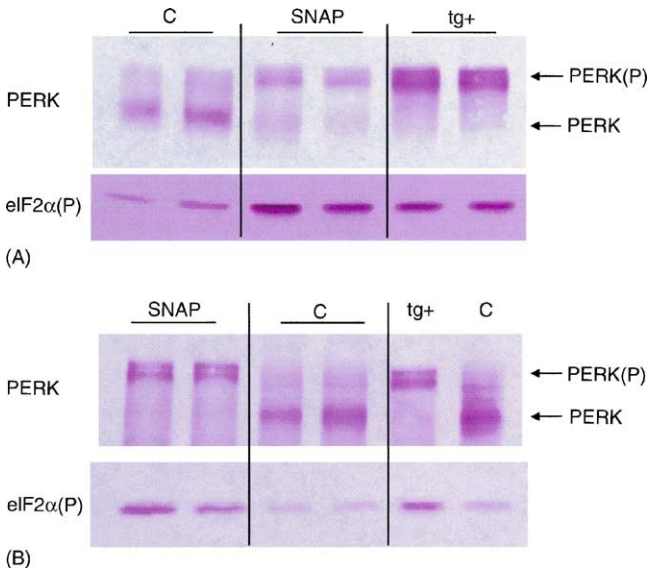


Fig. 5. SNAP activated PERK and phosphorylated eIF2 $\alpha$  in (A) differentiated and (B) undifferentiated NB104 cells. Following 3 h treatment in either SNAP or thapsigargin (tg+), NB104 cells underwent activation of PERK [PERK(P)] and phosphorylation of eIF2 $\alpha$ . In contrast, untreated control cells C showed predominantly inactive PERK, and lower levels of eIF2 $\alpha$ (P). Hence, nitric oxide (NO) activated PERK similarly to thapsigargin in cultured cells.

Several studies have indicated that NO can induce ER stress. Inhibition of protein synthesis [15,16] increased eIF2 $\alpha$ (P) [15], CHOP induction and apoptosis [16–18], depletion of ER Ca<sup>2+</sup> stores [16], and activation of another marker of ER stress, ATF6 [19] all occurred following increased NO in cell culture systems. To our knowledge, ours is the first demonstration of PERK activation following increased NO exposure of cells. The exact mechanism by which NO causes ER stress is unknown, but studies suggest that NO alters ER Ca<sup>2+</sup> fluxes [16,17]. However, in spite of PERK activation and eIF2 $\alpha$  phosphorylation following SNAP administration to cultured neuroblastoma cells, attenuation of NO production in vivo, as evidenced by increased MAP following L-NAME treatment, had no effect on eIF2 $\alpha$ (P) formation or PERK activation. We would expect if NO was an important proximal event in PERK activation following ischemia and reperfusion, that L-NAME treated animals would at least show a detectable decrease in eIF2 $\alpha$  phosphorylation. For all brain regions, and for GI tract, heart and kidney, the present study had >90% power to detect a 50% decrease in eIF2 $\alpha$ (P) formation from peak levels at 10R (Table 1), but no decrease was observed in L-NAME treated samples. Thus, we conclude that NO production is not the primary physiologic contributor to organ ER stress following ischemia and reperfusion.

We have divided the response of the various organs into three categories, reactive, moderately reactive and non-reactive (Fig. 3) for the following reasons. The responses of brain and kidney were robust and consistent: these organs showed high levels of eIF2 $\alpha$ (P) formation and clear evidence of PERK activation. GI tract showed robust eIF2 $\alpha$  phosphorylation, but we were unable to detect PERK in GI tract tissue. Liver and heart were less consistent in their demonstration of ER stress. Liver showed a large intra-sample variation for eIF2 $\alpha$ (P) formation, but showed clear evidence of PERK activation. Heart showed consistent eIF2 $\alpha$ (P) increases at 10R, but we were unable to detect PERK(P) in reperfused left ventricle (Fig. 6). Thus, it remains an open question if PERK at very low levels, or some other eIF2 $\alpha$  kinase, is activated in GI tract and heart following I/R. Nonetheless, it is intriguing that kidney and liver, the two peripheral organs showing PERK activation, are the organs often associated with dysfunction following cardiac arrest and shock [20].

Of all organs tested, only brain and GI tract continued to display ER stress at 90 min reperfusion. Other studies indicate that eIF2 $\alpha$  phosphorylation in brain persists for perhaps 6–8 h following ischemia durations typical of brain I/R studies [11,21]. The rapid reversibility of eIF2 $\alpha$  phosphorylation in kidney, liver and heart suggests that the 10 min ischemia duration in the present study was a relatively weak stress stimulus in these organs. However, our results are proof of the principle that cell stress occurs in these organs following I/R. Longer durations of ischemia associated with longer CPR, or that are typical of transplantation surgeries [22], would be expected to produce more intense cell stress in reactive organs. Thus, understanding the consequences of I/R-induced

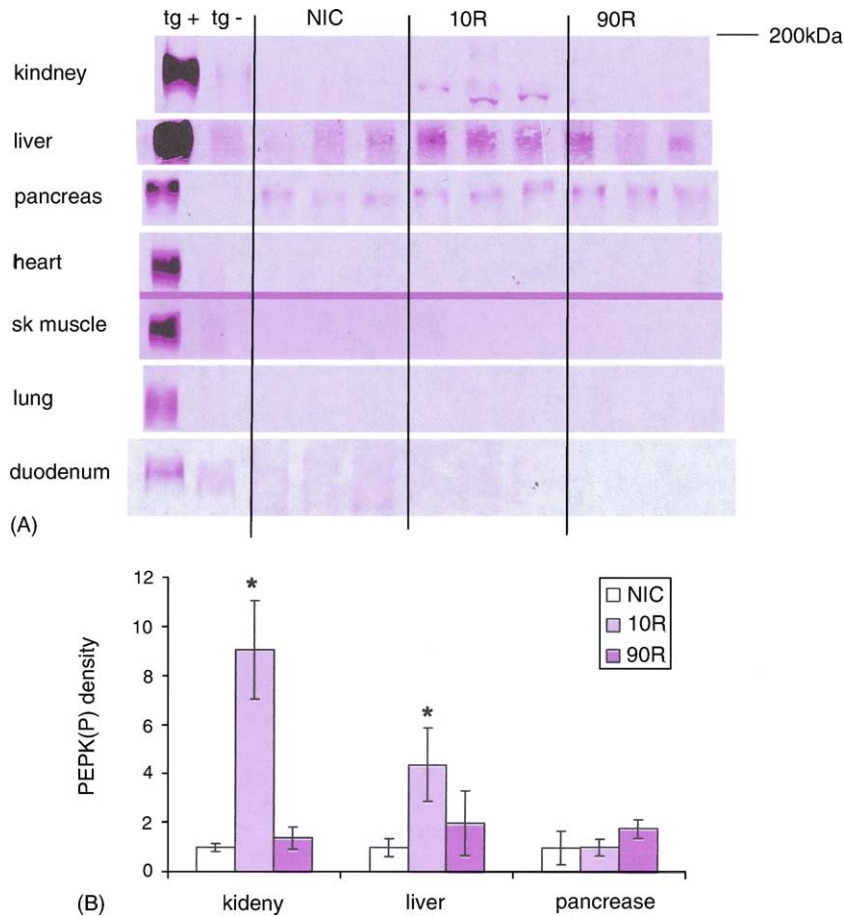


Fig. 6. Phosphorylation of PERK following ischemia and reperfusion of peripheral organs. (A) Western blots of immunoprecipitated phosphorylated PERK [PERK(P)] from organ homogenates as labeled. Positive control PERK(P) was from thapsigargin treated NB104 cells (tg+) and negative controls were from untreated NB104 cells (tg-). Kidney and liver homogenates showed increased PERK(P) at 10 min reperfusion. Pancreas homogenates showed constitutively high levels of PERK(P) that did not change with ischemia and reperfusion. PERK(P) was not detected in heart, skeletal muscle, lung or duodenum. (B) Scanning densitometry (mean  $\pm$  standard deviation) of kidney, liver and pancreas PERK(P) immunoblots shown in (A). Kidney showed a 9-fold increase ( $p=0.008$ ) and liver a 4.4-fold increase ( $p=0.03$ ) in PERK(P) at 10 min reperfusion (10R), as compared to nonischemic controls (NIC). PERK(P) in kidney and liver returned to control values at 90 min reperfusion (90R). Pancreas showed no change in PERK(P) levels ( $p=0.17$ ). Molecular weight marker position is shown for kidney and applies to all blots shown.

peripheral organ cell stress may contribute to improvements of functional outcome in the clinical settings of both CPR and transplantation. Further, it is possible that longer durations of ischemia activate ER stress in lung, pancreas and skeletal muscle, a possibility requiring further study.

We note that the responses of each tissue were independent of the ER stress machinery present in each tissue. Other investigators have quantified the relative levels of PERK mRNA and protein in various body organs. Shi et al. [23] showed PERK mRNA levels as: pancreas > lung > kidney  $\sim$  liver > brain > heart  $\gg$  skeletal muscle. Harding et al. [24] evaluated the expression of total PERK protein as: pancreas  $\sim$  lung > liver > kidney  $\sim$  brain  $\gg$  skeletal muscle. These results are consistent with our evaluation of eIF2 $\alpha$  levels (Fig. 3 and Table 2): pancreas  $\gg$  kidney  $\sim$  liver  $\sim$  GI tract > lung  $\sim$  cerebral cortex  $\gg$  heart  $\sim$  skeletal muscle. To our knowledge there is no report on PERK mRNA or protein levels in GI tract.

Thus, pancreas, the organ in which PERK was first discovered [23], clearly possess the greatest quantity of ER stress machinery, and is also the organ with perhaps the greatest protein synthesis demands, but it failed to demonstrate ER stress following ischemia and reperfusion. Likewise, lung also has high levels of PERK [24], but was also nonreactive under our I/R conditions. Two reactive organs, brain and kidney, are alike in having high ATP demands for the continued function of the Na<sup>+</sup>-K<sup>+</sup>-ATPase to maintain various transmembrane gradients, and both organs are functionally dependent on continuous blood flow. Like kidney, the GI tract is dependent upon the Na<sup>+</sup>-K<sup>+</sup>-ATPase to maintain transmembrane gradients used for transcellular transport, and in spite of undetectable levels of PERK, GI tract also showed a large production of eIF2 $\alpha$ (P). Interestingly, heart and skeletal muscle, two functionally similar tissues, show relatively low levels of ER stress machinery, in spite of abundant sarcoplasmic reticulum (SR), and heart demon-

strated a moderate cell stress response, but skeletal muscle did not.

Taken together, these observations indicate that it is not the amount of ER stress machinery present in a tissue, nor the relative proteins synthesis demands of a tissue that determines that tissue's response to I/R. More likely, there are tissue specific adaptations of the ER stress machinery that determine its activation following I/R. For example, the moderate response of heart and nonreactivity of skeletal muscle likely reflect the evolutionary modification of ER to SR, with its very high  $\text{Ca}^{2+}$  fluxes, leading to modification in regulation of the ER stress machinery. Similarly, the ER stress machinery in pancreas has been hypothesized to have been modified to meet the cyclical nutrient demands of insulin synthesis [25], which may preclude it from responding to ischemic stress in the typical fashion.

The role of ischemic ATP levels and reperfusion organ blood flow are likely to be complex contributing factors because: (1) visceral organs can reasonably tolerate up to 1 h ischemia under optimal conditions and retain function [3], and (2) low flow states have different effects on different viscera, with some able to meet tissue needs at as low as 20% flow (e.g. pancreas [26]). Brain ATP falls essentially to zero by 5 min ischemia [27] and renal ATP levels were 12% of controls at 10 min renal artery ligation [28]. Liver ATP levels drop to 25% control at 10 min ischemia [29]. Thus, the presence or absence of ER stress does not appear to correlate with absolute ATP levels, but the degree and duration of ER stress appears to roughly correlate with relative drops in ATP.

Following resuscitation, it is well appreciated that endogenous reflexes shunt blood to the main survival organs brain and heart, and catecholamine-mediated vasoconstriction severely limits visceral organ reperfusion [30]. For example, post-resuscitation blood flow to pancreas is well below pre-arrest baseline [31,32], and renal reperfusion ranged from 10 to 50% pre-arrest values [33,34]. It is possible that a constellation of factors, including ischemic ATP levels, other forms of ischemia-induced damage (e.g. protease activation, arachidonate production), CPR blood flows, post-resuscitation blood flows, post-resuscitation ATP levels and other concurrent damage mechanisms (e.g. free radical production) may contribute to the observed ER stress responses. Clearly, further detailed studies are required to tease apart the factors responsible for differential displays of ER stress in ischemic and reperfused body organs.

With respect to peripheral contributions to resuscitation outcome, the model of “self-intoxication” has guided research in this area [4,35]. The self-intoxication model suggests that collective dysfunction of peripheral organs responding to ischemia and reperfusion may contribute to poor neurological outcome following resuscitation. However, one detailed study to evaluate this model noted trends, but did not find correlations between visceral organ function and neurological outcome [36]. The present results do not contribute to a systemic view as offered by the self-intoxication model,

but instead provide a further mechanism—ER stress—that may or may not contribute to individual organ dysfunction following ischemia and reperfusion. However, it is of interest to note that the self-intoxication model posits the GI tract, particularly the intestines, as the “motor” or “engine” of multiorgan failure following shock and trauma [3], and in the present study, the duodenum was the only peripheral organ to display levels of eIF2 $\alpha$ (P) higher than controls at 90 min reperfusion. As eIF2 $\alpha$  phosphorylation is generally associated with cell stress, our result suggests that the GI tract experienced the strongest cell stress of all the peripheral organs tested in the present study. While our aim here was not to test the self-intoxication model, our result is consistent with an enhanced sensitivity of the GI tract to I/R-induced cell stress. The relationship between cellular stress in the GI tract, eIF2 $\alpha$ (P) phosphorylation, and the intestines as proximal mediators of organ dysfunction clearly merits further detailed study.

## 5. Conclusion

The present study has investigated the ER stress response, using PERK activation and eIF2 $\alpha$  phosphorylation as markers, in specific brain regions and several peripheral organs following a clinically relevant duration of ischemia, and reperfusion via standard critical care CPR. The novel findings are: (1) two peripheral organs, kidney and liver, demonstrated evidence of graded ER stress, but lung, pancreas and skeletal muscle did not following I/R, (2) GI tract and heart, while not showing clear evidence of ER stress, clearly showed evidence of cell stress via increased eIF2 $\alpha$  phosphorylation and (3) organs showing increased eIF2 $\alpha$ (P) following I/R were refractory to physiologic changes in NO levels.

In the clinical setting of resuscitation from cardiac arrest, cell stress in GI tract, kidney, liver and heart may be contributing factors to post-resuscitation dysfunction of these organs. Further studies are needed to determine the extent to which ER stress affects post-resuscitation function in kidney and liver. The cause of increased eIF2 $\alpha$ (P) in heart and GI tract also merits further serious investigation. If indeed ER stress, or other types of cell stress, contribute to post-resuscitation dysfunction, then therapies aimed at ameliorating the effects of these stresses may contribute to improvement in resuscitation outcomes via improvements in both cerebral and affected peripheral organ function.

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