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THE ROLE OF CALPAIN IN ONCOTIC CELL DEATH

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■ **Abstract** Numerous lines of evidence demonstrate that calpains, a family of 14 Ca^{2+}-activated neutral cysteine proteases, are involved in oncotic cell death in a variety of models. At this time, the biochemistry of most calpains and the specific roles of different calpains in physiology and pathology remain to be determined. A number of calpain substrates have been identified in cellular systems, including cytoskeletal proteins, and recent studies suggest that calpains mediate the increase in plasma membrane permeability to ions and the progressive breakdown of the plasma membrane observed in oncosis through the proteolysis of cystokeletal and plasma membrane proteins. Further, a number of reports provide evidence that the mitochondrial dysfunction observed in oncosis may be mediated by a mitochondrial calpain of unknown identity. Finally, a number of diverse calpain inhibitors have been developed that show cytoprotective properties in cellular systems and in vivo following diverse insults. It is suggested that future research be directed toward elucidation of the role(s) of specific calpain isozymes in physiological and pathological conditions; identifying and linking specific calpain substrates with altered cellular functions; and developing cell-permeable, potent, isozyme-selective calpain inhibitors.

CELL DEATH: ONCOSIS AND APOPTOSIS

Ischemia/hypoxia/reoxygenation or toxicants produce cell injury that can lead to cell death. Generally, cell death is thought to occur through two recognized pathways, necrotic cell death (oncosis) or apoptosis (1–4), although cell death containing markers of both oncosis and apoptosis has been reported (5). Apoptosis is a tightly controlled, organized process that usually affects scattered individual cells. Morphological changes in apoptosis include cell shrinkage, chromatin condensation, plasma membrane phosphatidylserine externalization, normal or condensed mitochondria, and apoptotic body formation. In some cases, a ladder-like pattern of DNA fragmentation can be visualized when genomic DNA is subjected to agarose electrophoresis (6–8). Oncosis, derived from the Greek word for swelling, is the common pattern of cellular changes in infarcts and zonal cell death

following chemical toxicity (1). Oncosis often affects many contiguous cells, with early manifestations consisting of plasma membrane blebbing, dilation of the ER, mitochondrial swelling, and clumping of nuclear chromatin. These morphological changes are followed by the breakdown of the plasma membrane, release of intracellular constituents, and inflammation.

Oncosis plays a critical role in tissue/organ injury produced by a variety of insults. This type of cell death occurs in numerous human diseases, such as acute myocardial infarction, acute renal and liver failure, and stroke. As the number of cells undergoing oncosis increases, organ dysfunction results. Further, the inflammation resulting from the release of the intracellular contents during oncosis may produce further tissue damage, exacerbate organ dysfunction, and contribute to chronic organ dysfunction. Consequently, understanding oncotic cell death in different cell types is essential for the development of effective measures to block this process and ameliorate human diseases resulting from oncotic cell death.

The mechanisms of oncosis are not completely understood and may vary significantly among different insults and tissues. Nevertheless, the different cellular targets are likely to converge into a common pathway(s). Along the common pathway, a "point of no return" is reached in which cells will die irrespective of any intervention. This raises the question, "When is a cell undergoing oncosis dead?" In our laboratory, we have defined oncotic cell death as the point in the oncotic process in which respiration (mitochondrial function and associated ATP formation) and ion homeostasis cannot be restored (9–11). This is in contrast to cytoprotective agents that block the breakdown of the plasma membrane without allowing the restoration of mitochondrial function.

Research in the past two decades has significantly advanced our understanding of oncosis and has revealed several of the major players and their interactions. Oncosis has been thought of as an unorchestrated catastrophic event, analogous to a balloon being blown up beyond its capacity and popping. However, as described below, there are identifiable sequences of events in cells undergoing oncosis that lead to the breakdown of the plasma membrane and cell death. Further, recent evidence suggests that the breakdown of the plasma membrane occurs in steps with progressive increases in plasma membrane permeability. In this review, we discuss recent findings concerning the roles of calpains in oncosis. Further, we review studies that have examined the effectiveness of calpain inhibitors in different animal models of injury.

MITOCHONDRIAL FUNCTION AND ATP

For those tissues that generate ATP exclusively through oxidative phosphorylation, the loss of mitochondrial function as a result of ischemia and toxicant exposure is catastrophic. In contrast, cells in tissues that are capable of generating ATP from oxidative phosphorylation and glycolysis are more resistant to the effects of ischemia and mitochondrial-directed toxicants. Therefore, caution should be exercised when studying cell death in cultured cells because most cultured cells

generate a significant amount of their ATP through glycolysis. Further, the dual source of ATP formation in cultured cells overemphasizes apoptosis following hypoxia/reoxygenation or toxicant exposure.

Following the loss of mitochondrial function, ATP levels decrease, and a number of studies have been conducted to determine the importance of ATP depletion in signaling cell death. In general, if decreases in ATP are less than 80%–85%, cells will either die by apoptosis or survive if mitochondrial function can be restored. However, if ATP depletion is greater than 80%–85%, oncosis will occur (12). The absolute decrease in ATP that is required to induce oncosis varies among different cell types.

Ca^{2+}

Ca^{2+} is a second messenger that plays a critical role in a variety of cellular functions. The distribution of Ca^{2+} within cells is complex and involves binding to macromolecules and compartmentalization within intracellular organelles, such as the endoplasmic reticulum (ER) and mitochondria. The cytosolic free Ca^{2+} (Ca^{2+}_f) concentration is tightly regulated and maintained at approximately 100 nM against a large extracellular/intracellular gradient with the assistance of a series of channels, exchangers, and ion pumps, including the smooth ER Ca^{2+}-ATPase (SERCA) and associated ER Ca^{2+} store (13).

Ca^{2+} has been implicated in cell death for some time, and the publication of Schanne et al. (14) stimulated a large number of studies in the 1980s and 1990s that examined the role of Ca^{2+} in cell death using the newly developed fluorescent Ca^{2+} indicators. At this time, increases in Ca^{2+}_f levels are thought to play a critical role in oncotic cell death in a variety of models using diverse injurious agents. The evidence to support this conclusion is usually based on one or more of the following types of experiments: Ca^{2+}_f increases following the insult, chelation of intracellular Ca^{2+}_f blocks cell death, depletion of the endoplasmic reticulum Ca^{2+} pool prior to the insult blocks cell death, inhibition of extracellular Ca^{2+} influx (via Ca^{2+} channel blockers or extracellular Ca^{2+} chelation) blocks cell death, and calpain inhibitors block cell death. Nevertheless, there are examples of oncosis in which the role of Ca^{2+} is reported to be minimal (15).

As discussed above, ATP levels must be depleted beyond 80%–85% to initiate oncosis. One question that has remained is what cellular changes occur at the 80%–85% ATP depletion transition. There are at least two possibilities: The concentration of ATP is below that needed for kinases to phosphorylate proteins needed for cell survival, and the concentration of ATP is below that needed for ATPase-dependent cellular functions. For example, Kobryn & Mandel (16) and Schnellmann et al. (17) provided evidence that the state of protein phosphorylation is important because the protein phosphatase (PP2A/PP1) inhibitor calyculin A maintained protein phosphorylation and decreased oncosis in renal proximal tubular cells. More recently, Harriman et al. (18) suggested that extensive ATP depletion results in inhibited SERCA activity, with an associated leakage of ER Ca^{2+}, leading to a sustained increase in Ca^{2+}_f concentrations and calpain activity.

This is consistent with previous data demonstrating that ER Ca^{2+} depletion, using the SERCA inhibitors thapsigargin or cyclopiazonic acid, prior to hypoxia or exposure to diverse toxicants is cytoprotective (18, 19). Consequently, the signaling pathway between ATP depletion and increased Ca_f^{2+} concentrations may be the inhibition of the SERCA and ER Ca^{2+} leakage.

CALPAIN

Since the first description of calpain (calcium-activated neutral protease, E.C., 3.4.22.17) in 1964 by Guroff (20), extensive progress has been made regarding the identity, structure, activity, localization, and physiological and pathological functions of calpains. To date, the number of identified mammalian calpain protease family members has grown to 14 (21). Based on their tissue expression patterns, calpains are classified as ubiquitous or tissue specific (22). Two ubiquitous isoforms, μ- and m-calpain, have been identified and studied extensively. As the names imply, purified μ- and m-calpains are activated by μM and mM Ca^{2+} concentrations in vitro, respectively (22–25). In an effort to understand the physiological functions of μ- and m-calpain, knockout mice were developed. Knockout mice lacking the μ-calpain large subunit gene [Capn1(−/−)] exhibited platelet dysfunction (26), whereas knockout mice lacking the m-calpain large subunit gene [Capn2(−/−)] or small subunit gene [Capn4(−/−)] were embryonic lethal (27). These results suggest that m-calpain is critical for embryogenesis, whereas μ-calpain is not. Additional physiological functions of calpains, including cell cycle progression (28), proliferation (29), differentiation (30, 31), migration (32–34), embryonic development (27), meiosis (35), and mitosis (36), have been described. The reader is referred to the following review articles concerning the molecular biology, biochemistry, and physiological functions of calpains (21–23, 25, 37). It is important to note that at this time, the structure, localization, regulation, and function of most of the calpain family members remains unknown.

Calpains in Oncosis

In a large number of studies, calpains have been implicated in the mediation of oncosis in different cellular models subjected to diverse isnsults and in a limited number of in vivo models (10, 11, 18, 38–64). Although calpain autolysis and/or hydrolysis of a known calpain substrate were used as markers of calpain activity in some of these studies, calpain inhibitors were used to demonstrate calpain involvement in the majority of the studies. For example, calpains are activated when Ca_f^{2+} concentrations reach 450 nM in cardiomyocytes (65), and an increase in calpain activity occurs prior to membrane damage using a fluorescent calpain substrate and calpain-mediated degradation of spectrin in rat renal proximal tubules (RPT) subjected to hypoxia (45–47). Further, calpain inhibitors are cytoprotective in primary cultures of rabbit RPT cells exposed to cyclosporine A or $HgCl_2$ (38, 40), in rat RPT subjected to hypoxia (46), and in rabbit hearts subjected

to ischemia/reperfusion (66, 67). In freshly isolated rabbit RPT, diverse calpain inhibitors blocked lactate dehydrogenase (LDH) release, a marker of oncosis, following exposure to a mitochondrial inhibitor (antimycin A), an alkylating quinone (bromohydroquinone), an oxidant (t-butylhydroperoxide), and a toxicant that forms a reactive electrophile [tetrafluoroethyl-L-cysteine (TFEC)] (54). These observations suggest a key role for calpains in the process of oncotic cell death produced by diverse insults.

Although numerous studies suggest that calpains play a critical role in oncosis, it is not clear which calpain isoform(s) are involved, how calpains are activated, which intracellular targets are modified by calpains, and how proteolysis of calpain targets leads to cell death. In the sections below, we review calpain activation and translocation, calpain inhibitors, calpain substrates, calpain-mediated disruption of ion homeostasis, calpain-mediated progressive plasma membrane permeability, and calpain-mediated mitochondrial dysfunction in oncosis.

CALPAIN ISOFORMS AND MECHANISMS OF ACTIVATION As stated above, μ- and m-calpain are ubiquitously and constitutively expressed and have been the major calpain isozymes studied in oncosis. Miyoshi et al. (51) and Rami et al. (68) suggested that activation of μ-calpain mediates oxidant-induced hepatocyte death and ischemic hippocampal neuronal death, respectively. Also, Edelstein et al. (46) suggested that rat renal proximal tubular cells only expressed μ-calpain and that μ-calpain may be the isozyme that mediates hypoxic cell death. However, Liu et al. reported that rabbit renal proximal tubular cells expressed both μ- and m-calpain and that it was not possible to implicate specifically μ- or m-calpain in oncosis using calpain inhibitors (10, 60). In contrast, Blomgren et al. (69) suggested that activation of m-calpain mediates hypoxic/ischemic brain tissue injury. Therefore, there is no consensus on the exact role of μ- or m-calpain in oncosis. Because most cell types express both isoforms, and currently available calpain inhibitors are not isoform-specific in cellular or in vivo systems, molecular approaches, such as inducible gene-knockout animal models, isoform specific antisense oligonucleotides, or small interference RNA technologies, are needed to answer this question. Fortunately, knockout mice lacking the μ-calpain large subunit gene [Capn1(−/−)] have been developed (26). Although knockout mice lacking the m-calpain large subunit gene [Capn2(−/−)], or small subunit gene [Capn4(−/−)], are embryonic lethal (27), certain types of cells may be isolated from the embryo and used for cell injury and death experiments. It is not known whether tissue-specific calpain isoforms or other calpains play a role in cell injury and death.

Both μ- and m-calpains are heterodimers and consist of an 80-kDa large subunit containing the active site and a 30-kDa regulatory subunit (21–23). The procalpains are predominantly localized in the cytosol and are long-lived proteins (29). Several cellular events can increase the activity of calpains: a rise in cytosolic Ca_f^{2+} concentration, translocation of calpains to membranes, autolysis of procalpains, dissociation of the calpain subunits, decreased levels of calpastatin (the endogenous inhibitor of calpain), and interaction with calpain activator proteins

or phospholipids (23, 70–73). The presence and interplay among these elements is critical for fine-tuning calpain activity.

Among these possible modifications, Suzuki & Sorimachi (73) proposed a dissociation/autolysis mechanism for the activation of calpain(s) at the membrane. In the autolysis/dissociation model, Ca^{2+} binds to calpain(s), inducing dissociation of the calpain subunits and two successive autolytic events (80 kDa to 78 kDa to 76 kDa); the autolyzed large subunit makes the dissociation irreversible (74–77). Numerous studies report calpain activation by measuring the appearance of an autolytic form of calpain during a biological event (34, 51, 68). However, an increasing body of evidence suggests that calpains are active without autolysis. For example, Molinari et al. (78) reported that μ-calpain is active in the erythrocyte membrane in its nonautolyzed 80-kDa form, and Elce et al. (79) demonstrated that a mutation in the autolysis site of the m-calpain large subunit prevented autolysis but had no effect on protease activity. Yoshizawa et al. (74) reported that the 80-kDa calpain subunit is enzymatically active and suggested that μ- and m-calpain function as monomers of the 80-kDa subunit in vivo. Koh & Tidball (80) demonstrated m-calpain mediates proteolysis of talin and vinculin without undergoing autolysis. Finally, immunoblot analysis was used to investigate whether μ- and/or m-calpain undergo autolysis during RPT oncosis (10). Under control conditions, most μ-calpain was present in the cytosol as the 80-kDa form and the membrane-associated μ-calpain was in the 78-kDa autolytic form, whereas m-calpain is present in the cytosol in the 80- and 76-kDa form. During mitochondrial inhibitor-induced oncosis, increased autolysis of either calpain isoform was not observed. Despite extensive investigation, the role of autolysis in calpain activation during oncosis is still unclear.

The translocation of calpain from the cytosol to the plasma membrane was proposed to be a critical step in the calpain activation process in platelets and red blood cells (24, 75, 81). For example, redistribution of μ-calpain from the cytosol to the membrane fraction after traumatic rat brain injury was detected using casein zymography (82). Using immunoblot analysis, no evidence of μ- or m-calpain translocation from the cytosol to the membrane fraction during mitochondrial inhibitor-induced renal cell oncosis was observed (10). Further, N-methyl-D-aspartic acid–induced calpain activation was independent of calpain translocation in primary rat cortical neurons (83). Finally, Blomgren et al. (84) reported that calpain immunoreactivity decreased in the cytosol with no significant changes in the membrane fractions of cortical tissue from neonatal rats subjected to cerebral hypoxia-ischemia. Therefore, whether translocation is responsible and/or needed for calpain activation during oncosis and other cellular events remains debatable.

In addition to autolysis and translocation, calpain activity is regulated by the endogenous inhibitor protein calpastatin (23) and reported calpain activator proteins (70–72). For example, decreases in calpastatin and, therefore, loss of calpain inhibition were reported in ischemia/reperfusion renal injury and neonatal cerebral damage (85, 86). Interestingly, the degradation of calpastatin is due to the actions of calpain(s) (85) or caspase(s) (86). In contrast, no changes in calpastatin were observed in renal proximal tubular cells during oncosis (10). Consequently, there

does not seem to be a consistent conclusion concerning the role of calpastatin in oncosis. A role for calpain activators during cell injury and death has not been reported.

Recent studies have shown that calpains have several phosphorylation sites recognized by protein kinases, suggesting that calpains also may be regulated by phosphorylation. For example, Imajoh et al. (87) described the presence of several evolutionarily conserved protein kinase A consensus sites in domain III of m-calpain, and Cong et al. (88) reported that freshly isolated calpains are highly phosphorylated. In addition, epidermal growth factor induces phosphorylation and activation of m-calpain through a protein kinase A and an extracellular receptor kinase signaling pathway (32, 89). At this time, it is not known whether calpains undergo phosphorylation or dephosphorylation during cell injury and death.

In summary, the calpain isozyme(s) involved in oncosis and the mechanism of calpain activation during oncosis remain unclear. The development of calpain "knockout" animals, antisense oligonucleotides, and small interfering RNA technologies may help resolve some of these questions.

CALPAIN INHIBITORS The rate-limiting step in determining the role of the calpain and calpain isoforms in cell physiology and pathology has been the lack of good pharmacological inhibitors. The active site of calpains contains characteristic residues of cysteine proteases and includes cysteine (Cys 105), histidine (His 262), and asparagine (Asn 286) (90). When a substrate is hydrolyzed, the active site Cys acts as a nucleophile and attacks the peptide carbonyl group, forming a tetrahedral intermediate that quickly collapses to release the amine product. The resulting acyl-enzyme is hydrolyzed to release the carboxyl product and regenerate the enzyme (90). Calpain proteolysis takes place preferentially at Leu, Val, or Ile (x*x) residues, with preference for Val, Leu, or Ile at the P2 position and a more tolerant residue preference at the P1 position (91). For example, Tyr, Gly, and Arg are also acceptable in the P1 position of substrates (91).

There are many diverse calpain inhibitors that can be described as either transition-state reversible inhibitors or irreversible inhibitors. The first transition-state inhibitors, peptide aldehydes (e.g., leupeptin), were isolated from Streptomyces species (92). Using these compounds as a starting point, new compounds were synthesized that exhibited improved membrane permeability and calpain specificity. Calpeptin (Z-Leu-Nle-H), one of these synthesized compounds, was 4-fold more potent than leupeptin for calpain I and 28-fold for calpain II (93). Other groups of inhibitors have since been discovered, including α-dicarbonyls (originally developed as serine protease inhibitors) (94–96), nonpeptide quinolinecarboxamides (97), and nonpeptide α-mercaptoacrylic acids and phosphorus derivatives (98). The α-mercaptoacrylic acid derivatives are particularly interesting because they mediate their inhibitory activity by interacting with the Ca^{2+}-binding domain of the calpain I and II large subunits (98).

Irreversible inhibitors include the epoxysuccinates (relatively nonspecific cysteine protease inhibitors) (99), acyloxymethyl ketones, and halomethylketones

(cell permeable and selective for calpain over cathepsins) (100), sulfonium methyl ketones (potent and highly calpain II selective) (101), and diazomethyl ketones (102). The epoxysuccinate inhibitor E64 is a commonly used irreversible calpain inhibitor, which was originally isolated from *Aspergillus japonicus* (99). E64 and its derivatives reportedly inhibit calpains irreversibly by the covalent binding of the active site cysteine thiol to the C-3 of the epoxysuccinyl group. Because of the irreversible nature of this bond, biotin-immobilized E64 derivatives also have been used to isolate novel calpains (103). Although E64 and its derivatives have been widely used as calpain inhibitors, they are generally nonselective, inhibiting a variety of cysteine proteases.

Calpain inhibition offers protection from ischemia and toxicant-induced cell death in diverse models. A variety of calpain inhibitors, including Z-Leu-Abu-CONHEt (AK275), E64 analogues, 27-mer calpastatin peptide, leupeptin, calpain inhibitor I, calpain inhibitor II, calpeptin, Cbz-Val-Phe-H (MDL28170), and 3-(4-iodophenyl)-2-mercapto-(Z)-2-propenoic acid (PD150606, a Ca^{2+}-binding site inhibitor), offer protection from ischemic and toxicant-induced cell death in diverse models (10, 11, 18, 42, 45–47, 54, 58, 60, 62–64, 68, 98). In our laboratory, calpain inhibitor I and II; PD150606; two nonpeptidyl inhibitors, including chloroacetic acid N′-(6,7-dichloro-4-phenyl-3-oxo-3,4-dihydroquinoxalin-2-yl)hydrazide (SJA-7029); and several peptidyl α-keto amide inhibitors protected renal proximal tubules from oncosis and promoted the recovery of mitochondrial function and active ion transport (10, 11, 18, 54, 60). However, at this time, pharmacological inhibitors of calpain are not capable of differentiating among different calpain isoforms in cellular systems or in vivo. Further, no calpain inhibitors are selective, potent, stable, and membrane permeable. The current diverse list of calpain inhibitors provides researchers with a starting point for characterizing the activity and function of calpains. The importance of calpains in disease will continue to stimulate the development of new and better inhibitors.

An alternate approach is the use of molecular biological techniques to selectively decrease specific calpain isoforms. This approach offers great potential with the cloning and sequencing of the calpain family and the development of knockout, transgenic, antisense, and small inhibitory RNA technologies. Zhang et al. (29) successfully employed antisense oligonucleotides to decrease expression of the calpain small subunit. Similar approaches may be used to specifically target each calpain isozyme prior to cell injury to determine the specific role of each isozyme in the process of oncosis.

CALPAIN SUBSTRATES (INCLUDING CYTOSKELETON) Calpains have a large number of substrates, including signaling molecules, membrane proteins, intracellular enzymes, and structural proteins (21–23, 25, 37, 104). Most of these substrates were identified using in vitro calpain cleavage assays and do not necessarily reflect calpain target proteins in the cell. Identification of intracellular calpain substrates during cell injury and death is needed to understand the role of calpain(s) and the target substrate(s) in oncosis. A few dozen intracellular substrates have been

TABLE 1 Calpain substrates during cell injury and death

Injury model	Calpain substrates[a]	Reference
Ischemia/reperfusion	spectrin	(86)
Mitochondrial inhibition	paxillin, vinculin, talin	(63)
Oxidant	talin, α-actinin	(51)
Hemorragic shock	$I^{\gamma}B\alpha$ and β	(64)
Traumatic spinal cord	spectrin and NF200	(58)
Apoptosis	paxillin, p130cas	(105)
Maitotoxin	cdk5 activator p35	(106)
Ca^{2+} ionophore	cdk5 activator p35	(107)
Apoptosis	CaMK IV	(108)
Ischemic/hypoxic	retina AAT	(109)
Degraded collagen	paxillin, talin, $pp125^{FAK}$	(110)
Ionophore	talin, vinculin	(80)
Hypoxia/ischemia	calpastatin	(85)
Hypoxia/ischemia	fodrin, MAP 2	(84)
Hypoxia/ischemia	caspase	(69)
Ischemia/reperfusion	Bid	(66, 67)

[a]Cdk, cyclin-dependent kinase; CaMK IV, calcium/calmodulin-dependent protein kinase IV; AAT, aspartate aminotransferase; MAP 2, microtubule associated protein 2.

identified (Table 1). Among these substrates are proteins involved in apoptosis (caspase 3, Bax, Bcl-X_L, and Bid). Calpain-mediated hydrolysis of these proteins leads to changes in their activity or intracellular localization. For example, in maitotoxin- or Ca^{2+} ionophore–treated neurons, calpain cleavage of the cdk5 activator p35 to a truncated form (p25) leads to redistribution of the cdk5 active complex to the soluble fraction (107), increased activity of cdk5, and phosphorylation of tau (106). Calpain hydrolysis of Ca^{2+}/calmodulin-dependent protein kinase IV (CaMK IV) leads to the loss of its enzymatic activity prior to cell death (108). In an ischemic/hypoxic rat retina injury model, Endo et al. (109) reported that calpain cleaved and decreased mitochondrial associated aspartate aminotransferase activity. Calpain also positively regulates its activity through cleavage of calpastatin in neonatal cerebral hypoxia/ischemia injury (85). Interestingly, m-calpain activates caspase-3 in neonatal hypoxia-ischemia cerebral injury (69).

Several calpain substrates listed in Table 1 are actin-associated proteins. Unlike other cysteine proteases, calpains tend to cleave substrates at interdomain boundaries, thereby serving to modulate the function of these proteins rather than simply digesting them (23). Indeed, unique proteolytic products of spectrin (150-kDa and 145-kDa fragments) have been used as markers of intracellular calpain activation and/or as a prognostic marker after traumatic brain injury (46, 86, 91, 111–113).

Other actin-associated proteins, including talin, paxillin, vinculin, and α-actinin, are calpain substrates (51, 63, 80, 105, 110). These proteins are important components of the actin cytoskeleton-plasma membrane junction and thereby play a major role in maintaining cell shape, retaining plasma membrane integrity, and supporting cell adhesion and migration. Calpain proteolysis of these proteins may lead to structural changes. For example, it has been reported that calpain hydrolysis of paxillin, talin, and $pp125^{FAK}$ leads to cell rounding and disassembly of focal adhesion complexes (110). Also, in mitochondrial inhibitor-injured renal proximal tubular cells, calpain cleavage of talin, paxillin, and vinculin is closely correlated with increased plasma membrane permeability (63), suggesting that modification of these structural proteins may lead to membrane damage and oncosis. In support of this hypothesis, calpain hydrolysis of talin and α-actinin precedes plasma membrane damage and is closely related to plasma membrane blebbing in oxidant-injured hepatocytes (51). Finally, calpain inhibitors block membrane blebbing in renal cells exposed to $HgCl_2$ (39).

The advancement of protein identification techniques (2-dimensional gel electrophoresis and mass spectrometry) should advance and accelerate the identification of additional calpain substrates. Some pioneering work by Santella et al. (35) identified α-tubulin, lamin B, dynamin, and two forms of actin as calpain substrates during meiosis in starfish oocytes. However, precaution should be taken when using this technique in oncosis experiments because compromised plasma membrane permeability may allow the leakage of intracellular proteins (10).

CALPAIN DISRUPTION OF ION HOMEOSTASIS As discussed in the Ca^{2+} section above, ER Ca^{2+} release plays an important role in oncosis. Harriman et al. (18) suggested that ER Ca^{2+} leakage initiates a signaling cascade during renal cell death. This raised the question, "What is the signaling cascade?" Chelation of intracellular Ca^{2+}prevented extracellular Ca^{2+} uptake and cell death in mitochondrial inhibitor-exposed renal proximal tubules. In addition, pretreatment with an ER Ca^{2+}-ATPase inhibitor, thapsigargin, blocked extracellular Ca^{2+} uptake in hypoxia- and mitochondrial inhibitor-induced cell death (19, 54). These results suggest a linkage among the early ER Ca^{2+} release, the increase in Ca_f^{2+} concentration, and the influx of extracellular Ca^{2+} in the late phase of renal proximal tubule oncosis. Recently, it was demonstrated that mitochondrial inhibition by antimycin A results in an early increase in calpain activity in conjunction with the release of ER Ca^{2+} (18) and that calpain inhibitors block the influx of extracellular Ca^{2+} in the late phase of RPT cell oncosis (54). These results suggest that calpain, but not capacitive Ca^{2+} entry or store-operated Ca^{2+} entry, functions as a signal to mediate the influx of extracellular Ca^{2+} following ATP depletion and ER Ca^{2+} release during cell injury and death (18). How calpain activation, subsequent to ER Ca^{2+} release, mediates the influx of extracellular Ca^{2+} remains unknown. Although it has been shown that calpains can cleave and modulate the function of plasma membrane Ca^{2+}-ATPase (104), Ca^{2+} channels (114), and other Ca^{2+}-regulating proteins, such as the inositol (1,4,5)-triphosphate (IP_3) receptor

(115, 116). Our current understanding of calpain-mediated influx of extracellular Ca^{2+} in oncosis remains unclear.

In addition to the early changes in Ca^{2+} that result from ATP depletion and ER Ca^{2+} leakage, the inhibition of the Na^+/K^+-ATPase results in increased intracellular Na^+ concentrations, decreased intracellular K^+ concentrations, depolarization of the plasma membrane, and a limited degree of cellular swelling. After a poorly studied lag period, Cl^- influx provides the osmotic force that triggers additional Na^+ and water influx and triggers the terminal cell swelling that leads to the breakdown of the plasma membrane (117–119). Because the Cl^- channel inhibitors IAA-94, niflumic acid, and NPPB inhibit antimycin A–induced Cl^- influx and LDH release (119) and are cytoprotective to rat RPT subjected to hypoxia (120), it is likely that Cl^- influx occurs through a Cl^- channel during the late phase of renal cell oncosis. Although the identity of the Cl^- channels and the exact mechanisms underlying extracellular Cl^- influx remain unclear, calpain inhibitors block extracellular Cl^- influx (19, 54), suggesting that extracellular Cl^- may enter the cell through a calpain-sensitive pathway.

Miller & Schnellmann (117) and Dong et al. (121) suggested that glycine and strychnine blocked increases in membrane permeability by acting on a glycine-sensitive Cl^- channel. Electrophysiological and biochemical measures are needed to further characterize these membrane proteins and determine whether a linkage to calpain exists.

CALPAIN MEDIATION OF PROGRESSIVE PLASMA MEMBRANE PERMEABILITY Plasma membrane damage, with increased permeability and altered morphology, is a hallmark of oncosis (10, 11, 15, 122–126). Although plasma membrane disruption during oncosis has been thought of as an all-or-none event, recent studies have demonstrated that it is a progressive process with a series of altered permeability states. For example, Chen et al. (125, 127) demonstrated three different permeability phases in freshly isolated rabbit RPT subjected to anoxia. The first phase allowed the entry of propidium iodide (a cell-impermeable DNA dye, MW 668 Da). The second phase allowed the entry of dextrans up to 3 kDa, and the last phase allowed the entry of 70-kDa dextrans and the release of cytosolic enzymes, such as LDH (MW 130 kDa). Similar plasma membrane permeability changes were observed in Madin-Darby canine kidney (MDCK) cells exposed to chemical hypoxia (124) and in hepatic sinusoidal endothelial cells subjected to chemical hypoxia (126). Although the biophysical basis of this increased plasma membrane remains unclear, there is evidence that the permeability to 70-kDa dextran and LDH could be blocked by several chemical cross-linkers, suggesting that alterations of certain molecules on the plasma membrane may be responsible (124, 125).

The mechanism underlying progressive plasma membrane permeability during cell injury and death remains poorly understood. However, growing evidence indicates that calpains play a critical role in this process. Two calpain inhibitors, PD-150606 and SJA-7029, prevented increased plasma membrane permeability to propidium iodide and LDH in the late stage of renal cell oncosis produced by

diverse toxicants or insults, suggesting that calpain mediates the increased plasma membrane permeability (10, 11, 42, 54, 60, 128). Recent studies have begun to link the progressive membrane permeability changes to calpain proteolysis of cytoskeletal proteins. For example, hydrolysis of paxillin occurs concomitantly with entry of propidium iodide and prior to LDH release, whereas hydrolysis of talin and vinculin occurred concomitantly with LDH release (63). Calpain inhibitors blocked the hydrolysis of all three cytoskeletal proteins and entry of propidium iodide and LDH release. Renal proximal tubules showed cell swelling and thinning and loss of the basal plasma membrane during oncosis, which was prevented by calpain inhibitors (X. Liu & R.G. Schnellmann, unpublished observations). These results support the idea that calpain proteolysis of paxillin may lead to increased plasma membrane permeability by destabilizing cytoskeletal support of the plasma membrane in the presence of an osmotic force.

Proteolysis of other cytoskeletal proteins also may occur and participate in other phases of plasma membrane permeability. For example, calpain proteolysis of spectrin contributes to ischemic neuronal membrane damage (129, 130). In many cell types, spectrin and ankyrin are associated with Na^+/K^+-ATPase and anchor the ion transporter to the basolateral membrane (131, 132). Ischemia produced significant loss of spectrin in rat brain, minimal loss of spectrin and profound loss of ankyrin in rat kidney, and no loss of spectrin or ankyrin in rat heart, suggesting that proteolysis of spectrin and ankyrin varies on the cell or tissue type (133). Proteolysis of spectrin and its related cytoskeleton may weaken the mechanical strength of the plasma membrane and make it more susceptible to the damage exerted by other factors, such as osmotic force. Although loss of cytoskeletal support to the plasma membrane alone is not sufficient for plasma membrane disruption, it is closely related to the formation of membrane blebs ATP-depleted renal cells (134, 135).

CALPAIN MEDIATION OF MITOCHONDRIAL DYSFUNCTION Mitochondria are involved in cell death when they are targets of an insult resulting in the loss of ATP production and through the release of apoptotic mediators [e.g., cytochrome c and apoptotsis inducing factor (AIF)] (136). The most common mitochondrial change during cell injury and death is the mitochondrial permeability transition (MPT) (137), which is defined as an increase in inner membrane permeability to solutes with a molecular weight $<$1500 Da (138). This phenomenon is observed after matrix accumulation of Ca^{2+} and is widely believed to be caused by the opening of a pore, a voltage-dependent and cyclosporin A–sensitive inner membrane channel of high conductance. The molecular composition of the pore remains uncertain, but it may be composed of the adenine nucleotide translocator (ANT) protein, cyclophilin D, and voltage-dependent anion chanel (VDAC) (139–142). Other proteins from the matrix, the intermembrane space, and the outer membrane also may contribute to the formation of the MPT pore (139, 141, 142). The pore is regulated at different sites by numerous factors, including depolarization, pH, membrane potential, matrix [Ca^{2+}] and [Mg^{2+}], conformation

of ANT, the oxidative status of glutathione, and the presence of cyclosporin A (136). Lemasters (137) suggested that the MPT is implicated in both oncosis and apoptosis.

The cellular mechanism leading to MPT during acute cell injury is not fully understood, although evidence indicates that Ca^{2+} and calpain may mediate MPT. For example, Peters et al. (143) observed a rise in cytosolic Ca^{2+} and collapse of mitochondrial potential in anoxic rat RPT. Aguilar et al. (144) demonstrated the presence of calpain-like activity in isolated liver mitochondria and that the calpain inhibitor Cbz-Leu-Leu-Tyr-CHN_2 inhibited Ca^{2+}- or TBHP-induced MPT in isolated mitochondria and delayed mitochondrial depolarization and cell death in t-butylhydroperoxide-exposed hepatocytes. In addition, Van Vleet et al. (145, 146) recently detected a mitochondrial calpain-like activity and associated this activity with mitochondrial dysfunction, inhibition of state 3 respiration. Gores et al. (147) demonstrated that a toxic bile salt caused oncosis of hepatocytes by inducing the MPT, and the Ca^{2+}-dependent MPT was associated with an increase in calpain-like protease activity, which was inhibited by calpain inhibitors in isolated liver mitochondria. Further, in an experimental model of cholestasis, mitochondrial calpain-like activity increased 1.6-fold, and the protease activity was found to be largely in the intermembrane space, suggesting that calpain-like protease modifies the external domains of proteins forming the MPT within the inner membrane (147).

How calpain mediates MPT during the oncotic death process is not clear. However, one recent line of evidence indicates that calpain may mediate MPT by modifying small proteins like Bcl-2, Bcl-X_L, Bax, or Bid (66, 148–151).

Is there a link among ER Ca^{2+}, calpain, and mitochondrial dysfunction during oncosis? Recent work showed that prior depletion of ER Ca^{2+} stores with thapsigargin, treatment with a Ca^{2+}-channel blocker (nifedipine), and calpain inhibition permitted the recovery of renal proximal tubule mitochondrial function following hypoxic injury (9–11; X. Liu & R.G. Schnellmann, unpublished observation). This suggests a possible link between ER Ca^{2+} release, calpain activation, and mitochondrial dysfunction. Based on the current available evidence, we propose a novel signal pathway in which the ER Ca^{2+} release leads to activation of calpain and influx of extracellular Ca^{2+}. Mitochondrial Ca^{2+} uptake leads to increased mitochondrial calpain activity, mitochondrial dysfunction, and cell death. Identification of the calpain-like protease and characterization of mitochondrial targets of this enzyme associated with mitochondrial dysfunction will greatly increase our understanding of oncotic cell death.

CALPAIN INHIBITORS IN IN VIVO MODELS Mounting evidence indicates that calpains are involved in ischemic/hypoxic-, toxicant-, and preservation/reperfusion-induced injury in the kidney, brain, liver, and myocardium. However, studies examining the protective effects of calpain inhibitors in animal models are limited (41–47, 50, 53–58, 61, 62, 64, 66, 67, 130, 152). Nevertheless, several of these studies have shown promising protective effects in vivo from dissimilar calpain

inhibitors. For example, the calpain inhibitor Cbz-Leu-Leu-Tyr-CHN_2 protected against ischemic and preservation-reperfusion liver injury and increased animal survival rates after liver ischemia and liver transplantation in rats (152, 153). In addition, a calpain inhibitor (Cbz-Val-Phe-H; MDL28170) protected against hippocampal neuron injury and death in Wistar rats and reduced focal cerebral ischemic injury in Sprague-Dawley rats (68, 154). Furthermore, a calpain inhibitor (CEP-4143) inhibited μ-calpain activation and cytoskeletal degradation, improved neurological function, and enhanced axonal survival after traumatic spinal cord injury in female Wistar rats (58). Finally, two recent reports showed evidence that calpain inhibitor I ameliorated both multiple organ failure produced by hemorrhagic shock and acute renal failure by ischemia/reperfusion in animal models (62, 64). These findings demonstrate that calpain(s) play a critical role in tissue injury and organ dysfunction in animal models and that calpain inhibitors may be potential therapeutic agents to reduce tissue injury and preserve organ functions.

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