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Redox regulation of ER and mitochondrial Ca^{2+} signaling in cell survival and death

Suresh K. Joseph*, David M. Booth, Michael P. Young, György Hajnóczky

MitoCare, Department of Pathology and Cell Biology, Thomas Jefferson University, Philadelphia, PA, 19107, USA

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ABSTRACT

Physiological signaling by reactive oxygen species (ROS) and their pathophysiological role in cell death are well recognized. This review focuses on two ROS targets that are key to local Ca^{2+} signaling at the ER/mitochondrial interface – notably, inositol trisphosphate receptors (IP_3Rs) and the mitochondrial calcium uniporter (MCU). Both transport systems are central to molecular mechanisms in cell survival and death. Methods for the measurement of the redox state of these proteins and for the detection of ROS nanodomains are described. Recent results on the redox regulation of these proteins are reviewed.

1. Introduction

Both Ca^{2+} and reactive oxygen species (ROS) are key players in mechanisms causing cell death. In many instances these factors do not operate independently, but work in a concerted manner to regulate multiple targets. Perhaps, the most important example is the role that Ca^{2+} and ROS play in the opening of the mitochondrial permeability transition pore (PTP) [1,2]. Mitochondrial oxidative metabolism is a major source of endogenous ROS and it is well established that mitochondrial Ca^{2+} accumulation enhances metabolic flux and ROS generation [3,4]. It is also clear that redox changes caused by ROS generation can regulate the activity of multiple Ca^{2+} transporting proteins and in particular, stimulates the activity of Inositol trisphosphate receptor (IP_3R) channels that traverse the ER membrane (reviewed in [5–8]), and the mitochondrial calcium uniporter (MCU) which mediates -selective access to Ca^{2+} in the mitochondrial matrix [9]. Thus, mitochondrial uptake of Ca^{2+} released from IP_3Rs promotes ROS generation, which in turn enhances MCU channel activity (an interplay depicted in cartoon form in Fig. 1). The membranes of the ER and the mitochondria are tethered in close proximity such that Ca^{2+} release and ROS generation occurs in a constricted microenvironment. This has the potential to amplify signaling or to engage a positive feedback loop that would promote the opening of the PTP, and trigger cell death. The amount of Ca^{2+} available for release from the ER is also determined by the activity of SERCA pumps that are also redox-sensitive [10]. There have been several recent reviews that have covered ROS regulation of distinct Ca^{2+} transport proteins and the general role of Ca^{2+} in cell death pathways [11–15]. The present review is confined to a discussion of recent studies that provide insights into the redox regulation of IP_3Rs and MCU channels.

2. Methods for the measurement of IP_3R redox state

It has generally been assumed that oxidative stress affects IP_3R channels by altering the redox state of critical thiols on the IP_3R protein. However, this has not been directly demonstrated, mostly because of the intrinsic experimental difficulties of measuring this parameter. The IP_3R family are large tetrameric proteins and in the case of the IP_3R1 isoform contains 60 cysteines/monomer. Any method to analyze IP_3R thiol redox state needs to minimize artefactual changes occurring during the experimental procedures. The benefits and disadvantages of a number of experimental methods that have been utilized are described below.

2.1. Gel-shift assays

Leichert and Jakob [16] have described a general procedure for the measurement of the redox state of a protein that can be applied to IP_3Rs. The method is outlined in Fig. 2A. The critical initial step is the lysis of cells directly in 10% TCA to prevent any alteration of thiol

Abbreviations: IP_3, Inositol-1,4,5-trisphosphate; IP_3R, IP_3 receptor; PTP, permeability transition pore; RyR, ryanodine receptor; CIB1, calcium and integrin-binding protein 1; Nox4, NADPH oxidase 4; Ask1, apoptosis signal-regulating kinase 1; Ire1α, inositol requiring enzyme 1; TMX, transmembrane thioredoxin-related protein; Traf2, TNF receptor associated factor 2; Jnk1, c-Jun N-terminal kinase; MPEG, Methoxypolyethylene glycol maleimide; ROS, reactive oxygen species

* Corresponding author at: MitoCare, Dept of Pathology, Thomas Jefferson University, Room 527E, 1020 Locust St, Philadelphia, PA19107, USA.
E-mail address: Suresh.joseph@jefferson.edu (S.K. Joseph).

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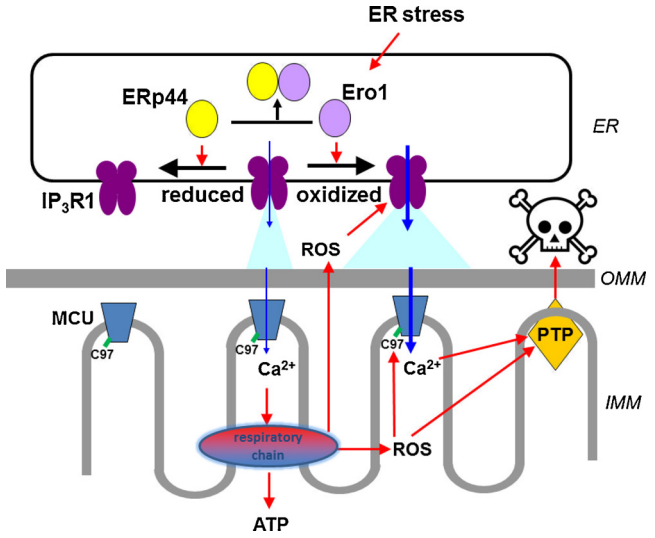

Fig. 1. Model of redox regulation of Ca^{2+}signaling at the ER-mitochondrial interface.
IP_3Rs are shown as undergoing reduction or oxidation. The binding of the ER luminal chaperone ERp44 favors the reduced species and inhibits channel function. Oxidative conditions induced by Ero1α stimulate channel function by altering the redox state of IP_3R intraluminal thiols and/or by binding to ERp44. Oxidation of critical thiols in the cytosolic domain of the IP_3R makes a major contribution to stimulation of channel activity. Enhanced efflux from the ER is directed into the mitochondria facilitated by MCU which itself is redox sensitive by a mechanism involving the cysteine-97 residue. Depending on the magnitude of the mitochondrial Ca^{2+}uptake and the concomitant ROS production, the mitochondria can generate more ATP or open the PTP as a prelude to cell death.

status. The precipitated protein is solubilized under strongly denaturing conditions (0.5% SDS, 6 M Urea) and the reduced pool of free thiols are blocked irreversibly by reaction with 10 mM iodoacetamide (IAM). The samples are then reprecipitated with TCA and the oxidized subset of thiols is reduced by addition of 10 mM DTT. A further TCA precipitation is used to remove the DTT and the lysates can then be subjected to several alternative procedures. One option is to react the free thiols (corresponding to the oxidized thiols in the original acid quenched protein) with Polyethyleneglycol maleimides (MPEG) which are available in different sizes. The resulting gel shifts can be measured on SDS-PAGE after immunoblotting for IP_3R. An example using the 2 and 5 kDa MPEG is shown in Fig. 2B. The data show that small gel shifts are observed for IP_3R1 transfected into HEK293 cells under basal conditions with both MPEGs. The shifts are enhanced when the cells are treated with the oxidant thimerosal, an organomercurial compound well documented to sensitize IP_3R channels [17–19]. Smaller effects are observed in response to H_2O_2 treatment of the cells indicating reaction with fewer IP_3R1 thiols. The exact number of reactive thiols is difficult to quantitate from the gel shifts because of anomalous migration of MPEGs on SDS-PAGE [20,21]. Thus, the method provides only a qualitative assessment of IP_3R redox state. Additional disadvantages include a low sensitivity and the possibility that oxidized cysteines are missed because of lack of reactivity to bulky MPEGs, despite the use of denaturing conditions. Nevertheless, gel-shift assays provide a simple first step for the analysis of multiple samples allowing the redox state of IP_3Rs to be monitored in their native environment in an intact cell. A recent modification of the method uses single-stranded DNA maleimide as the gel-shift agent [22]. This avoids the anomalous mobility of the MPEG molecule on SDS PAGE. The further development of UV-sensitive in-gel detachment of the DNA has also improved the transfer of modified proteins for immunoblotting [23].

2.2. Mass-spectrometry

The procedure used for mass-spectrometry is the same as for gel-shift assays except that biotin maleimide (0.5 mM; 16 h) is used as the reactant to label free thiols in the final step after DTT treatment (Fig. 2A). The sample is then subjected to gel filtration on a PD-10 column to exchange the denaturing buffer with an alternative buffer that allows efficient immunoprecipitation of the IP_3R. Immunocomplexes are processed on 5% SDS-PAGE and stained with silver or with colloidal blue. The prominent IP_3R band can be excised for in-gel trypsin digestion and LC/MS-MS. Variable modifications to be identified in the peptide spectra include cysteine alkylation by iodoacetamide (+57.02 Da), for reduced thiols, and reaction with biotin maleimide (+451.54 Da), for oxidized thiols. An example of the results from these analyses is shown in Fig. 2C. Clearly mass spectrometry provides the most detailed information but also has several drawbacks. The principal problem is the incomplete sequence coverage of this large protein (71 ± 2% (n = 10)). The lack of coverage was not random since the same peptides were consistently missing in multiple trials.

2.3. Functional assays

A straightforward approach to identifying redox-sensitive thiols is to mutate selected cysteines and transfect them into a cell which has no IP_3R isoforms. Kurosaki and coworkers utilized the unique DNA recombination properties of the chicken DT40 B-cell line to generate a cell line lacking all 3 IP_3R isoforms (3KO) [24]. These have proven a valuable resource in the Ca^{2+} signaling field [25]. However, these cells transfect poorly and stable cell lines are difficult to generate for large numbers of cysteine mutants. In addition, the cells are likely to display regulatory features that are unique to the avian lymphocyte which may not be generally applicable to mammalian cells. More recently, the Yule laboratory have used CRISPR technology to produce a 3KO human cell line (HEK293) lacking all 3 IP_3R isoforms [26]. These cells are easy to transfect and can be used to design functional experiments to screen cysteine mutants. The 3KO cells show no cytosolic Ca^{2+}elevations in response to carbachol acting on endogenous Ca^{2+}mobilizing muscarinic M3 receptors (not shown; [26,27]). Transfection with IP_3R1 restores the response and pretreatment with low concentrations of thimerosal for short periods (10μM for 2 min) enhances the response to a sub-saturating concentration of carbachol (0.25μM) (Fig. 3A). Thimerosal potentiation of the carbachol response is lost when cells are transfected with an IP_3R1 construct in which 12 cysteines in the N-terminus have been mutated to serine [28] (Fig. 3B). Both wild-type and Cys-less mutant are expressed in equivalent amounts (Fig. 3C). These findings are discussed in more detail in Section 4.

2.4. IP_3R-linked probes and ROS nanodomains

The closely apposed ER and mitochondria form a physically tethered interface separated by a restricted volume of cytosol to which IP_3Rs are preferentially exposed [29,30]. This volume hosts elevated concentrations of Ca^{2+}, as revealed by sensors targeted to the outer mitochondrial membrane [31]. It is also possible to fuse sensors into a drug-inducible synthetic linker that concentrates fluorescent probes specifically to organellar contact sites, such as the ER-mitochondrial interface. An example of this strategy is to exploit the FKBP12/FRB protein association induced by rapamycin [32]. Such targeting promotes dramatic enrichment of information from interface regions that are below the spatial resolution of conventional fluorescence microscopy. This approach was used to directly measure Ca^{2+} from agonist-evoked IP_3R release events at > 10-fold the levels of the bulk cytosol [33]. To probe the redox environment within the ER-mitochondrial interface, the H_2O_2 sensor HyPer [34] was substituted into the linker system to make measurements at rest and during physiological IP_3-linked Ca^{2+} signals [35]. The interface hosts modest elevations of H_2O_2

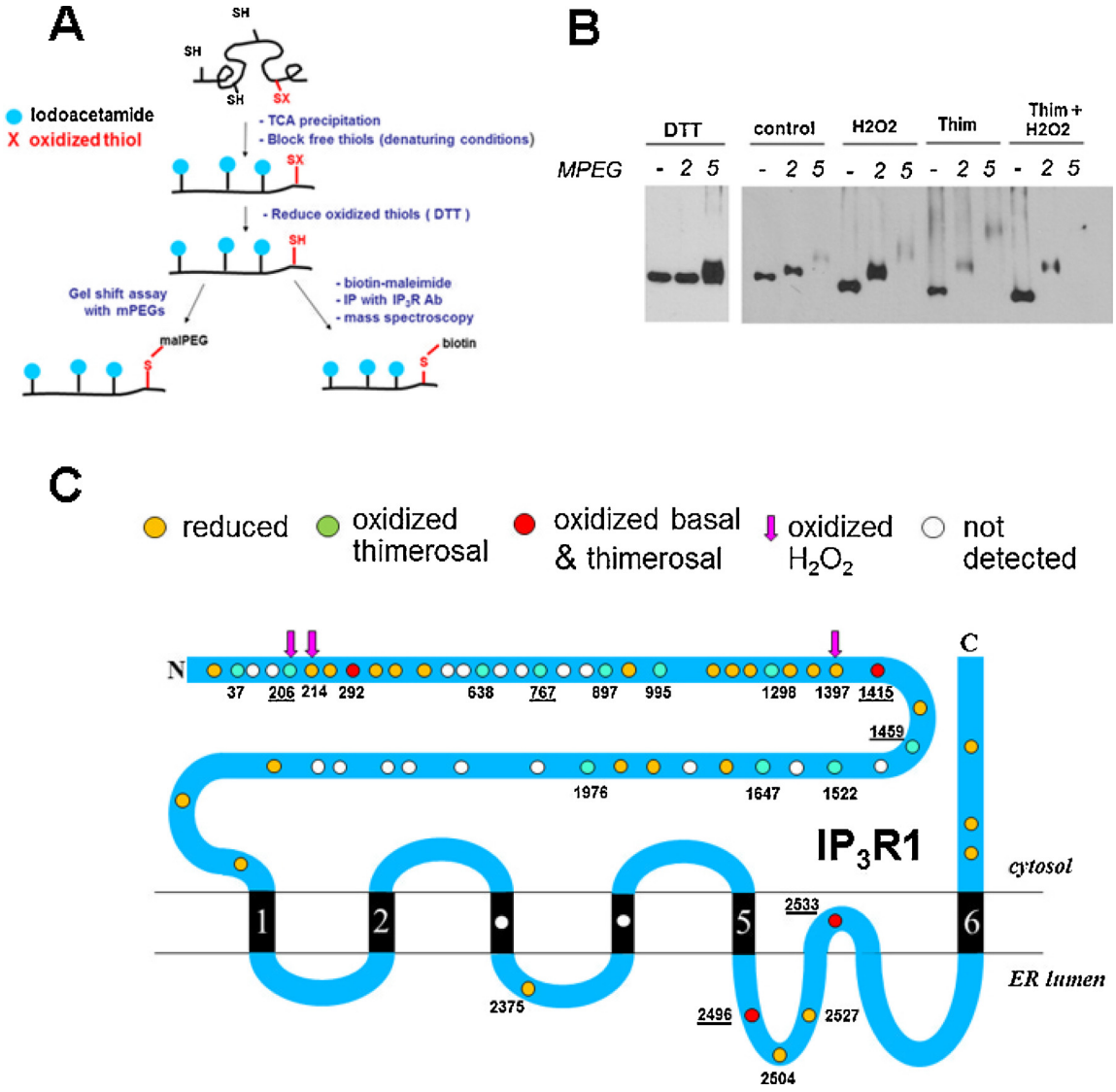

Fig. 2. Methods for measuring the redox state of IP_3Rs in situ.
Panel A. Work-flow diagram showing the steps used for measuring the redox state of IP_3Rs by gel-shift assay or LC–MS/MS. *Panel B.* Representative gel-shift assays of HEK293 cells transfected with IP_3R1 and treated for 10 min with H_2O_2 (0.5 mM) or thimerosal (50μM). TCA precipitated lysates were processed as described in Panel A and reacted with for 1 h with 0.5 mM MPEG-2 or MPEG-5. A control is also shown in which the cells were pretreated for 30 min with DTT (10 mM) before lysis. *Panel C.* Cartoon showing the redox state of cysteines in IP_3R1 determined by LC–MS/MS. Redox lysates were prepared from transfected HEK293 cells treated in the presence and absence of thimerosal (50μM) and processed sequentially with iodoacetamide (IAM), DTT and biotin maleimides as depicted in Panel A. The lysates were processed on a sephadex-G25 column to exchange the sample into a buffer suitable for immunoprecipitation which was carried out with a C-terminal IP_3R1 Ab. The immunoprecipitates were processed on 5% SDS-PAGE and the silver-stained IP_3R1 was excised and processed for LC–MS/MS. Spectra were analyzed with the SEQUEST search engine for tryptic peptides containing cysteines modified with IAM (reduced) or biotin maleimide (oxidized). Underlined residues were identified in each of 3 independent trials. Oxidized residues observed in H_2O_2 (0.5 mM) treated cells are indicated by arrows. Open circles are residues that were not detected in the analysis. For additional details see Ref. [68].

at rest and pronounced agonist-evoked H_2O_2 transients not observed in the bulk cytosol. The H_2O_2 derives from the mitochondrial respiratory chain and acutely modulates the IP_3-linked agonist sensitivity. Since the IP_3Rs are concentrated to the volumes that host local H_2O_2, the conditions may promote direct oxidation of thiols in the cytosolic face of the IP_3R.

To directly test the redox environment of the IP_3R, the opportunity exists to create recombinant IP_3R linked to a genetically-encoded redox sensor. Previously, fusion of roGFP with the $p47^{Phox}$subunit of NADPH oxidase has allowed direct, real-time assessment of the redox status of this specific protein [36]. Extension of this principle to the IP_3R is feasible since functionality of IP_3R1 and IP_3R3 tagged with GFP at the N-terminus has been demonstrated [37,38]. There are two candidate probes for an IP_3R redox indicator:

roGFP. Both roGFP, and related roGFP2, are bright, pH-stable ratiometric probes that are similar to existing GFP variants used in IP_3R constructs. They possess a thiol pair and responses rely upon endogenous thiol oxidase and reductase enzymes to modulate their fluorescence in response to redox shifts [39]. Since IP_3R can exist in physically constrained environments, non-uniform access to the necessary adapter proteins may limit sensitivity. To solve this, versions of roGFP modified with enzymes have been developed to convey specificity to roGFP, including H_2O_2 sensitive Orp1-roGFP2 and Grx1roGFP2, which equilibrates with the GSH:GSSG ratio [40,41]. Both are excellent candidates with caveats. Grx1roGFP2 will perform poorly in experiments where total glutathione is depleted, as in permeabilized cell models. Orp1roGFP2 responds slowly, over several minutes, to changing H_2O_2, whereas the observed H_2O_2 transients at the ER-

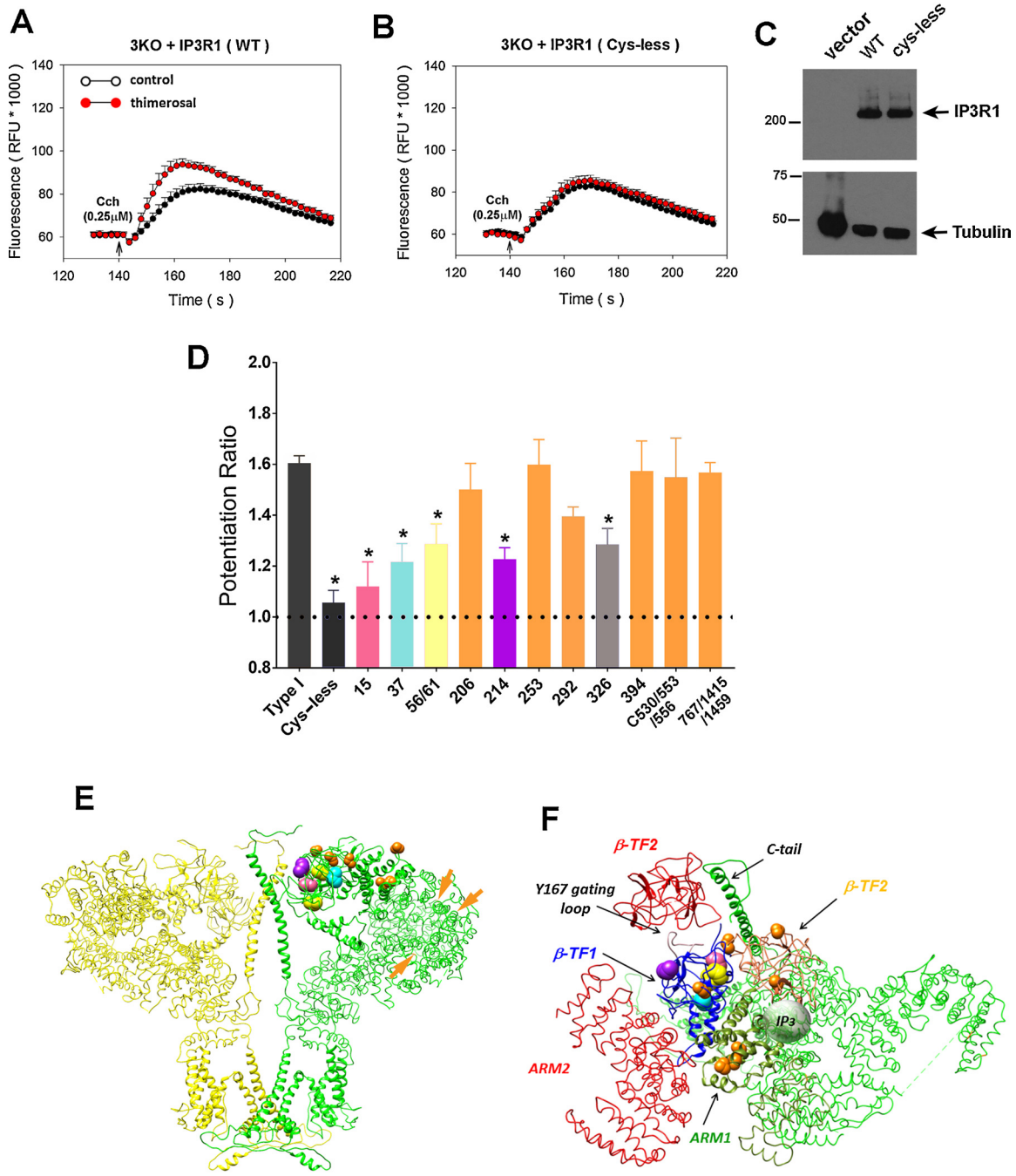

Fig. 3. Location of functionally relevant redox-sensitive cysteines in IP_3R1.

Panel A & B. Representative traces illustrating the approach to measuring redox potentiation described in Section 2.3 are shown. HEK293 cells in which all 3 IP_3R isoforms have been deleted by CRISPR were transfected with wild-type IP_3R1 or a mutant in which the first 13 N-terminal cysteines are substituted with serine (cys-less) [28]. The cells were loaded with Fluo-8 AM and the cytosolic Ca^{2+} response to a subsaturating dose of carbachol (0.25μM) was measured in the absence of extracellular Ca^{2+} with and without a 2 min preincubation with 10μM Thimerosal. The data were obtained with a flex station plate reader and are the mean ± S.D. of 4 wells for each condition. Responses to maximal doses of carbachol were also quantified and were not significantly different between wild-type and cys-less mutant (not shown). *Panel C.* The expression of the cys-less mutant is shown by immunoblotting with IP3R1 Ab. *Panel D* The thimerosal-induced potentiation of the individual cysteines corresponding to the cys-less mutant were measured as described in panels A &. The amplitude of the Ca^{2+} response to 0.25μM carbachol in the presence or absence of thimerosal treatment was quantified as a "potentiation ratio". All data are the mean ± S.E.M. of n = 3–6 independent experiments. * ; $P < 0.001$. All the mutants were responsive to maximal concentrations of carbachol (not shown). *Panel E* Side-view of the cryo-EM structure of IP_3R1 [69] showing the location of the cysteine mutants used in Panel D. For clarity only two opposing subunits of the tetramer are shown. The residues with diminished redox potentiation are color-coded as given in Panel D: C15 (red), C37 (blue), C56/61 (yellow), C214 (purple). Residues having no significant effects on redox potentiation are colored orange: C206, C253, C292, C394, C530, C553, C556, C767, C1415, C1459. Orange arrows are used for residues where the side-chains were not resolved (C767, C1415, C1459). C326 is located in the SI splice site which was not present in the cryo-EM structure. *Panel F* A close-up of the top view of the cytosolic surface of the channel showing the 3 domains that constitute the ligand-binding site of a single subunit (colored green) with the approximate position of IP_3 indicated by a sphere. The domains are the β-trefoil 1 domain (β-TF1), β-TF2 and the armadillo solenoid fold-1 (ARM-1). The structure shows the segregation of the redox-sensitive residues to a small region of β-TF1. Other domains from an adjacent subunit that are in contact with β-TF1 are shown in red. For additional details see text and Ref. [68].

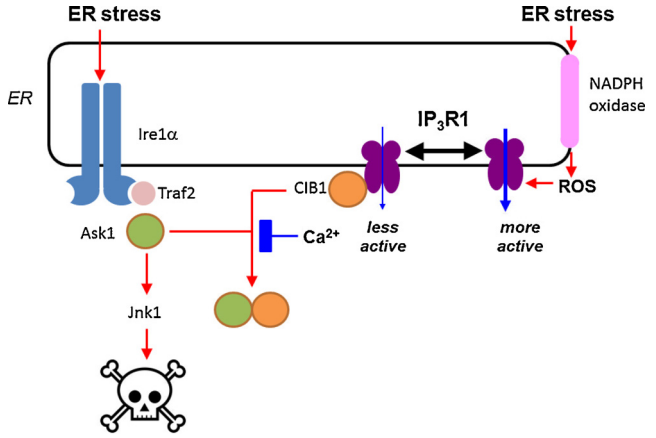

Fig. 4. Alternative hypotheses linking ER stress and IP_3R-mediated Ca^{2+} signaling.
Shown are some alternative mechanisms of regulating IP_3R channel activity during ER stress independently of changes in ER luminal redox state involving ERp44 or Ero1α. The EF-hand containing protein CIB1 is depicted as partitioning between IP_3Rs or Ask1, a kinase which is a required component of the MAP-kinase cascade linking the ER stress sensor, Ire1α, to apoptotic and inflammatory pathways. CIB1 binding inhibits Ask1 kinase activity and CIB1/Ask1 complex formation is blocked by Ca^{2+} binding to CIB1. This provides one mechanism by which IP_3R-mediated Ca^{2+} signaling can regulate ER-stress induced apoptosis. Also shown is the possible regulation of IP_3Rs by local ROS generated by NADPH oxidases in the ER.

mitochondrial interface resolve in seconds. However, should time resolution be unimportant, this represents an excellent choice.

HyPer. A HyPer-IP_3R fusion protein is also likely to be functional, since its ER-surface targeted variant performs well [35]. The probe would likely be exposed to the dynamic redox nanodomains already observed at the ER-mitochondrial interface and would prove useful in confirming to what extent the IP_3R is exposed to such conditions. Recent modifications of HyPer have become available. HyPer2, HyPer3 and HyPer Red feature increased dynamic range, brightness and spectral shifts respectively [42,43]. HyPer2/3 are likely unsuitable however, since their function is accompanied by formation of multimers, which may compromise sensing activity and/or channel function. HyPer Red is the only red shifted option, but lacks dynamic range and is not ratiometric. The original HyPer remains most promising for the measurement of rapid changes in H_2O_2, but pH must be a consideration in any experimental protocol. During IP_3R opening, rapid shifts in pH are to be expected as Ca^{2+} displaces H^+ from buffering species. To control for this sensitivity, a redox-desensitized derivative, SypHer-IP_3R is an essential control for paired experiments [44].

3. Intraluminal redox regulation of IP_3Rs

There are 5 cysteines in the IP_3R1 sequence that would be predicted to be intraluminal (Fig. 2C). Most attention has focused on the cysteines located in the 3rd intraluminal loop which consists of C2496, C2504 and C2527. An additional cysteine (C2533) is located at the luminal end of a helix that forms part of the pore of the IP_3R channel. Previous studies have identified that C2533 is essential since its mutation inactivates channel function [45]. An additional cysteine (C2375) is present in the small 2nd intraluminal loop of IP_3R1 but is not conserved in any of the other isoforms. It is generally accepted that the ER lumen is maintained in a more oxidized state than the cytosol [46]. Based on 1H-NMR studies using a recombinant peptide encoding the 3rd intraluminal loop, it was suggested that C2496 and C2504 formed a disulfide bond [47]. The mass-spectrometry data (Fig. 2C) however, show that only 2 of the 5 intraluminal cysteines are constitutively oxidized (C2496 and C2533). Therefore, C2496 and C2504 are unlikely to form disulfide bonds either under basal or oxidized conditions in live cells. It remains possible that C2496 and C2533 may be in disulfide linkage which could be related to structural stabilization of the pore. The lack of luminal oxidation of C2504 is in line with the suggestion that steric hindrance may be caused by the adjacent asparagine being a site of N-glycosylation in IP_3R1 and IP_3R2 (but not IP_3R3) [14].

Higo et al. [48] demonstrated that IP_3R1 (but not IP_3R2 or IP_3R3) interacted with ERp44, an intraluminal ER chaperone of the thioredoxin family. Reducing conditions and low Ca^{2+} concentrations within the lumen favored the interaction which was inhibitory to channel function. Mutagenesis suggested that C2496, C2504 and C2527 were all important IP_3R residues for ERp44 interaction. Thus, a model could be proposed in which an oxidative shift in the redox poise of the ER lumen would lead to a diminished IP_3R1/ERp44 interaction and an enhanced IP_3R1-mediated Ca^{2+} release (Fig. 1). A pathophysiological situation in which this model could potentially be involved is ER stress, a condition which is associated with many human diseases [49]. It has been suggested that ER stress may cause an oxidative shift in the ER lumen by a mechanism involving a CHOP-dependent increase in Ero1α expression [50]. The effects of the redox shift on IP_3R1/ERp44 interaction and/or the direct binding of Ero1α to ERp44 is proposed to enhance IP_3R mediated delivery of Ca^{2+} to the mitochondria and trigger ER stress-induced apoptosis [51] (Fig. 1). This model has gained wide acceptance in the literature [7,52–55]. However, there are a number of experimental observations that are inconsistent with this model. Firstly, the redox poise of the ER lumen appears remarkably stable to perturbations, including redox stress [56]. Secondly, even a 20-fold overexpression of Ero1α does not alter intraluminal glutathione redox state [57]. Finally, there is no evidence from direct measurements of IP_3R redox state that oxidants influence the redox state of the 3 intraluminal thiols implicated in ERp44 binding (Fig. 2C). It should be noted that an exception to the redox stability of the ER lumen is the reproducibly documented ~20 mV reductive shift occurring when luminal Ca^{2+} levels fall in response to agonist stimulation [58–60]. Avezov et al. have provided an explanation for this phenomenon by showing that ER Ca^{2+} depletion causes the sequestering of the oxidative enzyme protein disulfide isomerase (PDI) in a low mobility complex with the abundant ER chaperone calreticulin [61]. Whether the small reductive shift in the ER accompanying IP_3-mediated Ca^{2+} release has any regulatory impact on the oxidation state of the luminal IP_3R thiols remains to be determined.

Alternative models connecting IP_3R signaling to ER stress-induced apoptosis, without a direct involvement of changes in ER luminal redox state can be proposed (Fig. 4). The ER harbors several sources of locally generated ROS that could potentially target IP_3R-mediated Ca^{2+} signaling. This includes ROS generated as a by-product of protein folding or the activity of enzymes such as NADPH oxidases (Nox) [62]. There is evidence that ER stress increases the activity of ER-localized Nox4 [63]. Local ROS generated by these enzymes could also target the cytosolic redox-sensitive thiols of IP_3Rs (Fig. 4). CIB1 is an ER localized, EF-hand containing protein which binds to IP_3Rs and inhibits the IP_3R mediated Ca^{2+} signal [64,65]. Knockdown of the ER-stress sensor Ire1α in SH-SY5Y neuronal cells induced ER stress and resulted in an IP_3R-mediated increase in basal cytosolic [Ca^{2+}], mitochondrial Ca^{2+} overload, increased ROS production and cell death [66]. CIB1 was observed to dissociate from IP_3R1 after Ire1α knockdown, while more CIB1 was found in complexes with Ask1, a kinase that is activated by interaction with Ire1α and is an essential component in the Jnk pathway that stimulates apoptosis and inflammatory responses to ER stress (Fig. 4). The association of CIB1 with Ask1 inhibits the kinase activity of Ask1 and the association is directly blocked by Ca^{2+} binding to the EF-hands of CIB1 [67]. These findings point to multiple regulatory circuits that may connect ER stress, ROS generation and IP_3R Ca^{2+} signaling.

4. Cytosolic redox regulation of IP_3R isoforms

Which of the 55 cytosol-facing cysteines in IP_3R1 is involved in conferring redox sensitivity to the channel? Our laboratory has recently utilized the methods outlined in Section 2 to address this question [68] and these data can be summarized as follows. The mass-spectrometry approach shows that two of the cytosolic cysteines are already oxidized under basal conditions (C292, C1415) and an additional 11 residues become oxidized after thimerosal treatment of HEK293 cells (Fig. 2C). Not all 11 residues were observed to be oxidized in each experiment. However, 3 residues were consistently oxidized by thimerosal (C206, C767, C1459). H_2O_2 treatment oxidized 3 residues, two of which were unique (C214, C1397), and the other was also oxidized by thimerosal (C206) (Fig. 2C). Taylor and colleagues showed that mutation of the first 12 cysteines in the N-terminus was sufficient to block the stimulatory effect of thimerosal on [^{3}H]-IP_3 binding to IP_3R1 [28]. Our functional analysis of this cysteine-less IP_3R1 mutant using HEK293 IP_3R-3KO cells are entirely consistent with these findings (Fig. 3B) [68]. A more detailed investigation into the redox responses of individual cysteine mutants is shown in Fig. 3D. This includes each of the 12 cysteines within the Cys-less construct, an additional cysteine (C326) present within the SI splice site of the wild-type construct, and the readily oxidized residues detected by mass-spectroscopy (C767, C1415, C1459).

Recently, several cryo-electron microscopy (cryo-EM) structures of IP_3Rs have been published. This includes the native IP_3R1, in both the apo-form [69], and bound to the high-affinity IP_3 analog adenophostin-A [70]. The structure of the IP_3 and Ca^{2+} bound forms of recombinant IP_3R3 have also been reported [71]. The location of the mutant cysteines have been mapped to the cryo-EM structure of apo-IP_3R1 in Fig. 3E. The SI splice site was not included in the structure and therefore the C326 residue is omitted from Fig. 3E. Cysteines are color-coded according to Fig. 3D with residues showing no effects on redox potentiation depicted in orange. The ligand-binding domain of the IP_3R is made up of three modular domains consisting of two successive β-trefoil domains (β-TF1 and β-TF2) and an armadillo solenoid fold-1 (ARM1) (Fig. 3F). It is apparent that the functionally important redox-sensitive residues are clustered together in a particular region of β-TF1 (also referred to as the "suppressor domain" (SD) (Fig. 3F). The deletion of this domain results in a channel that loses function but retains high-affinity IP_3 binding [72]. It is therefore generally accepted that β-TF1 is critically important for coupling IP_3 binding to channel gating. Examination of apo and IP_3 bound forms of IP_3R1 do not indicate large scale motions of the β-TF1 domain upon IP_3 binding. Instead, the β-TF1 domain is viewed as a pivot around which the β-TF2 and ARM1 domain move to clamp IP_3 in the binding pocket [70]. However, two regions within β-TF1 make inter-molecular contacts with adjacent subunits and these interfacial regions are altered in the presence of IP_3 [70]. Additional interactions involving βTF2 and ARM1 are proposed to be part of a complex allosteric network that transmits the energy of IP_3 binding to the opening of the IP_3R1 channel [70,71]. Although none of the redox-sensitive cysteines in β-TF1 are directly located at subunit interface sites, it can be envisaged that reaction of critical cysteines with bulky oxidants could have indirect effects on the conformational transitions in the ligand-binding domain that lead to an enhanced efficacy of the gating mechanism.

There have been very few studies on how redox regulation impacts the single-channel gating properties of IP_3Rs. In bilayers, thimerosal induced higher subconductance levels and increased channel open times [19]. In nuclear patch clamp studies thimerosal was added after an initial treatment with DTT. Under these conditions the thiol agent affected the recruitment of channels in the patch and produced channel rundown, an effect that was not observed with H_2O_2 treatment [73]. A detailed mechanistic understanding of these effects awaits further detailed biophysical characterization of the alterations in channel gating induced by redox active agents. A final point with regard to the functional measurements is that cysteine residues that are reproducibly oxidized in mass-spectrometry studies (e.g. C767, C1459) turn out to have no role in potentiation, which probably reflects only their ease of accessibility and reactivity, rather than their role in channel regulation.

Not all IP_3R isoforms are equally sensitive to redox regulation, with IP_3R1 and IP_3R2 retaining the most sensitivity and IP_3R3 being relatively insensitive [7,28,74,75]. IP_3R3 also has the lowest affinity for IP_3 [8]. These observations on the different isoforms have mechanistic and physiological implications. Attempts to tease out the redox-sensitive cysteines by comparing the conserved and non-conserved residues between isoforms have not been successful [28,75]. A further layer of complexity is that the same isoform from different species may have dissimilar redox sensitivities (e.g. rat and chicken IP_3R3 [75]). If redox regulation of IP_3R channels was linked to altered regulation by other factors (such as sensitivity to Ca^{2+} [76]) then the differential redox responses of IP_3R isoforms may depend on more than the location of cysteines within the primary sequence. The possibility of selective localization of particular IP_3R isoforms towards or away from sites of local ROS production (e.g. IP_3R3 at ER/mitochondrial junctions [77]) could also influence whether the localized Ca^{2+}-signaling enhances metabolism or triggers cell death.

Ryanodine receptors (RyR) are also redox-sensitive channels and respond to reactive oxygen and nitrogen species, glutathione redox state and changes in oxygen tension [78,79]. In the case of RyR1 which contains 101 cysteines/monomer, the oxidation of ~10 cysteines has no effect on channel function [80], which agrees with the findings in IP_3Rs that readily oxidizable residues may not necessarily have functional significance. The further oxidation of ~15 cysteines increases channel activity and more extensive oxidation of an additional ~10 cysteines inactivates the channel [80]. Multiple studies have used endogenous or exogenous oxidants combined with mass-spectroscopy approaches to identify redox-sensitive thiols [81–84]. Surprisingly, there is only a limited overlap of cysteines observed when comparing these studies. Mutagenesis of cysteines in RyR1 indicates that functionally relevant redox-sensitive RyR1 thiols are distributed throughout the molecule [83], unlike IP_3Rs where they appear to cluster in the β-TF1 domain. Several high resolution cryoEM structures of open and closed states of RyRs have been published recently (reviewed in [85,86]). Although the β-TF1 of IP_3R1 has an equivalent domain in RyRs [87], the conservation of key β-TF1 cysteines between the two families of channels is poor. Two mass-spectroscopy studies detected C36 as the sole redox-sensitive residue in the β-TF1 domain of RyR1 [82,84]. The structural data suggest that the detailed mechanism of channel gating of IP_3Rs [69] and RyRs [85,86] are dissimilar and therefore the mechanism of thiol potentiation of the two channels is also likely to be different.

5. Chemical nature of the thiol modifications of IP_3Rs

None of the methods for measuring the redox state of IP_3Rs described in Section 2 identify the chemical nature of the modification occurring upon oxidation of the cysteine. A common modification to be expected is the formation of disulfide bonds, at least when the oxidative stress has endogenous origins in the form of increased ROS. However, although redox sensitive cysteines appear in spatial proximity in the 3D structure, none of the sulfur atoms are orientated to be within the cutoff distance of 2.3 Å expected for formation of a disulfide bond in the apo or ligand-bound IP_3R1 channels [69,70]. In the absence of adjacent cysteines, the reactive thiols can also undergo reversible S-glutathionylation. The formation of this modification on IP_3R1 has been demonstrated in vascular endothelial cells subjected to oxidative stress [76,88]. Hydrogen sulfide has been shown to inhibit IP_3Rs in airway smooth muscle but the type of modification involved is not known [89]. Similarly, although effects of nitric oxide on IP_3Rs have been reported [90], the direct S-nitrosylation of the protein remains to be demonstrated. A recently described modification of IP_3R thiols that can be added to the list is S-palmitoylation [91]. Three cysteines in IP_3R1 are

proposed to be so modified by a ER-localized complex of palmitoyl acyltransferase (DHH6) and selenoprotein-K [92]. This reversible modification stabilizes the IP_3R1 protein and prevents degradation by the proteasome pathway. Variable levels of S-palmitoylation are viewed as a means of fine-tuning Ca^{2+} signaling, particularly in immune cells where this modification has been characterized [93]. Interestingly, RyR1 is also S-palmitoylated and the modification on RyR1 occurs on many of the same cysteines that are oxidized [94]. If this proves to be applicable to IP_3Rs, it would provide a mechanism for modulating the redox sensitivity of IP_3Rs and possibly contribute to differences in redox sensitivity of individual IP_3R isoforms [75]. It has also been demonstrated that palmitoylation targets the ER proteins calnexin and TMX thioredoxin to the mitochondria-associated membrane fraction (MAM) [95,96]. These findings raise the question of whether this modification could play a similar role in the enrichment of IP_3Rs at this location.

6. Redox regulation of mitochondrial Ca2+ uptake

The possibility that mitochondrial Ca^{2+}uptake may be redox sensitive has been investigated by Dong et al. [9]. Using gel-shift assays similar to those described in Section 2.1, they demonstrated that ROS generation in intact cells leads to the oxidative modification of the MCU pore-forming complex but not the other ancillary proteins, such as MICU1, MCUb or EMRE which are part of the functional "uniplex" that mediates Ca^{2+} transport across the mitochondrial inner membrane. Additional studies indicate that the C97 position of MCU, which is absent in the MCUb homolog, is subject to S-glutathionylation. Using multiple approaches, it was demonstrated that mutation of the C97 residue to an alanine or methionine enhanced the activity of MCU channels [9]. This somewhat unexpected finding was interpreted as indicating that the C97 mutations mimicked the structural changes induced by S-glutathionylation. Expression of the C97 mutant in pulmonary vascular endothelial cells leads to enhanced mitochondrial ROS production, lower ATP levels and oxygen consumption rates, and sensitization of the cells to H_2O_2-induced cell death. All of these effects are prevented by ectopic expression of mitochondrial antioxidant enzymes [9]. These studies re-enforce the positive feedback roles of Ca^{2+} and ROS generation in promoting the funneling of Ca^{2+} from the ER into the mitochondrial matrix associated with the activation of cell death pathways. Recently, multiple studies have reported the cryo-EM structure of MCU from several fungal species [97–100]. However, the C97 residue is not conserved in fungi.

Another potential target for redox regulation of mitochondrial Ca^{2+}uptake are the Ca^{2+} binding regulatory proteins MICU1 and MICU2. Both have conserved cysteines which form intermolecular disulfides bonds that yield homo and heterodimers [101]. This process is facilitated in the intermembrane space by Mia40, a protein which functions in a redox relay to promote disulfide bond formation with a number of client proteins [102]. There have been many differing models of the functional effects of homodimers and heterodimers of MICU1 and MICU2 on MCU channel regulation [101–107]. However, it seems unlikely that redox modifications of the disulfide bond status of MICU1 and MICU2 could be of potential regulatory significance. In part, this is because of the extreme resistance to reductants observed for the MICU1/2 disulfide bonds in the mature proteins [101,102], which probably accounts for the lack of MPEG gel-shifts in MICU1 observed previously [9]. In addition, MICU1 mutants without the critical cysteines can still assemble [108], and retain partial function (Melanie Paillard, SKJ, GH; unpublished observations), which suggests that other factors, besides disulfide bonds, may contribute to the oligomerization of these regulatory molecules. In the crystal structure of the MICU1 oligomer, obtained in the absence of Ca^{2+}, the cysteines are too far apart to form disulfide bonds [108]. Since they clearly do form disulfides [101,102], the results suggest that the crystal structure may not be an accurate depiction of MICU1 in its native environment.

7. Future directions

The oxidant-induced regulation of IP_3Rs and MCU proteins discussed in this review represent only two components of an extensive network of redox-sensitive proteins that play a role in cellular Ca^{2+} homeostasis [14,15]. The ability of ROS to regulate these channels may have evolved out of the necessity to reversibly communicate the bioenergetic status of the mitochondria with the protein biogenesis and signaling functions of the ER. As yet, there have been few studies examining the redox regulation of IP_3R and MCU where they concentrate at the ER/mitochondrial interface, particularly in view of the enhanced Ca^{2+} and ROS signaling in this region [33,35]. At the molecular level, a greater understanding of the specific thiols involved in the redox sensitivity of IP_3R channels would allow the engineering of redox-insensitive channels that can be used to explore the role of redox regulation in physiological and patho-physiological cellular responses. Recent advances in cryo-EM studies of IP_3Rs suggests that we may be able to obtain the structure of redox modified IP_3Rs and gain a more detailed understanding of how ROS regulates the gating of these channels. At the level of the mitochondria, it has been observed that the oxidation of a single C97 residue in the MCU channel radically alters its activity and its higher order structure [9]. Further mechanistic studies will require more information on the stoichiometry and composition of functional MCU complexes. No doubt, additional insights into the physiological relevance of redox regulation of MCU will be obtained from "knock-in" animal and cell models of the MCU-C97 mutant. The role of redox stress as a contributing factor in many human diseases is increasingly recognized [109–111]. More detailed investigations into how redox and Ca^{2+}-signal networks interact at the level of individual proteins may aid in developing new therapeutic strategies for the treatment of disease.

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