Calcium/calmodulin-dependent protein kinase IV limits organ damage in hepatic ischemia-reperfusion injury through induction of autophagy

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LIVER ISCHEMIA-REPERFUSION (I/R) injury is a complex process that results in organ damage as a consequence of several pathophysiological factors (44). It is encountered clinically during elective liver surgery, organ transplantation, and hypovolemic shock. Both direct tissue hypoxia and the subsequent proinflammatory immune response determine the ultimate extent of damage. Several pathogenic mechanisms have been identified, including activation of resident Kupffer cells, neutrophil recruitment, upregulation of proinflammatory cytokines, mitochondrial damage, hepatocyte apoptosis, and damage-associated molecular pattern (DAMP) signaling (1, 36, 44, 50). While the roles of Kupffer cells (23) and neutrophils (9, 32, 43) have been extensively studied, the role of DAMP molecules and their relationship to innate immune activation remains an active area of investigation. The prevailing paradigm that links DAMP signaling to organ damage involves the recognition of circulating endogenous molecules by pattern recognition receptors on immune cells, promoting a proinflammatory response. DAMP molecules, including S100 (31), ATP, urate (30), hyaluronic acid (52), and DNA (3), have also been shown to contribute to organ damage during I/R; additionally, our group has identified key roles for the nuclear protein high mobility group box 1 (HMGB1) (42), as well as histone proteins (21) as circulating DAMPs critical to I/R injury.

While several cellular responses after I/R contribute to organ damage, protective signaling pathways are also known to be activated. One such protective signaling mechanism that has gained increasing attention recently is autophagy. Autophagy is a process whereby cytoplasmic proteins are degraded or recycled in lysosomes and serve to remove long-lived or damaged organelles and provide energy substrates during acute cellular stress (29). While basal levels of autophagy are maintained to achieve homeostasis during normal physiological functioning, acute cellular stressors, including nutrient deprivation (49) and infection/sepsis (6), induce rapid and profound changes in autophagic signaling in hepatocytes. Autophagic signaling is also known to be regulated by oxidative stress (24), and this process has been well studied in cardiac I/R injury (18). However, the role of autophagy is less clear in hepatic I/R, as it has been implicated as both damaging and protective in separate studies (33). While questions regarding the function of autophagy in hepatic I/R remain unanswered, even less is known about the mechanisms that regulate autophagic signaling during I/R.

The calcium/calmodulin-dependent protein kinases (CaMKs) are a family of proteins activated in response to a variety of cellular stimuli. Members of the multifunctional CaMKs include CaMKI, CaMKII, and CaMKIV. CaMKI and CaMKIV are activated by upstream CaMK kinases (CaMKK) (39), while CaMKII is activated directly by calmodulin or by direct oxidation (14). While the physiological roles of the CaMKs have been extensively studied, their function in sterile inflammatory injury is not well delineated. Our group previously demonstrated the activation of CaMK signaling cascades in ischemic liver injury (41). However, the roles of individual CaMK isoforms in this process remain to be elucidated.

We undertook this study to investigate the function of CaMKIV in hepatic I/R injury and show that this protein serves primarily a protective role by activating the prosurvival process of autophagy in hepatocytes. CaMKIV knockout (KO) mice subjected to a model of warm liver I/R had significantly greater organ damage compared with wild-type (WT) control mice and displayed significantly lower levels of autophagy in the liver. We also found that induction of autophagy protects livers from I/R injury, and its restoration in CaMKIV KO mice reduces damage to WT levels. Lastly, we show that CaMKIV activates autophagy in vitro in hepatocytes and prevents oxidative stress-induced cell death.
MATERIALS AND METHODS

Reagents. cAMP response element binding protein (CREB) inhibitor was obtained from EMD. Transfection reagent and lactate dehydrogenase (LDH) assay kit were obtained from Roche. β-Actin and Flag M2 antibodies were obtained from Sigma. All other antibodies, including LC3B, beclin 1, phospho (p)-CREB (Ser133), CREB, and p-P70S6K, were purchased from Cell Signaling.

Animals. Male CaMKIV KO mice (Camk4tm1Tch/Camk4tm1Tch), CaMKK KO mice (Camkk2tm1Tch/Camkk2tm1Tch), and the WT counterparts C57BL/6J mice (8–12 wk old) were purchased from Jackson Laboratory (Bar Harbor, ME). The animals were maintained in a laminar-flow, specific pathogen-free atmosphere. The research project was approved by the University of Pittsburgh Animal Care and Use Committee and was in compliance with the National Institutes of Health Guidelines for the Use of Laboratory Animals.

Hepatic I/R. A nonlethal model of partial (70%) hepatic warm ischemia and reperfusion was used, as previously described (42). Mice between 10 to 12 wk old were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). A midline laparotomy was performed, the hepatic hilum was dissected, and an atrumatic vascular clamp was used to interrupt the portal circulation to the median and left lateral lobes of the liver. The abdomen was covered with a piece of saline-soaked gauze, and mice were placed on a heating pad (37°C). The clamp was subsequently removed after 60 min to initiate hepatic reperfusion. After 6 h of reperfusion, mice were anesthetized with isoflurane inhalation (Baxter Healthcare, Deerfield, IL), the abdomens and thoraces were opened, and whole blood samples were collected by cardiac puncture before the removal of the left and midliver lobes for protein analysis, reverse transcription (RT)-PCR, and histological analysis. Sham surgeries were performed by following similar procedure, but without vascular occlusion.

Isolation, culture, and treatment of hepatocytes. Mouse hepatocytes were isolated by a modified in situ collagenase (type IV, Sigma) perfusion technique, as previously described (47). Cells were seeded with Williams’ medium E containing 10% calf serum, 15 mM HEPES, 2 mM l-glutamine, and 100 U/ml penicillin and streptomycin. Cells per well (5 × 10^5) were seeded on six-well plates, and 1.5 × 10^5 cells were plated per dish on 60-mm cell culture dishes. Following 24-h incubation, cells were treated under normoxia (21% O2), 1% hypoxia, or 500 μM H2O2 at indicated time points. For experiments involving hypoxia, the medium was replaced with hypoxic medium (equilibrated with 1% O2, 5% CO2, and 94% N2) and placed into a modular incubator chamber (Billups-Rothenberg), which was flushed with the same hypoxic gas mixture. The cells were exposed to hypoxia for determination of induction of autophagy or LDH release in the supernatants.

Plasmid construction. A plasmid encoding a constitutively active CaMKIV (CaMKIV-dCT) was used in the study and was a gift from M. Rosengart. CaMKIV-dCT contains a COOH-terminally truncated CaMKIV (CaMKIV-dCT) was used in the study and was a gift from M. Rosengart.

Western blot analysis. Liver slice was minced with an electric homogenizer (Omini) with a midspeed at 4°C in 500 μl of lysis buffer (ThermoFisher) with protease inhibitors (Sigma) and phosphatase inhibitors (Pierce). Protein concentration was determined using a bicinchoninic acid protein assay (Pierce). Samples (50 μg protein) were separated onto SDS-polyacrylamide gels and probed with the following primary antibodies: rabbit polyclonal antibody to LC3; rabbit polyclonal antibody to beclin-1; p-CREB, CREB, p-P70S6K (Cell Signaling); mouse monoclonal antibody to FLAG; and rabbit polyclonal antibody to β-actin (Sigma). Blots were analyzed using the NIH image software. Data were normalized to β-actin.

Cell transfection. Murine hepatocytes at a concentration of 2.0 × 10^6 were seeded in 6-cm dishes. Hepatocytes were allowed to adhere overnight. Cells were washed and transfected for 6 h in Opti-Mem serum-free medium with small interfering RNA (siRNA) for CaMKIV (Santa Cruz Biotechnology) or a scrambled, control siRNA (Santa Cruz Biotechnology, sc-37007) using lipofectin transfection reagent (Invitrogen).

Liver damage assessment. Serum alanine aminotransferase (sALT) levels were determined by using the DRI-CHEM ALT Slides (Heska). The ALT values were expressed as international units per liter.

Histopathology. Formalin-fixed liver samples were embedded in paraffin and cut to 6-μm-thick sections. Tissues were stained with hematoxylin-eosin and assessed for inflammation and tissue damage.

RNA isolation and reverse transcription. Hepatocytes (5 × 10^5) well of six-well plates) were treated with appropriate experimental reagents for desired time periods. At the end of the experimental period, cells were washed two times with ice-cold PBS. Total RNA was isolated using the Qiagen RNeasy Mini Kit (Qiagen), according to the manufacturer’s protocol. Total RNA concentration was determined by absorbance at 260/280 nm using Nanodrop (ThermoFisher). The RT was carried out using the Sprint RT Complete-Double PrePrimed kit (Clontech) following the manufacturer’s instruction.

SYBR green real-time PCR. The real-time PCRs were carried out using the AB StepOnePlus real-time PCR system and the SYBR Green PCR Master Mix kit (Applied Biosystems), following thermal cycling conditions, as described by the manufacturer. Primers applied were LC3B and the housekeeping gene β-actin. Gene expression was normalized to β-actin mRNA content.

Transmission electron microscopy. Mice were subjected to warm I/R. At the end of 3 h of the reperfusion period, mice were perfused with cold PBS, then with 2% glutaraldehyde in 0.1 mol/l phosphate buffer (pH 7.4), and processed for transmission electron microscopy, as described previously (40). After dehydration, thin sections (70 nm) were stained with uranyl acetate and lead citrate for observation under a JEM 1011CX electron microscope (JEOL, Peabody, MA). Images were randomly selected from a pool of 18 fields throughout at least five grid squares under each condition. Quantification of autophagic vacuoles was based on the method described previously (15).

Statistical analysis. Results are expressed as the mean ± SE. Statistical analysis was performed using Student’s t-test or one-way ANOVA test. All statistical analyses were performed using Sigma Stat version 3.5 (Systat Software, Chicago, IL).

RESULTS

CaMKIV deficiency results in increased organ damage following liver I/R injury. To investigate the role of CaMKIV in an in vivo model of liver I/R, we first performed a time course using 60 min of ischemia and various points of reperfusion and measured CaMKIV activation by Western blot. CaMKIV was activated immediately after ischemia and was sustained throughout reperfusion up to 24 h (Fig. 1A).

Next, we subjected CaMKIV KO and WT mice to I/R and compared organ damage by measuring sALT levels and examining histopathology of liver sections. Hepatocyte injury in CaMKIV KO mice was significantly greater than that of the WT counterparts (Fig. 1B). Liver histology correlated the greater liver damage in CaMKIV KO mice with more severe sinusoidal congestion (Fig. 1C) and significantly greater necrotic areas (Fig. 1D). We found no significant activation of...
apoptosis as measured by cleaved caspase-3 in either WT or CaMKIV KO mice after I/R (Fig. 1E). To further confirm the function of CaMKIV in hepatic I/R, we used CaMKK KO mice and subjected them to I/R. CaMKK is directly upstream of CaMKIV, and we hypothesized that CaMKK KO mice would display a similar pattern of organ damage as CaMKIV KO mice. Similar to CaMKIV KO mice, CaMKK KO mice exhibited greater damage after I/R, as evidenced by higher systemic ALT concentrations, compared with WT mice (Fig. 1F).

Hepatocyte autophagy is deficient in CaMKIV KO mice after I/R. Our laboratory has previously reported that induction of autophagy is protective in the setting of hepatic I/R (7), and
deficient autophagic signaling has been shown to contribute to mitochondrial dysfunction in anoxic hepatocytes (25). Autophagy induction has been linked to CaMKK in a model of cardiac I/R (19), and since CaMKIV is a direct downstream substrate of CaMKKK, we hypothesized that autophagy might play a role in CaMKIV-mediated protection during I/R. We observed that autophagy, as measured by the conversion of LC3B-I to LC3B-II, increased in a time-dependent manner following I/R. However, CaMKIV KO mice had markedly lower levels of LC3B-II at all time points, suggesting that autophagic signaling was deficient in these mice compared with WT mice. In addition, levels of beclin 1, another protein involved in the induction of autophagy, were also lower in CaMKIV KO mice at all time points following I/R compared with WT mice (Fig. 2A).

To determine if CaMKIV-mediated autophagy activation protects hepatocytes from cell death, we stimulated primary mouse hepatocytes with 1% hypoxia or 0.5 mM H2O2 after transfection with CaMKIV-dCT and measured cell death by release of LDH. Hepatocytes transfected with CaMKIV-dCT displayed significantly lower level of LDH release compared with control cells (Fig. 4D). These findings suggest that CaMKIV protects hepatocytes from oxidative stress-induced cell death.

**CREB signaling is deficient in CaMKIV KO mice.** To elucidate the mechanism by which CaMKIV regulates autophagy, we examined CREB, a direct downstream substrate of CaMKIV (28). CREB has been linked to induction of autophagy in cerebral ischemia (8), and we hypothesized that this signaling axis might also play a role in hepatic I/R. We found that the level of p-CREB was significantly lower in CaMKIV KO mice compared with WT mice following I/R in vivo (Fig. 5A). In vitro, CaMKIV knockdown with siRNA reduced CREB activation (Fig. 5B), whereas transfection with the activating

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**Fig. 2.** Autophagy is deficient in CaMKIV KO mice. A: Western blot for LC3B and beclin 1 on liver tissues of WT and CaMKIV KO mice after I/R. The top bar graph represents the conversion rate of LC3B-I to II, and bottom graph indicates relative density of becln 1 to β-actin. Blots were representative of three similar experiments. *P < 0.05, WT vs. KO at the same time point. B: LC3B mRNA levels after 6 h of reperfusion in WT and CaMKIV KO mice. *P < 0.05 WT vs. KO. C: transmission electron microscopy images of liver sections from WT and CaMKIV KO mice after 3 h of reperfusion. Inset shows representative images. D: autophagic vacuole (AV) volume in liver sections of WT and CaMKIV KO mice after 3 h of reperfusion. For details refer to MATERIALS AND METHODS. Values are means ± SE. *P < 0.01.
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C

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construct CaMKIV-dCT increased CREB signaling (Fig. 5C). Furthermore, autophagy activation by CaMKIV-dCT was abolished with treatment with a CREB inhibitor (Fig. 5D), suggesting that the mechanism of CaMKIV-mediated autophagy regulation is through CREB signaling. Taken together, our collective results demonstrate that CaMKIV activation following liver I/R injury in vivo or oxidative stress in vitro activates the protective autophagy pathway in hepatocytes to limit organ damage and hepatocyte cell death.

DISCUSSION

I/R injury is a complex phenomenon involving an injurious inflammatory response. Our laboratory’s previous study dem-
CaMKIV AND AUTOPHAGY IN LIVER I/R INJURY

G195

Fig. 4. CaMKIV activates autophagy in vitro in hepatocytes. A: cultured hepatocytes were subjected to normoxia (N; 21% O2) for 6 h (control), or hypoxia (H; 1% O2) for various time points. Whole cell lysate protein was examined by Western blot for autophagy by LC3B. Figure is a representative image of similar experiments repeated three times. B: hepatocytes were transfected with small interfering RNA (siRNA) against CaMKIV and subjected to hypoxia for analysis of autophagy by LC3B Western blot. C: hepatocytes were subjected to hypoxia, with or without the CaMKK inhibitor STO609. D: hepatocytes were transiently transfected with control vector (pDNA 3.1+) or dominant-active mutant CaMKIV-dCT, and Western blot was performed for LC3B. E, top: hepatocytes were transfected with control vector or CaMKIV-dCT and exposed to either normoxia, 1% hypoxia for 12 h, or H2O2 (500 μM) for 8 h before lactate dehydrogenase assay was performed on the cell culture supernatants. Values are means ± SE. OD, outer diameter. Bottom: the FLAG blot indicates expression of plasmids in hepatocytes.

onstrated a role for the family of CaMK in the activation of immune pathways and their role on I/R-induced hepatocellular injury (41). Although a number of mechanisms have been implicated in I/R injury, the role of autophagy as a protective process has been unclear. Our results provide evidence that autophagy is a protective process during I/R and reveal a novel link between CaMKIV and autophagy induction.

The role of autophagy in hepatic I/R is controversial, as it has been described as both damaging and protective (33). However, experimental models to study this process vary greatly. Furthermore, studies have employed models of both cold I/R and warm I/R, and these two processes should be interpreted separately. Autophagic signaling has been shown to increase rapidly after warm reperfusion in transplanted livers.
following 24 h of cold ischemia and preservation in nutrient-deficient University of Wisconsin solution (27). Additionally, hepatocytes with strong LC3 staining colocalized with Kupffer cell markers in immunofluorescent-stained livers after reperfusion, suggesting that high levels of autophagy were associated with hepatocyte cell death and phagocytosis by Kupffer cells. Pharmacological inhibition with phosphatidylinositol 3-kinase inhibitors, wortmannin, or LY-294002 also increased survival in a model of orthotopic liver transplantation (17). In light of these studies, the process of autophagy in cold I/R was interpreted as damaging, promoting hepatocyte cell death and graft dysfunction after transplantation. In human studies, the use of steatotic grafts for liver transplantation is an increasingly encountered clinical challenge (11), and induction of autophagy in ischemic-preconditioned steatotic livers was associated with fewer episodes of rejection and better outcomes (10), suggesting a possible protective role.

In warm hepatic I/R, decreased levels of autophagy proteins Atg7 and beclin 1 were observed in anoxic rat hepatocytes in vitro and in liver tissue in vivo, employing a model of 45 min of warm ischemia followed by 30 min of reperfusion (25). However, no time points beyond 30-min reperfusion were examined in this study. This study also suggested a protective role for autophagy during anoxia/reoxygenation in hepatocytes in vitro; both augmentation of autophagic signaling with nutrient depletion before anoxia and overexpression of autophagy proteins Atg7 or beclin 1 protected hepatocytes from cell death. A study from our group also suggested a protective role for autophagy (7), as pharmacological treatment with cisplatin induced autophagy and protected livers from I/R damage. More recently, autophagy was found to be a protective mechanism in human hepatocytes stimulated with hypoxia/reoxygenation by preventing apoptosis. Induction of autophagy was dependent on mitochondrial ROS production (4). In addition to hepatic I/R, clues from other I/R models, notably cardiac I/R, have implicated autophagy as protective (19) and several upstream regulatory mechanisms have been explored (18). One such mechanism showed a role for intracellular calcium, CaMKK-β and mTOR (20). While no studies have investigated this pathway in hepatic I/R, we hypothesized that CaMKK downstream signaling molecules such as CaMKIV might also be involved in regulation of autophagy. The CaMK’s have been shown to play a role in several inflammatory conditions, and their function appears to be tissue and cell specific. CaMKII and CaMKIV silencing reduces cytokine secretion in macrophages (34), and CaMKIV activation in macrophages induces release of the DAMP molecule HMGB1 (54). In vivo, silencing of CaMKII protects mice from death in a model of endotoxemia in a mechanism dependent on reduced proinflammatory cytokine and HMGB1 secretion (53). Furthermore, CaMKII activation has been extensively linked to cell death and injury in cardiac I/R (35, 45), and inhibition of this signaling cascade is an attractive therapeutic strategy. Interestingly, our group is also investigating...
the role of CaMKII in hepatic I/R, and our preliminary studies show that redox-dependent activation of this protein promotes organ damage through release of the DAMP molecule HMGB1 (unpublished data). Taken together, it is clear that the family of CaMK plays diverse roles in several acute, inflammatory conditions.

Downstream targets of the CaMKs are diverse and serve several different cellular functions. CaMKIV has been shown to interact with the mitogen-activated protein kinase cascade (13) and also activates the transcription factor CREB by phosphorylation at serine 133 (39). This signaling pathway has been linked to the survival of dendritic cells (22) and as a survival factor in neonatal hypoxic-ischemic brain injury (26). Furthermore, the role of CREB has been extensively studied in the liver and is considered a master regulator of gene transcriptional activity (37). In this study, we show that CaMKIV is upstream of autophagy activation, and our in vitro data suggest that CREB signaling may also play a role in this process by regulating the transcription of autophagy proteins, such as LC3B.

In conclusion, our studies implicate a role for autophagy in limiting organ damage after ischemic liver injury in a mechanism dependent on signaling through CaMKIV. Organ damage is significantly greater in CaMKIV KO mice due to deficient autophagy signaling in hepatocytes. Augmentation of autophagy in these mice restores organ damage to WT levels, and in vitro constitutive CaMKIV activation leads to autophagy activation. These findings represent a novel approach to reduce organ damage during I/R: in addition to aiming to inhibit pathways that promote organ damage, augmentation of protective pathways, such as autophagy, represent additional strategies to reduce organ damage.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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