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Mitochondrial Calcium and the Permeability Transition in Cell Death

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Abstract

Dysregulation of Ca^{2+} has long been implicated to be important in cell injury. A Ca^{2+} -linked process important in necrosis and apoptosis (or necroptosis) is the mitochondrial permeability transition (MPT). In the MPT, large conductance permeability transition (PT) pores open that make the mitochondrial inner membrane abruptly permeable to solutes up to 1500 Da. The importance of Ca^{2+} in MPT induction varies with circumstance. Ca^{2+} overload is sufficient to induce the MPT. By contrast after ischemia-reperfusion to cardiac myocytes, Ca^{2+} overload is the consequence of bioenergetic failure after the MPT rather than its cause. In other models, such as cytotoxicity from Reye-related agents and storage-reperfusion injury to liver grafts, Ca^{2+} appears to be permissive to MPT onset. Lastly in oxidative stress, increased mitochondrial Ca^{2+} and ROS generation act synergistically to product the MPT and cell death. Thus, the exact role of Ca^{2+} for inducing the MPT and cell death depends on the particular biologic setting.

Keywords

calcium; cyclosporin A; mitochondrial permeability transition; necroptosis; oxidative stress

INTRODUCTION

Free Ca^{2+} is a key signaling agent in eukaryotic cells. Hormonal and other stimuli activate phospholipase C, inositol triphosphate formation and mobilization of Ca^{2+} from the endoplasmic reticulum [3,5]. In excitable tissues, membrane depolarization also causes Ca^{2+} entry through voltage gated Ca^{2+} channels. Increased cytosolic free Ca^{2+} then activates protein kinases and in striated muscle stimulates release of Ca^{2+} from sarcoplasmic reticulum to activate muscle contraction. Influx of Ca^{2+} into the cytosol also leads to mitochondrial accumulation of Ca^{2+} and activation of respiratory dehydrogenases and oxidative phosphorylation [35]. Most unstimulated cells tightly regulate free Ca^{2+} in the range of 100 to 200 nM in both the cytosol and the mitochondria through action of membrane Ca^{2+} pumps in the plasma membrane and endoplasmic/sarcoplasmic reticulum, Na/Ca exchange across the plasma membrane and, to a lesser extent, mitochondrial Ca^{2+} uptake. Thus, cytosolic free

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Ca^{2+} is substantially lower than the 2 mM free Ca^{2+} present in extracellular fluids. With physiologic stimulation, however, free Ca^{2+} in both cytosol and mitochondria can rapidly and transiently increase by 10 to 20-fold as occurs on a beat to beat basis in cardiac myocytes due to mitochondrial Ca^{2+} uptake [48].

Dysregulation of Ca^{2+} homeostasis has long been implicated to play an important role in cell injury. Pathological Ca^{2+} overload and calcification are frequently features of tissue ischemia and infarction, and increased Ca^{2+} activates a number of phosphatases, proteases and nucleases. Early experimental studies in hepatocytes showed that removal of extracellular Ca^{2+} protects against various hepatotoxicants, suggesting that influx of extracellular Ca^{2+} is responsible for irreversible cell injury [13,44]. Since maintenance of Ca^{2+} gradients across the plasma membrane and between cellular compartments depends on ATP-driven reactions, metabolic disruption by injurious stresses may quickly perturb cellular Ca^{2+} homeostasis. In particular, release of Ca^{2+} from the endoplasmic reticulum may flood the cytosol with free Ca^{2+} , possibly leading to activation of degradative processes and dysfunction of other organelles, particularly mitochondria [39]. However, other early work indicated that cytosolic free Ca^{2+} does not rise preceding hypoxic cell death and therefore may not contribute to the pathogenesis of lethal cell injury after ATP depletion [32].

MITOCHONDRIAL PERMEABILITY TRANSITION

A Ca^{2+} -linked process important in many instances of both necrotic and apoptotic cell death is the mitochondrial permeability transition (MPT). In the MPT, large conductance permeability transition (PT) pores open that make the mitochondrial inner membrane abruptly permeable to all solutes of molecular weight up to about 1500 Da [2,21,25]. Ca^{2+} , numerous reactive chemicals and oxidative stress induce PT pore opening, whereas cyclosporin A (CsA) and pH less than 7 block opening. As a consequence of the MPT, mitochondrial depolarization, uncoupling of oxidative phosphorylation and large amplitude mitochondrial swelling driven by colloid osmotic forces occur.

The composition of PT pores remain controversial. In one model, the adenine nucleotide transporter (ANT) from the inner membrane, the voltage dependent anion channel (VDAC) from the outer membrane, the CsA binding protein cyclophilin D (CypD) from the matrix, and possibly other proteins form PT pores (reviewed in [10,17]). However, recent genetic knockout studies challenge the validity of this model by showing that the MPT still occurs in mitochondria that are deficient in ANT, VDAC and even CypD, although some properties of the MPT are altered [2,23,28,29]. For example in ANT deficient mitochondria, the ANT ligand, atractyloside, no longer promotes PT pore opening, whereas in CypD deficient mitochondria, MPT induction requires greater Ca^{2+} and is not blocked by CsA.

An alternative model proposes that damage by reactive chemicals, oxidative stress, thiol crosslinking and other perturbations cause misfolding of integral membrane proteins [18]. Misfolding creates hydrophilic surfaces that face the lipid bilayer, a thermodynamically unfavorable condition. Consequently, misfolded membrane proteins aggregate at these hydrophilic surfaces to create aqueous transmembrane channels, which are the nascent PT pores. To prevent catastrophic permeabilization and uncoupling of oxidative phosphorylation, CypD and other molecular chaperones acting from both sides of the inner membrane block conductance through these nascent PT pores. CypD like other cyclophilins is a cis-trans peptidyl proline isomerase, namely a foldase, which may have a physiological role of promoting refolding of the misfolded proteins back to their native state [51]. However, CypD binding also makes PT pores sensitive to reversible Ca^{2+} -induced opening, an action inhibited by CsA. When nascent PT pores formed by misfolded protein aggregates exceed the content of chaperones available to block their conductance, pore opening occurs in an unregulated

fashion. Such unregulated pore opening is insensitive to CsA inhibition and no longer requires Ca^{2+} to occur. In addition to CypD, other as yet poorly characterized chaperones are involved in regulating conductance by nascent PT pores. Indirect evidence suggest that the Rieske iron sulfur protein (RISP) and the heat shock protein Hsp27 may participate in regulating PT pore conductance [19,20]. In particular, dephosphorylation of RISP may be an important event promoting pore opening.

In the protein misfolding model, any membrane protein in principle might contribute to pore formation, but abundance and propensity to misfold determine the likelihood of a particular membrane protein to contribute to the MPT. As the most abundant inner membrane protein on a molar basis, ANT frequently is the target of damage causing misfolding. Moreover, ANT undergoes large conformation changes, and the atractyloside-stabilized conformation appears to be particularly vulnerable to damage leading to misfolding and PT pore formation (reviewed in [33]). Other proteins can also misfold and contribute to PT formation. For example, both old and recent evidence suggests that other anion transporters, such as the aspartate-glutamate and phosphate carriers, participate in formation of large conductance pores [11,12,33,34,45]. Because PT pores can form from these other membrane proteins, the MPT still occurs in ANT deficient mitochondria [28].

The misfolded protein model also accounts for why completely exogenous pore-forming peptides like mastoparan and alamethicin form CsA-sensitive PT pores [18,41]. Chaperones recognize and regulate mastoparan and alamethicin pores in the same way as endogenous PT pores. When pores formed by exogenous pore forming peptides are at low concentration, chaperones block their conductance, but these regulated pores open in response to increased matrix Ca^{2+} in a CsA sensitive fashion. However, when the concentration of exogenous pore formers exhaust the supply of chaperones, constitutively open CsA insensitive pores form even in the absence of Ca^{2+} . Such behavior for mastoparan and alamethicin is confirmed experimentally [18]. Also as predicted by the misfolded protein model, mitochondria from CypD knockout mice are desensitized to Ca^{2+} induction of the MPT and require much higher Ca^{2+} to induce mitochondrial swelling [1], which is consistent with the proposed role of CypD as the Ca^{2+} sensor of the MPT.

Kinetic analysis of mitochondrial swelling after the MPT suggests that the diameters (conductances) of individual pores are heterogeneous [36]. Such variation is consistent with a variable protein composition for individual PT pores. Protein misfolding promoting PT pore formation should not be equated with irreversible protein denaturation. Indeed, reversal of protein misfolding may be an important physiological function of CypD. When ANT is the principal misfolded membrane protein contributing to PT pore formation, bongkreikic acid by inducing the more stable m-conformation of ANT may also be effective in reversing misfolding and pore opening [17].

MULTIPLE ROLES OF CALCIUM IN PERMEABILITY TRANSITION-DEPENDENT CELL INJURY

Many chemicals and radicals promote the MPT. Typically the effect of such inducers is to decrease the threshold amount of Ca^{2+} needed to cause PT pore opening. In pathological settings where the MPT contributes to cell killing, Ca^{2+} may have several roles. First, increased Ca^{2+} alone and its uptake into mitochondria may cause MPT onset. Second, other stressors may decrease the threshold for the Ca^{2+} -induced MPT such that Ca^{2+} need not change but is still permissive for MPT onset. Lastly, stressors and increased Ca^{2+} may act synergistically to induce the MPT.

Induction of the MPT and cell death by calcium overload

That increased Ca^{2+} alone can cause the MPT and cell death is supported by experiments with Br-A23187, a Ca^{2+} ionophore [42]. In cultured rat hepatocytes, Br-A23187 causes rapid mitochondrial depolarization and inner membrane permeabilization, as shown by confocal microscopy from mitochondrial release of tetramethylrhodamine methylester (TMRM), a potential-indicating fluorophore, and mitochondrial uptake of calcein, a 623 Da fluorophore that is normally impermeant to the inner membrane. After the Br-A23187-induced onset of the MPT, necrotic cell death occurs from ATP depletion. However, if an alternative source of ATP as from glycolysis, then necrosis is prevented. Instead, caspase-dependent apoptosis occurs secondary to release of cytochrome *c* and other proapoptotic factors from the mitochondrial intermembrane space after outer membrane rupture. CsA prevents both necrosis and apoptosis induced by Br-23187, confirming the important role of the MPT in both phenotypes of cell death. Similarly after MPT-dependent ischemia-reperfusion injury, the presence or absence of ATP determines whether apoptosis or necrosis occurs [27]. The term, necroapoptosis, describes such death processes that begin with a common death signal or toxic stress, progress by shared pathways, and culminate in either plasma membrane failure (necrosis) or programmed cellular resorption (apoptosis) depending on other modifying factors [30,31].

Permissive role of calcium in cell death caused by Reye-related toxicants

Reye's and Reye-related syndromes are often fatal illnesses characterized by high fever, fulminant hepatic failure and encephalopathy in children and sometimes adults in association with ingestion of aspirin, valproic acid, unripened fruit of the ackee tree and other toxicants [40]. Electron microscopy of liver and brain from these patients shows large-amplitude mitochondrial swelling, suggestive on onset of the MPT. In isolated liver mitochondria, Reye-related toxicants do not by themselves induce the MPT but instead lower the Ca^{2+} threshold for the MPT [49].

Salicylate, the active metabolite of aspirin, is directly cytotoxic to cultured rat hepatocytes [50]. CsA blocks this cytotoxicity, and confocal microscopy shows that CsA-sensitive mitochondrial depolarization and inner membrane permeabilization occur after salicylate exposure. A permissive role of Ca^{2+} in salicylate toxicity is shown by cytoprotection with the Ca^{2+} channel blocker, verapamil, an effect associated with decreased mitochondrial-free Ca^{2+} measured by confocal microscopy using the Ca^{2+} indicator, Rhod-2. Similarly in support of a permissive role for Ca^{2+} , increased extracellular Ca^{2+} promotes salicylate-induced cell killing. Synergism between salicylate and Ca^{2+} may help account for the idiopathic nature of Reye's syndrome. The vast majority of individuals taking aspirin experience no liver or brain toxicity, but children with a prodromal viral illness may incur higher mitochondrial Ca^{2+} loading, predisposing them to MPT-dependent illness after exposure to salicylate, valproic acid and other Reye-related toxicants.

CALCIUM AND OXIDATIVE STRESS-INDUCED INJURY

The short-chain hydroperoxide, *tert*-butylhydroperoxide (TBH), is an analog of the lipid hydroperoxides that form during oxidative stress and ischemia-reperfusion. TBH is detoxified to *t*-butanol with oxidation of glutathione, NADPH, and NADH by the concerted action glutathione peroxidase, glutathione reductase, and the mitochondrial NADP/NAD transhydrogenase [4,46]. In isolated mitochondria, TBH is a potent inducer of the MPT [16]. Similarly in cultured hepatocytes, TBH induces MPT-dependent cell killing [22]. After TBH, confocal microscopy reveals sequential mitochondrial NAD(P)H oxidation, increased mitochondrial free Ca^{2+} , intramitochondrial generation of reactive oxygen species (ROS), mitochondrial depolarization and inner membrane permeabilization, as monitored with autofluorescence, Rhod-2, dichlorofluorescein, TMRM and calcein, respectively (Fig. 1) [6,

37,38]. These events culminate in ATP depletion and cell death. Chelation of intramitochondrial Ca^{2+} with BAPTA-AM prevents the increase of mitochondrial free Ca^{2+} and also blocks increased mitochondrial ROS formation but not early oxidation of mitochondrial NAD(P)H. By contrast, inhibition of ROS generation with desferal or diphenylphenylene diamine delays, but does not block, increased mitochondrial Ca^{2+} after TBH. Thus, NAD(P)H oxidation leads to mitochondrial Ca^{2+} loading, which in turn promotes ROS formation and the MPT. Indeed, Ca^{2+} loading of isolated mitochondria promotes ROS formation, although the mechanism for this remains obscure [7]. In this way, increased mitochondrial Ca^{2+} and ROS generation act synergistically to induce the MPT in this model of oxidative stress.

CALCIUM, OXIDATIVE STRESS AND MPT-DEPENDENT ISCHEMIA-REPERFUSION INJURY IN CARDIAC MYOCYTES

In cardiac myocytes, both cytosolic and mitochondrial free Ca^{2+} increase transiently during the contractile cycle as part of excitation-contraction coupling and possibly to stimulate mitochondrial ATP production on a beat to beat basis [8,48]. Since MPT-dependent ischemia-reperfusion injury is associated with Ca^{2+} overload and hypercontracture, a reasonable expectation is that Ca^{2+} dysregulation promotes MPT onset and ensuing cell death. After about 20 to 40 min of reperfusion of ischemic cardiac myocytes, the MPT occurs as shown by mitochondrial depolarization and movement of calcein through the mitochondrial inner membrane (Fig. 2) [26]. MPT onset after reperfusion is preceded by mitochondrial ROS formation and followed quickly by hypercontracture and cell death (Fig. 3). Antioxidants and MPT blockers like CsA and its nonimmunosuppressive analog, N-methyl-4-isoleucine cyclosporin (NIM811), prevent the MPT, depolarization, hypercontraction, and cell killing (Fig. 2). Antioxidants but not MPT blockers inhibit ROS formation after reperfusion, signifying that ROS generation is upstream of MPT induction (Fig. 3).

Although both cytosolic and mitochondrial free Ca^{2+} increase markedly during ischemia, both Ca^{2+} pools recover to near normal in the first few minutes after reperfusion (Fig. 4). Late after reperfusion, mitochondrial and cytosolic Ca^{2+} overloading again occur, but CsA and NIM811 block this late Ca^{2+} overloading (Fig. 4). Moreover, intramitochondrial Ca^{2+} chelation by cold loading/warm incubation of BAPTA did not decrease cell death after reperfusion. These data indicate that mitochondrial ROS generation after reperfusion promotes MPT onset and subsequent myocyte death. Unexpectedly, late Ca^{2+} overloading is not a factor inducing the MPT after reperfusion. Rather, Ca^{2+} overloading is a consequence of bioenergetic failure following MPT onset. Since mitochondrial BAPTA loading fails to suppress cell death after reperfusion, mitochondrial Ca^{2+} appears not to have even a permissive role for onset of the MPT after reperfusion of cardiac myocytes [26].

ROLE OF THE MITOCHONDRIAL PERMEABILITY TRANSITION IN STORAGE-REPERFUSION INJURY TO TRANSPLANTED LIVER GRAFTS

Because cardiac myocytes experience constant fluctuations of free Ca^{2+} , their mitochondria may adapt to become resistant to Ca^{2+} induction of the MPT. Such an adaptation may not occur in non-excitatory tissues, such as liver, which are also vulnerable to MPT-dependent ischemia-reperfusion injury [27,43]. A clinically important form of ischemia-reperfusion injury to liver is that associated with cold ischemic storage of livers for transplantation surgery and which can lead to liver graft dysfunction and failure. When rat livers are stored 18 h in University of Wisconsin cold storage solution and transplanted, intravital (*in vivo*) confocal/multiphoton microscopy reveals mitochondrial depolarization and loss of cell viability of hepatocytes at 4 h after implantation in association with transaminase release and subsequent development of

histological necrosis [47]. Within individual hepatocytes, mitochondria depolarization is an all or nothing phenomena. Graft and recipient treatment with the non-immunosuppressive MPT blocker, NIM811, prevents mitochondrial depolarization, decreases cell killing and transaminase release and improves graft survival. NIM811 also decreases apoptosis after transplantation as assessed by deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) and caspase 3 measurements. Thus, the MPT plays an important role in storage-reperfusion injury to liver grafts.

Minocycline is a semisynthetic tetracycline antibiotic shown to be protective in neurodegenerative disease, trauma, and hypoxia–ischemia [9,14,15,24,52–54,54,55]. Cytoprotective mechanisms proposed for minocycline include inhibition of apoptotic pathways, decreased mitochondrial release of pro-apoptotic factors such as cytochrome *c* [55], upregulation of antiapoptotic proteins [52], inhibition of caspase-1 and caspase-3 activation and suppression of cytochrome *c* release [52,55]. After cold storage, reperfusion and transplantation of rat livers, minocycline but not tetracycline decreases graft injury, improves graft survival, minimizes mitochondrial depolarization and decreases cell death as effectively as NIM811. In isolated rat liver mitochondria, minocycline and NIM811 but not tetracycline block the MPT. By contrast to NIM811 which does not block mitochondrial Ca^{2+} uptake, minocycline blocks onset of the MPT by inhibiting mitochondrial Ca^{2+} uptake (Fig. 5). Thus, minocycline represents a new inhibitor class for the mitochondrial electrogenic Ca^{2+} uniporter. An implication of minocycline protection against the MPT after storage-reperfusion graft injury is that Ca^{2+} plays a role in MPT induction in this model. So far, it has not been possible to visualize cytosolic and mitochondrial free Ca^{2+} in this *in vivo* model, but previous work in cultured hepatocytes indicates that cytosolic free Ca^{2+} does not increase during hypoxia [32]. Thus, Ca^{2+} may be permissive in MPT onset after storage-reperfusion with other factors such as ROS formation decreasing the threshold for a Ca^{2+} -induced MPT.

CONCLUSION

While a role for the MPT in the pathobiology of cell death is strongly supported by experimental data for a number of models of cell injury, the importance of Ca^{2+} in MPT induction seems to vary with the particular set of circumstances. Certainly, Ca^{2+} overload when it occurs can be sufficient to induce the MPT, as occurs with cytotoxicity from the Ca^{2+} ionophore Br-A23187. By contrast after reperfusion of ischemic cardiac myocytes, Ca^{2+} overload is the consequence of bioenergetic failure after MPT onset rather than its cause. Even intracellular Ca^{2+} chelation does not suppress MPT onset and subsequent cell death after reperfusion of cardiac myocytes. In other models, such as cytotoxicity from Reye-related agents and storage-reperfusion injury to liver grafts, Ca^{2+} appears to be permissive to MPT onset. Reye-related toxicants lower the threshold for Ca^{2+} induction of the MPT, and modulation of cellular Ca^{2+} up or down has a corresponding effect on Reye toxicant-induced cell death. In liver grafts, minocycline protects against MPT onset and graft failure apparently by inhibiting mitochondrial Ca^{2+} uptake. The implication is that Ca^{2+} is at minimum permissive for MPT onset in the liver grafts. Lastly in a model of oxidative stress with TBH, increased mitochondrial Ca^{2+} and ROS generation act synergistically to product the MPT and cell death. Thus, Ca^{2+} can be involved in MPT onset in a number of ways, but the exact role of Ca^{2+} depends on the particular biologic setting.

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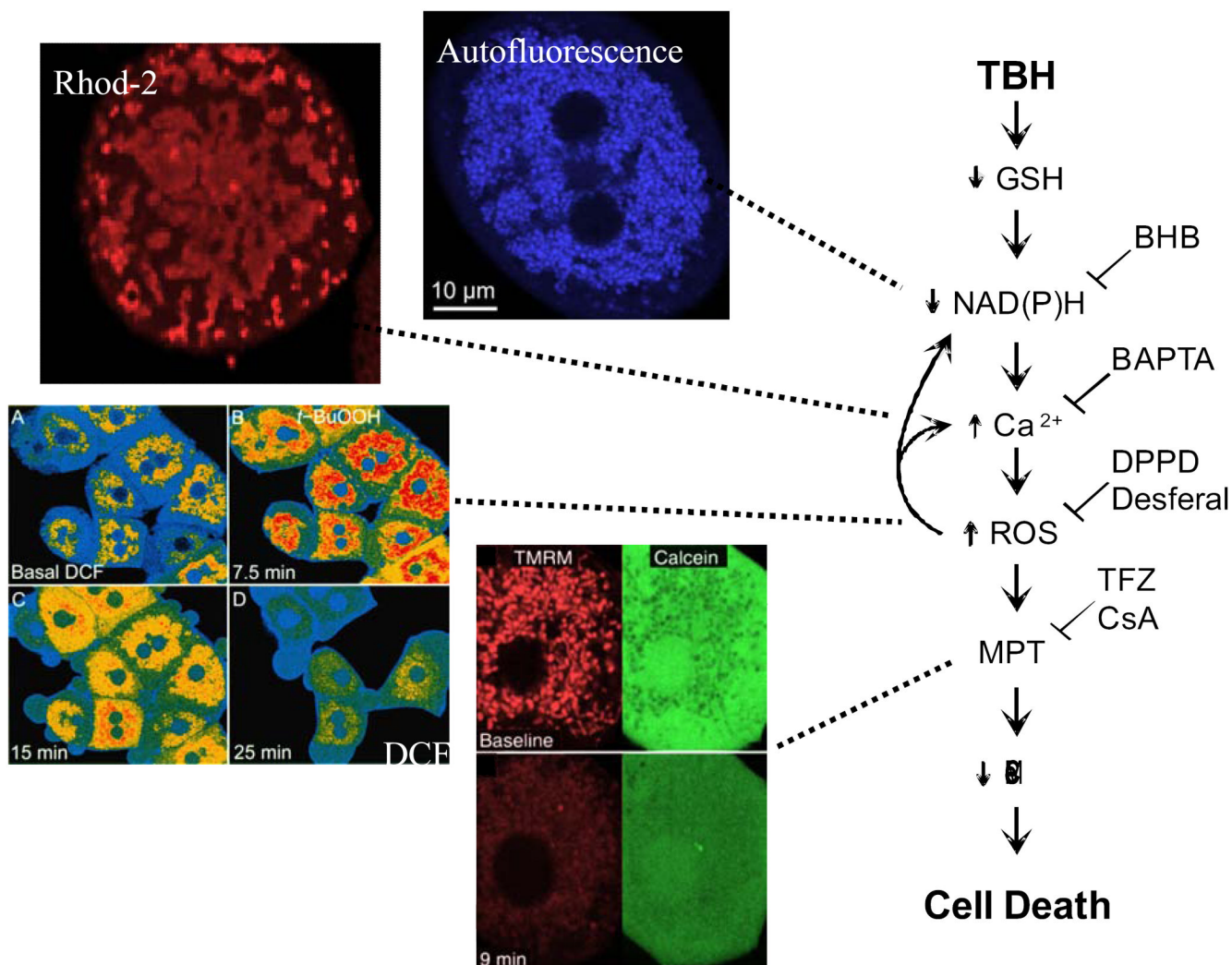


Figure 1. Changes leading to onset of the MPT after oxidative stress to hepatocytes

After exposure of hepatocytes to TBH, a sequence of events occurs: oxidation of GSH and NAD(P)H (autofluorescence), increased mitochondrial free Ca^{2+} (Rhod2), increased mitochondrial ROS formation (dichlorofluorescein [DCF]), the MPT with inner membrane permeabilization (calcein) and mitochondrial depolarization (TMRM), and cell death. The MPT is prevented or delayed by increased reduction of NAD(P)H with β-hydroxybutyrate (BHB), mitochondrial Ca^{2+} chelation with BAPTA, antioxidants (diphenylphenylene diamine [DPPD] and desferal) and blockers of the MPT (trifluoperazine [TFZ] and CsA). Adapted from [6,37,38].

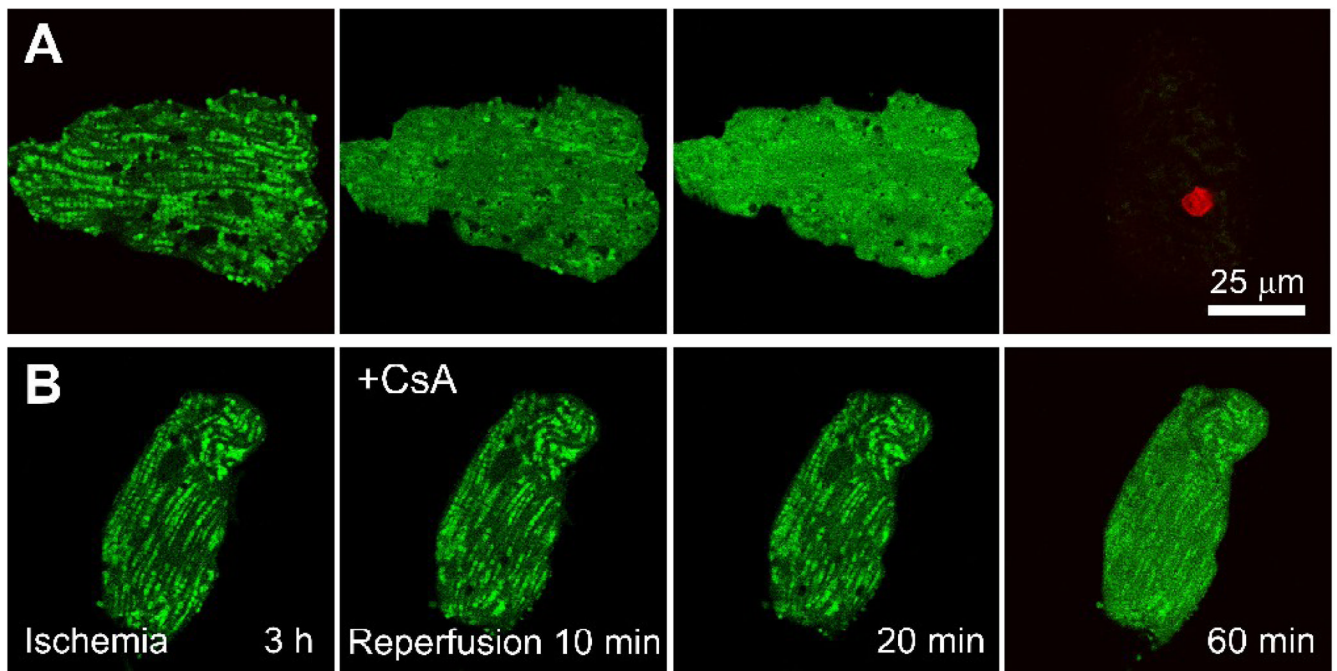


Figure 2. The MPT and cell death after ischemia-reperfusion to adult rat cardiac myocytes Mitochondria of myocytes were loaded with calcein by a cold-loading/warm incubation procedure, and the cells were subjected to 3 h of anoxia at pH 6.2 (ischemia) followed by reoxygenation at pH 7.4 (reperfusion) in the absence (A) and presence (B) of 1 μ M CsA. Nuclear staining by red-fluorescing propidium iodide (PI) detected loss of cell viability. Note that green mitochondrial calcein fluorescence was retained at the end of ischemia, indicating that PT pores were closed. After reperfusion in the absence of CsA (A), mitochondria released calcein into the cytosol beginning after as early as 10 min. After 60 min, all cellular calcein was lost, and PI nuclear staining signified loss of cell viability. In the presence of CsA added at reperfusion (B), mitochondrial release of calcein was greatly diminished and viability was maintained for more than an hour. Adapted from [26].

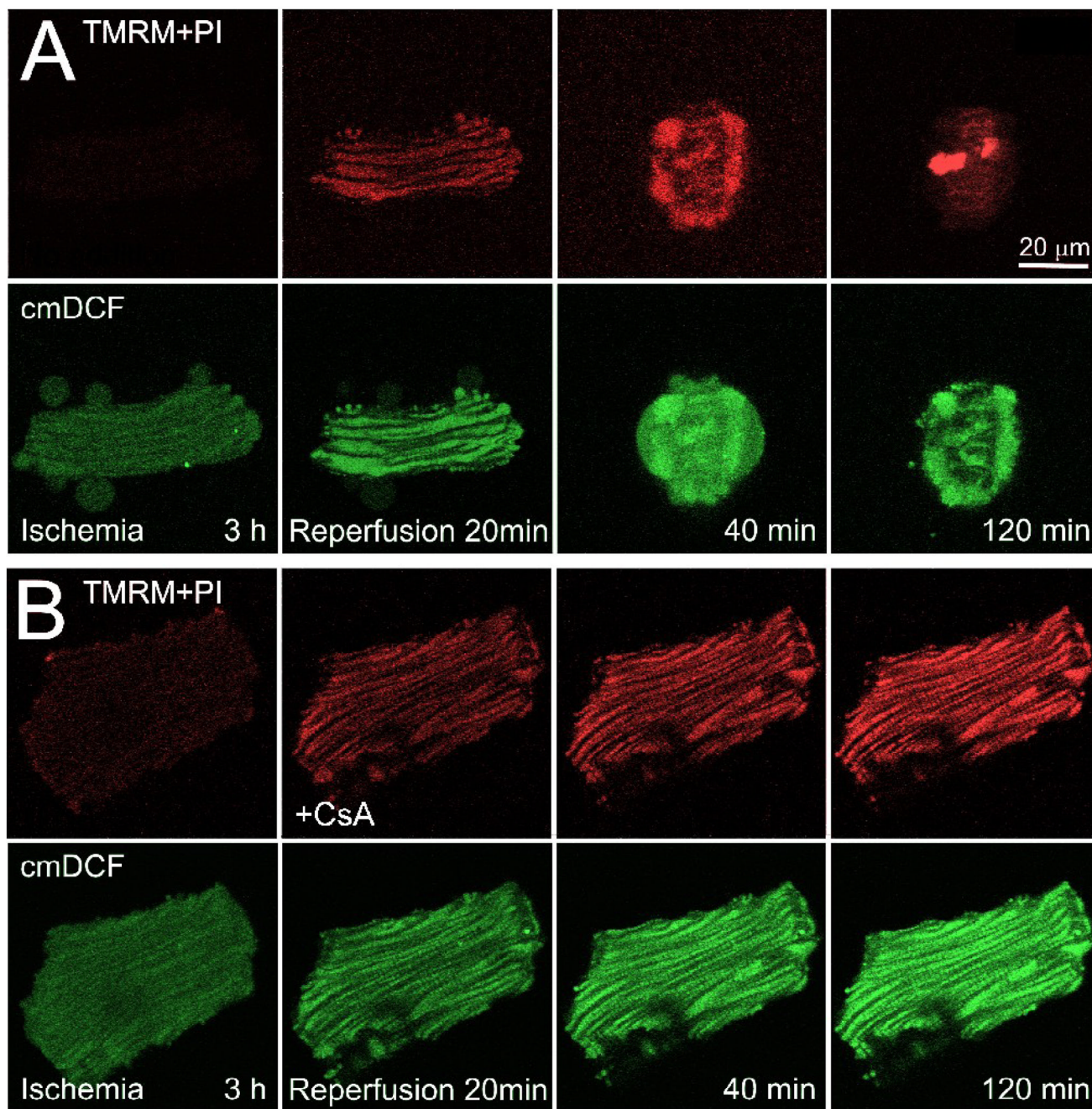


Figure 3. Cyclosporin A-insensitive formation of mitochondrial ROS after reperfusion
 Myocytes were co-loaded with TMRM and chloromethyl-dihydrodichlorofluorescein (cmDCF) to monitor mitochondrial membrane potential and ROS formation, respectively, and subjected to ischemia-reperfusion as described in Fig. 2. Note loss of red TMRM fluorescence after 3 h of ischemia, signifying depolarization. After reperfusion, mitochondria accumulated TMRM, indicating repolarization, and green cmDCF fluorescence increased progressively inside mitochondria. In the absence of CsA (**A**), the myocyte hypercontracted and depolarized after 40 min, and lost viability after 120 min, as shown by nuclear PI labeling. When myocytes were reperfused in the presence of CsA (**B**), mitochondria repolarization was sustained, and cell death did not occur, but mitochondrial cmDCF still increased. Adapted from [26].

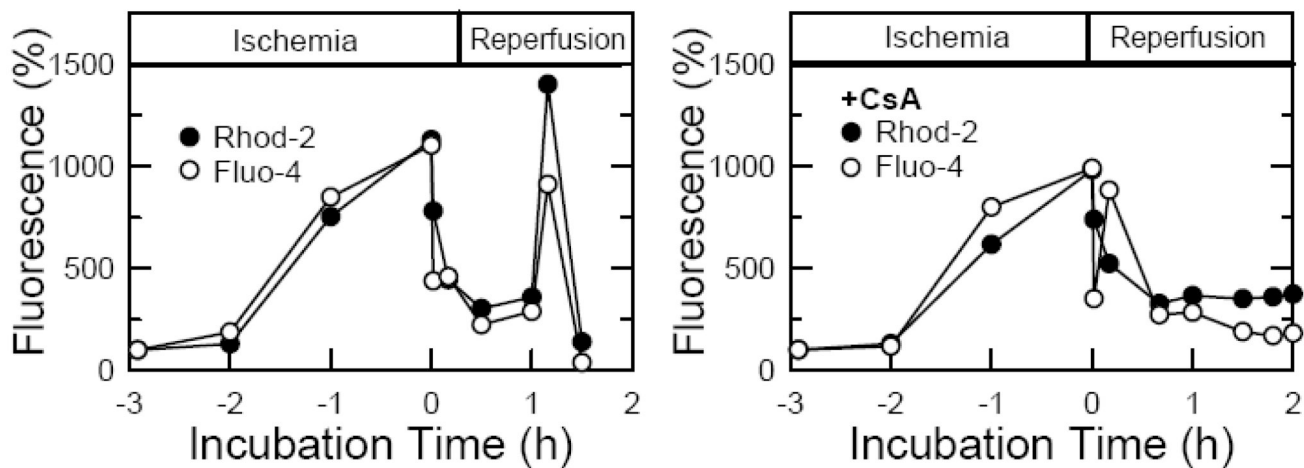


Figure 4. Change of cytosolic and mitochondrial free Ca^{2+} changes during ischemia-reperfusion to cardiac myocytes

Relative changes of mitochondrial and cytosolic Ca^{2+} were monitored with Rhod-2 and Fluo-4, respectively, during ischemia-reperfusion to cardiac myocytes. Adapted from [26].

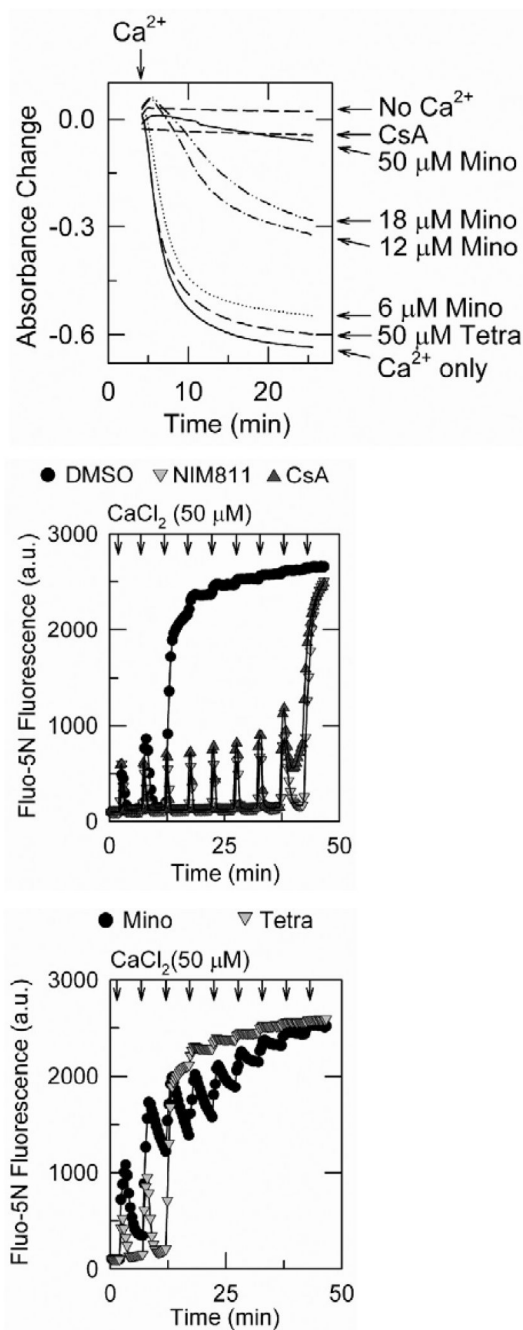


Figure 5. Minocycline inhibits calcium uptake and onset of the MPT in isolated rat liver mitochondria

In the top panel, onset of the MPT was monitored by mitochondrial swelling as determined from decreased absorbance. CaCl₂ (250 μM) was added as indicated in mitochondria pretreated with minocycline (Mino), tetracycline (Tetra), 1 μM CsA or no addition. In the middle and lower panels, extramitochondrial free Ca²⁺ was measured by Fluo-5N fluorescence. As indicated, mitochondria were pre-incubated with vehicle, 5 μM NIM811, 1 μM CsA, 18 μM minocycline or 18 μM tetracycline. CaCl₂ was then added in 50 μM increments. Note that minocycline inhibits mitochondrial swelling (the MPT) in a dose-dependent fashion. Minocycline also inhibits mitochondrial accumulation after each Ca²⁺ pulse. In the absence of

minocycline, mitochondria rapidly accumulate each pulse of Ca^{2+} until onset of the MPT when all accumulated Ca^{2+} is abruptly released. Adapted from [47].