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The Role of TRP Channels in Oxidative Stress-induced Cell Death

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Abstract. The transient receptor potential (TRP) protein superfamily is a diverse group of voltage-independent calcium-permeable cation channels expressed in mammalian cells. These channels have been divided into six subfamilies, and two of them, TRPC and TRPM, have members that are widely expressed and activated by oxidative stress. TRPC3 and TRPC4 are activated by oxidants, which induce Na^+ and Ca^{2+} entry into cells through mechanisms that are dependent on phospholipase C. TRPM2 is activated by oxidative stress or TNFα, and the mechanism involves production of ADP-ribose, which binds to an ADP-ribose binding cleft in the TRPM2 C-terminus. Treatment of HEK 293T cells expressing TRPM2 with H_2O_2 resulted in Ca^{2+} influx and increased susceptibility to cell death, whereas coexpression of the dominant negative isoform TRPM2-S suppressed H_2O_2-induced Ca^{2+} influx, the increase in $[Ca^{2+}]_i$, and onset of apoptosis. U937-ecoR monocytic cells expressing increased levels of TRPM2 also exhibited significantly increased $[Ca^{2+}]_i$ and increased apoptosis after treatment with H_2O_2 or TNFα. A dramatic increase in caspase 8, 9, 3, 7, and PARP cleavage was observed in TRPM2-expressing cells, demonstrating a downstream mechanism through which cell death is mediated. Inhibition of endogenous TRPM2 function through three approaches, depletion of TRPM2 by RNA interference, blockade of the increase in $[Ca^{2+}]_i$ through TRPM2 by calcium chelation, or expression of the dominant negative splice variant TRPM2-S protected cell viability. H_2O_2 and amyloid β-peptide also induced cell death in primary cultures of rat striatal cells, which endogenously express TRPM2. TRPM7 is activated by reactive oxygen species/nitrogen species, resulting in cation conductance and anoxic neuronal cell death, which is rescued by suppression of TRPM7 expression. TRPM2 and TRPM7 channels are physiologically important in oxidative stress-induced cell death.

Key words: Apoptosis — Intracellular Ca^{2+} — Oxidative stress — TRPC3 — TRPM2 — TRPM7

Introduction

The transient receptor potential (TRP) protein superfamily is a diverse group of voltage-independent calcium-permeable cation channels expressed in mammalian cells (Harteneck, Plant & Schultz, 2000; Montell, 2001, 2005; Minke & Cook, 2002; Clapham, 2003). They are structurally related to the transient receptor potential channels first described in *Drosophila*. In *Drosophila*, the *transient receptor potential (trp)* locus is involved in phospholipase C-dependent visual transduction, and mutant flies have a transient rather than sustained response to light and display defects in light-induced Ca^{2+}-influx (Montell & Rubin, 1989; Hardie & Minke, 1992). Mammalian TRP channels have been organized into six protein subfamilies based on sequence identity. These have been designated C (canonical), V (vanilloid receptor), M (melastatin), A (ANKTM), P (polycystin), and ML (mucolipin) (Clapham, 2003). Most mammalian cells express a number of TRP channel family members, since many TRP channels are ubiquitously expressed and most have splice variants. Mammalian isoforms have six putative transmembrane domains similar to the structure of many pore-forming subunits of voltage-gated channels except they lack positively charged residues necessary for the voltage sensor (Fig. 1). TRP channels are proposed to function as tetramers, with the pore formed by loops between the fifth and sixth transmembrane domains. The regulation of TRP channels is complex, but a number of unifying themes have emerged. These include regulatory roles for: (1)

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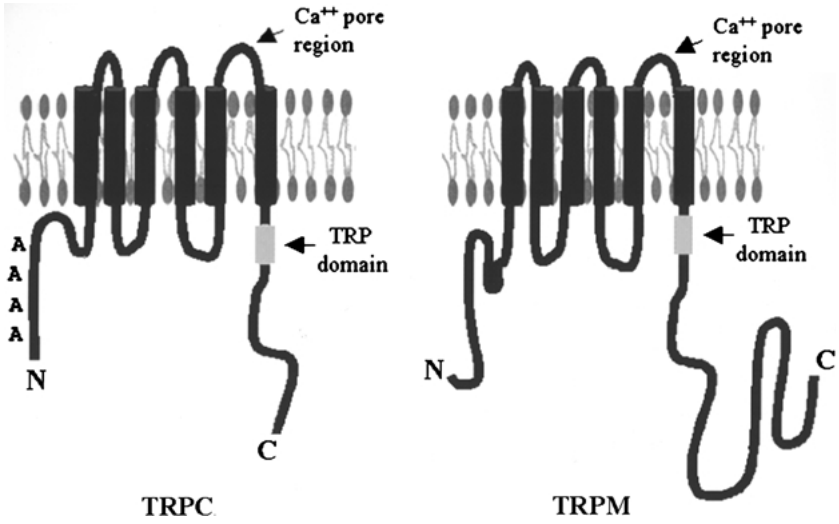

Fig. 1. Proposed scheme of membrane localization of TRPC and TRPM channels. TRPC and TRPM channels have intracellular N- and C-termini. TRPC channels have ankyrin repeat domains in their N-terminus, which may mediate protein/protein interactions. The six transmembrane domains and the proposed cation pore formed by loops between the fifth and sixth transmembrane domains in tetramers are shown. The function of the conserved intracellular TRP domain is not known.

extracellular signals, (2) second messengers, (3) channel subunit assembly, and (4) macromolecular complex formation. This review will focus on two subfamilies of TRP channels, TRPC and TRPM, which have members activated by oxidative stress.

Tissue damage caused by oxidative stress has a role in a number of physiological processes including aging, cancer, acute and chronic neurodegenerative disorders (Alzheimer's and Parkinson's diseases), diabetes mellitus, atherosclerosis, ischemia/reperfusion injury, and autoimmune disease (Chandra, Samali & Orrenius, 2000; Langley & Ratan, 2004). Oxidative stress is a disturbance in the balance between oxidants and antioxidants, which leads to cell injury depending on severity and duration (Chandra et al., 2000; Langley & Ratan, 2004). Production of reactive oxygen species (ROS) occurs naturally during respiration generated by the mitochondrial electron transport chain, following activation of the arachidonic acid cascade in the cytosol, or exposure to a number of extracellular agents and events including ionizing radiation, cytotoxic drugs, and infections which activate neutrophils and phagocytes. Free radical intermediates are produced including superoxide anion ($O_2^{\cdot-}$), H_2O_2, which in the presence of $O_2^{\cdot-}$ or reduced transition metals can produce the more damaging hydroxyl radical ($^{\cdot}OH$), and nitric oxide ($NO^{\cdot}$). These radicals contribute to DNA and protein oxidation and lipid peroxidation and damage. Biological antioxidants including α-tocopherol and ascorbic acid, and antioxidant enzymes including catalase, glutathione peroxidase, and superoxide dismutases, reduce ROS levels. Oxidative stress results in activation of a number of complex and interrelated signaling events (Chandra et al., 2000; Gopalakrishna & Jaken, 2000). It increases the activity of a number of enzymes including phospholipases A2, D, and C, and protein kinases including tyrosine kinases, mitogen-activated kinases (MAPKs), and protein kinase C. Oxidants further influence tyrosine phosphorylation by inactivating tyrosine phosphatases (Sattler et al., 2000). H_2O_2 has been shown to induce apoptosis through several pathways. H_2O_2 can upregulate Fas/Fas ligand, resulting in enhanced activation of the "extrinsic" cell death pathway involving caspase 8 and its downstream effectors caspase 3, caspase 7, and endonucleases (Bauer et al., 1998; Cabaner et al., 1999; Matsura et al., 1999). Alternatively, H_2O_2 can act upon the mitochondria to cause release of cytochrome c (Stridh et al., 1998). Cytosolic cytochrome c binds to Apaf-1 (apoptotic protease activating factor 1), forming the apoptosome and activating caspase 9, followed by caspases 3, 7, and PARP cleavage. Taken together, recent data suggest that the predominant pathway through which oxidative stress mediates cell death involves opening of the mitochondrial permeability transition pore (PT) (Crompton, 1999). The PT is a nonspecific pore that allows passage of solutes up to 1.5 kDa and can be activated by factors including high concentrations of Ca^{2+} in the mitochondrial matrix (Scorrano et al., 2001). H_2O_2 stimulates an increase in the intracellular free calcium concentration ($[Ca^{2+}]_i$), activating MAPKs and protein kinase C; these act in synergy to increase phospholipase A2 activity (Leslie, 1997; Herson et al., 1999; Petronilli et al., 2001; Scorrano et al., 2001; Han et al., 2003; Klohn et al., 2003; Green & Kroemer, 2004;). Elevated cytosolic $[Ca^{2+}]_i$ results in elevated mitochondrial matrix $[Ca^{2+}]_i$, which participates with arachidonic acid, produced by activation of phospholipase A2, to open the mitochondrial PT pore (Crompton, 1999). PT pore activation results in uncoupling of oxidative phosphorylation, which prevents ATP production, and in cytochrome c release into the cytosol, both contributing to cell death (Petronilli et al., 2001; Scorrano et al., 2001; Halestrap, McStay & Clarke, 2002). Several different

mechanisms have been proposed for the H_2O_2-mediated increase in $[Ca^{2+}]_i$, including a role for voltage-dependent calcium channels and alteration in Na^+-Ca^{2+} exchange, but due to conflicting data there is no consensus (Herson et al., 1999). Recent evidence suggests that calcium influx through TRP channels is an important mechanism through which oxidative stress mediates cell death, and this will be reviewed here.

Regulation of the TRPC Channels by Oxidative Stress

Involvement of TRP channels in anoxic cell death was first shown with *Drosophila* light-sensitive channels TRP and TRPL. Anoxia, treatment with mitochondrial uncouplers, or ATP depletion rapidly activated TRP and TRPL in the dark (Agam et al., 2000). In contrast, double mutation of both TRP and TRPL channels eliminated Ca^{2+} influx in photoreceptor cells in response to anoxia, confirming that these channels are targets of metabolic stress. A physiological role for these TRP channels was demonstrated by mutations that caused their constitutive activation, resulting in massive photoreceptor cell death *in vivo* (Yoon et al., 2000).

The TRPC subfamily is the most closely related to *Drosophila* TRP, and members are activated by stimulation of G–protein-coupled receptors and receptor tyrosine kinases. Mammalian TRPC (1–7) proteins share greater than 30% amino acid sequence identity in the N-terminal ~750–900 amino acids, but display great variability in the C-terminus. TRPC conductances are nonselective cation channels with differences in the permeability of Ca^{2+}, Na^+, and other cations. Interaction of membrane receptors with their ligands can activate phospholipase C (PLC), resulting in production of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). A common feature of TRPC channels is that their activation mechanisms involve PLC, and several models of TRPC regulation through PLC-mediated pathways have been proposed and are supported by experimental evidence. (1) IP_3 interaction with its receptor (IP_3R) can mediate release of Ca^{2+} from the endoplasmic reticulum and depletion of Ca^{2+} from internal stores, triggering Ca^{2+} entry across the plasma membrane through TRPC (Boulay et al., 1999; Vazquez et al., 2003). TRPC1, 2, 3, 4, and 5 may be activated through store-operated mechanisms. (2) Independently of store depletion, TRPC3, 6, and 7 can be regulated by an increase in DAG (Hofmann et al., 1999; Venkatachalam et al., 2001). (3) IP_3R bound to IP_3 may be able to activate TRPC through direct interaction; calmodulin and IP_3R binding sites are present in the C-terminus of all TRPC (Tang et al., 2001; Tong et al., 2004). For most TRPC, the contribution of each of these PLC-mediated pathways in channel activation is not unequivocally resolved. For TRPC3, data suggests that the mechanism of activation depends on the level of channel expression (Vazquez et al., 2003). In addition to interaction with second messengers, other factors contribute to TRPC channel activation. One critical mechanism involves regulation of TRPC cell surface expression. PLCγ1 has recently been shown to control cell surface expression of TRPC3 through pleckstrin homology (PH) domains (van Rossum et al., 2005), and epidermal growth factor has been shown to induce vesicular translocation and membrane insertion of TRPC5 (Bezzerides et al., 2004). In native cells, TRPC3 may largely be localized to the intracellular compartment (Strubing et al., 2001; Buniel, Schilling & Kunze, 2003). Extracellular factors that modulate physiological membrane insertion of TRPC3 and most other TRPC still need to be identified. Channel subunit composition is another important factor in mediation of channel activation. TRPC are proposed to assemble as tetrameric channels, whose characteristics differ with the TRPC proteins which associate to form the channel (Hofmann et al., 2002). Evidence suggests that these channels assemble based on structural similarities reflected in proximity of their phylogenetic relationships (Fig. 2 Montell, 2005). Multimeric channel formation has been reported for TRPC1/4/5 and TRPC3/6/7 (Strubing et al., 2001; Goel, Sinkins & Schilling, 2002; Hofmann et al., 2002). Heteromeric TRP channels have functional characteristics different from their homomeric counterparts. For example, coexpression of TRPC1 and TRPC3 suppresses carbachol-induced Ca^{2+} entry and abolishes 1-oleoyl-2-acetyl-*sn*-glycerol–induced Sr^{2+} entry observed through TRPC3 homomonomers (Lintschinger et al., 2000).

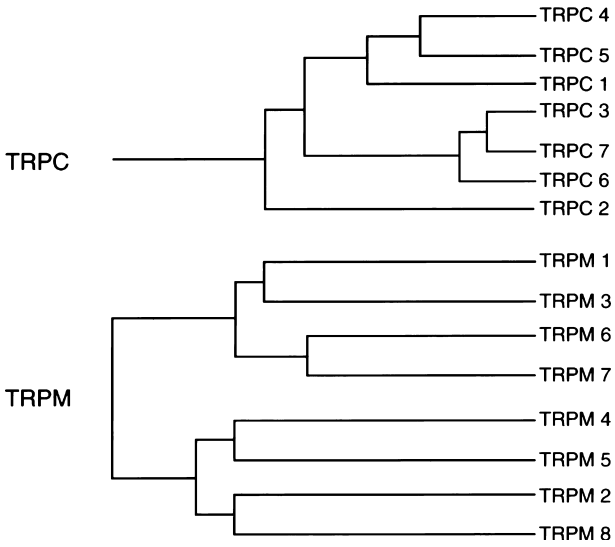

Fig. 2. Phylogenetic relationships between members of the TRPC and TRPM subfamilies.

Two TRPC channels have been identified that are activated by oxidative stress, TRPC3 and TRPC4. TRPC3 was first shown to be physiologically important in oxidant-activated cation current in porcine aortic endothelial cells. A dominant negative N-terminal splice variant of TRPC3 inhibited cation conductance and membrane depolarization induced by the oxidant tert-butylhydroperoxide (Balzer, Lintschinger & Groschner, 1999). In subsequent experiments using HEK 293T cells overexpressing TRPC3 or TRPC4, tert-butylhydroperoxide induced an increase in basal membrane conductance that was mostly carried by Na^+. These results support the conclusion that TRPC3 and TRPC4 form redox-sensitive cation channels that participate in Na^+ loading and membrane depolarization during oxidative stress (Groschner, Rosker & Lukas, 2004). Additional experiments suggested that in HEK 293T cells, Ca^{2+} entry into TRPC3-expressing cells involves reverse mode Na^+/Ca^{2+} exchange (Rosker et al., 2004), but the generalized use of this pathway in other cell types has not yet been shown. Cation currents induced in TRPC3-expressing cells by oxidative stress were inhibited by the PLC inhibitor U73122, demonstrating that phospholipase C activity is required for activation of TRPC3 by reactive oxygen species. Oxidative stress induced disruption of caveolin-rich lipid raft domains, but did not alter the localization of TRPC3 in the cell membrane (Groschner et al., 2004). Whether disruption of lipid raft structures affects the signaling mechanisms of TRPC3 or influences channel activity in response to oxidative stress is under investigation. Activation of TRPC3 by the muscarinic receptor and by DAG is abolished by pharmacological inhibition of tyrosine kinases and specifically Src kinases (Vazquez et al., 2004). In addition, TRPC3 is not activated following coexpression with dominant-negative Src in HEK 293T cells or when TRPC3 is expressed in a Src-deficient cell line, and wild-type Src restored normal TRPC3 regulation (Vazquez et al., 2004). Whether tyrosine phosphorylation is involved in TRPC3 activation by ROS is not yet known, but is a likely possibility. In summary, oxidative stress has been shown to activate TRPC3 and TRPC4. Because of common activation mechanisms and structural similarities among TRPC family members, other TRPC channels may be involved in oxidative stress as well. However, the importance of oxidative stress-induced activation of TRPC including TRPC3, in sustained Ca^{2+} influx leading to cell death is unknown.

Oxidative Stress Modulates Cell Death Through TRPM Channels

The TRPM subfamily was named after the first described member, melastatin (TRPM1), a putative tumor suppressor protein (Duncan et al., 1998). TRPM1 is expressed on melanocytes, and its expression level correlates inversely with melanoma aggressiveness and the potential for metastasis. Other members of the TRPM subfamily also have important roles in cell proliferation and survival, including TRPM2 (Hara et al., 2002; Zhang et al., 2003; Fonfria et al., 2005), TRPM5 (Prawitt et al., 2000), TRPM7 (Aarts et al., 2003), and TRPM8 (Tsavaler et al., 2001). Members of the TRPM subfamily have the same six-transmembrane-loop structure as TRPC, and share a region of unknown function in the N-terminus and a region of high coiled coil character (CCR) in the C-terminus, that may play a role in ion channel subunit multimerization or in recruitment of regulatory proteins (Schmitz & Perraud, 2005). Otherwise, the C-terminus displays considerable variability, and three of these channels have the unique C-terminal enzymatic domains, TRPM2, TRPM6, and TRPM7. A role for two of these channels, TRPM2 and TRPM7, in oxidative-induced cell death has been documented and will be reviewed here.

TRPM2

TRPM2, also called LTRPC-2, was the second member of the TRPM subfamily to be described. It is expressed in many cell types including brain and hematopoietic cells (Nagamine et al., 1998; Sano et al., 2001). TRPM2 channels are permeable to sodium, potassium, and calcium, and several pathways involved in activation have been identified.

(1) *Extracellular Signals*. Two extracelluar signals are known to activate TRPM2, oxidant stress and TNFα (Hara et al., 2002; Wehage et al., 2002).
(2) *Second Messengers*. Intracellular ADP-ribose (ADPR) activates TRPM2 by binding to the TRPM2 C-terminal NUDT9-H domain, which has homology with NUDT9 ADPR hydrolyase but a low level of ADPR hydrolase activity (Fig. 3) (Wehage et al., 2002; Heiner, Eisfeld & Luckhoff, 2003b; Perraud, Schmitz & Scharenberg, 2003; Kraft et al., 2004; Kolisek et al., 2005; Perraud et al., 2005). The preponderance of evidence suggests that it is not the enzymatic activity of the NUDT9-H domain that is critical for ADPR activation of TRPM2 but rather ADPR binding to this site (Kuhn & Luckhoff, 2004; Perraud et al., 2005). Although patch-clamp studies suggest that nicotinamide adenine dinucleotide (NAD) also can directly induce opening of TRPM2 (Sano et al., 2001; Hara et al., 2002), other evidence suggests this is secondary to conversion to or contamination by

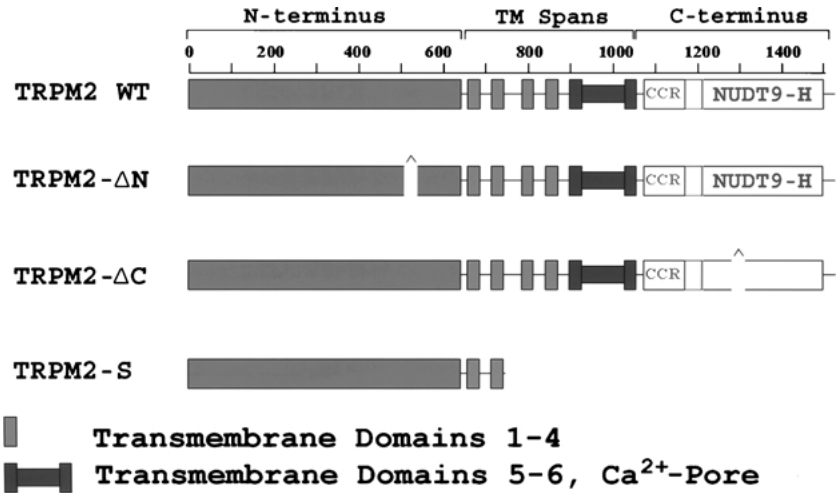

Fig. 3. Schematic representation of TRPM2 isoforms. Membrane spanning domains 1–4 and the putative pore region including transmembrane domains 5–6 are indicated. CCR represents the coiled coil region which may mediate protein/protein interactions. NUDT9-H represents the NUDT9 ADP-ribose hydrolase domain. TRPM2-ΔN has a deletion of aa 538–557 in the N-terminus. TRPM2-ΔC has a deletion of aa 1292-1325 in the C-terminus. TRPM2-S is missing four of six transmembrane domains and the putative calcium pore.

ADPR (Wehage et al., 2002; Perraud et al., 2005). Cyclic adenosine diphosphoribose (cADPR) can also gate the TRPM2 channel by itself at high concentrations, and at lower concentrations potentiates the effects of ADPR (Kolisek et al., 2005). Arachidonic acid has been shown to activate TRPM2 (Hara et al., 2002). TRPM2 channel currents activated by ADPR have a strong requirement for Ca^{2+} at the intracellular surface of the plasma membrane (McHugh et al., 2003), and evidence from our laboratory suggests that the Ca^{2+}-sensor for TRPM2 activation is calmodulin (*unpublished observation*).

(3) *Channel Tetramer Formation*. TRPM channels are also thought to function as tetramers, and subunit composition is a factor in regulation of TRP channel opening. Four physiological splice variants of TRPM2 have been identified (Fig. 3): TRPM2-L (full length or wild type), TRPM2-S (Zhang et al., 2003), TRPM2-ΔN (Wehage et al., 2002), and TRPM2-ΔC (Wehage et al., 2002). TRPM2-S (short) has a deletion of the entire C-terminus, including four of six C-terminal transmembrane domains and the putative Ca^{2+}-permeable pore (Zhang et al., 2003). TRPM2-S suppresses Ca^{2+} influx through TRPM2-L and inhibits cell death induced by oxidative stress. TRPM2-ΔN has a deletion of amino acids 538–557 in the N-terminus, and HEK 293 cells expressing TRPM2-ΔN failed to respond to H_2O_2 or ADPR, suggesting that the TRPM2-ΔN mutation dominantly disrupts channel gating, channel assembly, or surface trafficking (Wehage et al., 2002). TRPM2-ΔC has a deletion of amino acids 1292–1325 in the C-terminal CAP-domain of NUDT9-H, decreasing affinity for ADPR (Wehage et al., 2002). HEK cells expressing TRPM2-ΔC responded to H_2O_2 but not to intracellular application of ADPR, suggesting that oxidative stress may be able to activate TRPM2 through a mechanism independent of ADPR.

(4) *Macromolecular Complex Formation*. In *Drosophila*, TRP channels assemble into macromolecular complexes with the PDZ domain-containing protein INAD providing a molecular scaffold (Li & Montell, 2000). PDZ domains are 80–100 amino acid repeated sequences that are important protein/protein interaction sites for clustering and organization of signaling molecules, particularly those involved in ion transport. Mammalian TRP channels are also organized into macromolecular complexes, either through scaffolding proteins including those with PDZ domains (Tang et al., 2000; Voltz, Weinman & Shenolikar, 2001; Goel et al., 2002) or through lipid rafts (Lockwich et al., 2000). For the TRPM channel subfamily, little is known about signaling complexes involved in their activation, although these likely exist.

Substantial evidence supports the conclusion that TRPM2 is an important factor in cell death induced by oxidative stress (Hara et al., 2002; Zhang et al., 2003; Fonfria et al., 2005; McNulty & Fonfria, 2005; Zhang et al., 2006). Initial evidence was obtained in heterologous expression systems. Oxidative stress induced a rise in Ca^{2+} influx and in $[Ca^{2+}]_i$ in HEK 293 cells expressing TRPM2, which was associated with an increase in susceptibility to cell death (Hara et al., 2002; Wehage et al., 2002; Zhang et al., 2003; Perraud et al., 2005). Coexpression of the dominant negative TRPM2-S splice variant suppressed H_2O_2-induced Ca^{2+} influx, susceptibility to cell death, and onset of apoptosis (Zhang et al., 2003). TRPM2-S and TRPM2-L were shown to directly interact, but the precise mechanisms through which TRPM2-S

inhibits TRPM2-L function in oxidative stress remains to be identified. Proposed mechanisms include the following: TRPM2-S may participate in heterodimer formation, altering the tertiary structure of the TRPM2-L tetramer required for cation permeability. Alternatively, TRPM2-S, which is missing the functional C terminus, could compete with localization of TRPM2-L in signaling complexes, or act as a dominant negative blocking an unknown aspect of TRPM2-L regulation critical for calcium channel activation. Experiments were then performed to look at endogenous TRPM2 function. H_2O_2 induced Ca^{2+} influx in microglia, which are host macrophages in the central nervous system and express TRPM2 (Kraft et al., 2004). Other studies were performed with antisense oligonucleotides targeted to TRPM2 to reduce endogenous TRPM2 levels. These showed suppression of H_2O_2 and TNFα-induced Ca^{2+} influx and cell death in rat insulinoma RIN-5F cells and in the monocytic cell line U937, demonstrating a critical role for TRPM2 (Hara et al., 2002).

The function of TRPM2 in oxidative stress-induced cell death was studied further in the monocytic cell line U937-ecoR using multiple different approaches (Zhang et al., 2006). These U937 cells stably express the ecotrophic receptor for retrovirus, which was used to up-and down-regulate expression of TRPM2 isoforms. In response to oxidative stress, U937-ecoR cells expressing increased levels of TRPM2-L exhibited significantly increased $[Ca^{2+}]_i$, decreased viability measured by trypan blue exclusion, and increased apoptosis measured by annexin V binding to early apoptotic cells quantitated by fluorescent microscopy and FACS analysis. Dramatically increased cleavage of caspase 8, 9, 3, 7, and PARP inactivation was observed, demonstrating downstream effector mechanisms involving both the intrinsic and extrinsic cell death pathways through which death is mediated. This is consistent with previous observations that caspases 8, 3, and PARP as well as mitochondrial cell death pathways are involved in hydrogen-peroxide-induced apoptosis (Jones et al., 2000; Denning et al., 2002; Ma et al., 2003). The intracellular Ca^{2+} chelator BAPTA inhibited the rise in $[Ca^{2+}]_i$, blocked caspase and PARP cleavage and inhibited cell death following exposure of TRPM2-expressing cells to H_2O_2. This data demonstrated that the rise in $[Ca^{2+}]_i$ is critical in cell death mediated by oxidative stress, and the viability of TRPM2-expressing cells is largely preserved when the increase in $[Ca^{2+}]_i$ is blocked. Inhibition of endogenous TRPM2 function by two additional approaches, depletion of TRPM2 in U937-ecoR cells by RNA interference or expression of the dominant negative TRPM2-S splice variant by retroviral infection, significantly protected cell viability after exposure to H_2O_2. These data strongly support the conclusion that TRPM2 is physiologically important in oxidative stress-induced cell death. The study of oxidant-induced death of hematopoietic cells from TRPM2 knockout mice is planned.

H_2O_2 and amyloid β-peptide have also been shown to induce cell death in primary cultures of rat striatal cells, which express TRPM2 endogenously (Fonfria et al., 2005). Amyloid β-peptide is a main component of senile plaques and is important in the pathogenesis of Alzheimer's disease. Amyloid β-peptide has been proposed to cause neuronal injury through the generation of oxidative stress (Butterfield, 2003). In addition, the striatum is vulnerable to ischemia and reperfusion injury, where oxidative stress is also implicated as a main factor (Lipton, 1999). In striatal cells, the dominant negative splice variant TRPM2-S inhibited both amyloid β-peptide and H_2O_2-induced increases in $[Ca^{2+}]_i$ and protected cell viability, but did not affect ROS generation (Fonfria et al., 2005). Reduction in endogenous TRPM2 levels by RNA interference also significantly enhanced cell viability after amyloid β-peptide and H_2O_2 treatment. These data provide significant support for the conclusion that TRPM2 is involved in oxidative stress-induced injury to striatal cells, and through activation by amyloid β-peptide may be involved in the pathogenesis of Alzheimer's disease.

The mechanisms through which TRPM2 is activated in oxidative stress-induced cell death need clarification. H_2O_2 but not ADPR application can activate the TRPM2-ΔC isoform, suggesting that oxidant stress can gate TRPM2 independently of ADPR (Wehage et al., 2002), but this has been disputed (Perraud et al., 2005). Although activation of TRPM2 by ADPR or NAD is a reasonable hypothesis since ADPR and NAD are produced in significant quantities following oxidant treatment (Chakraborti et al., 1999; Davidovic et al., 2001), the role of endogenous ADPR is not completely clear. Few direct measurements of intracellular free ADPR have been made due to technical difficulties, and little is known about whether endogenous ADPR levels can reach that required to activate TRPM2 (Perraud et al., 2001). A related issue is whether oxidant stress can directly gate TRPM2 or whether a second messenger (ADPR or NAD) is required. TRPM2 opening in patch-clamp studies using the perforated patch, which limits dilution of cytoplasmic contents, has been documented following oxidative stress, supporting the conclusion that diffusible messengers are involved (Hara et al., 2002). But in some studies using the whole-cell configuration, which allows dilution and minimizes the contribution of intracellular factors due to the large patch-clamp reservoir, oxidant stress also gates TRPM2, suggesting that diffusible factors are not required (Wehage et al., 2002). Recent data suggests that oxidative stress induces production of ADPR within the mitochondria, which is then released in the cytosol to activate TRPM2 (Perraud

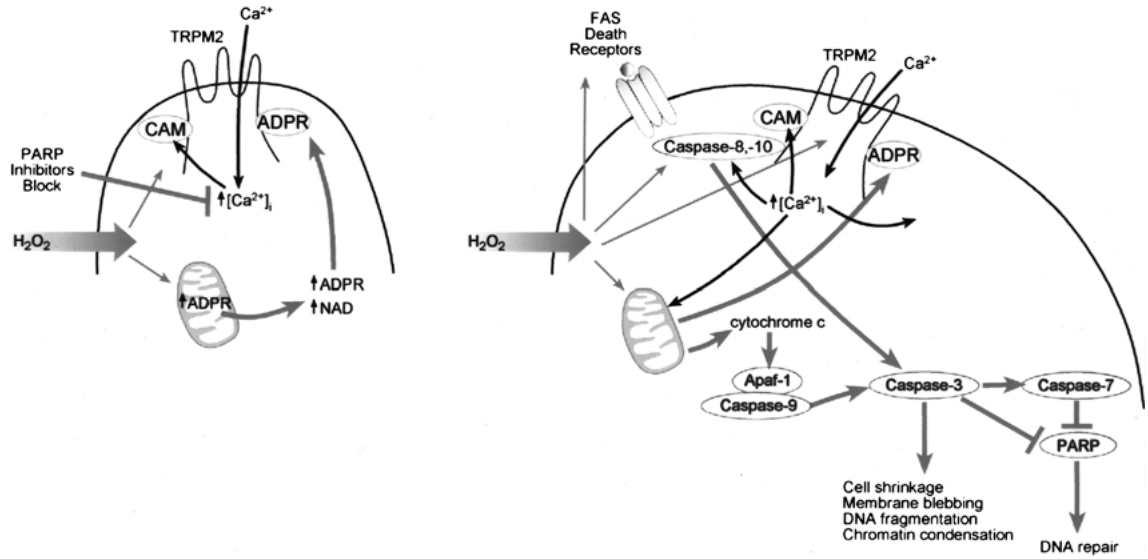

Fig. 4. Proposed signaling mechanisms of TRPM2 activation and induction of cell death by H_2O_2. *Left panel:* TRPM2 activation. H_2O_2 activates mitochondrial production of ADP-ribose (ADPR), which is released into the cytosol. The increase in ADPR (and possibly NAD) activates TRPM2 by binding to the C-terminal NUDT9-H domain. Ca^{2+} influx ensues, which enhances calmodulin (CAM) binding to TRPM2 and channel opening. PARP inhibitors block TRPM2 activation. *Right panel:* Induction of cell death. $[Ca^{2+}]_i$ rises, and in association with other oxidative stress-induced signals results in activation of extrinsic and intrinsic cell death pathways, leading to caspase 3 activation and PARP cleavage and inactivation.

et al., 2005). Induction of NUDT9 ADPR pyrophosphatase, which degrades ADPR, suppressed H_2O_2-induced calcium transients, providing strong support for a requirement for ADPR binding in TRPM2 gating.

CD38 is a transmembrane glycoprotein that is expressed in many tissues including lymphoid and myeloid cells, and has NAD-glycohydrolase/ADP-ribosyl cyclase/cADPR hydrolase activity (Heiner et al., 2003b). Approximately 97% of its product is ADPR, and the rest is cADPR, both of which are able to activate TRPM2 (Kuhn & Luckhoff, 2004; Kolisek et al., 2005). A role for CD38 in TRPM2 activation in immune cells is supported by the observation that Ca^{2+} influx is almost abolished in CD38 knockout mice (Partida-Sanchez et al., 2001). A perplexing issue that needs to be resolved is whether CD38 is involved in regulation of intracellular ADPR levels, since the enzymatic activity of CD38 is extracellular, whereas TRPM2 ADPR and cADPR binding sites are intracellular, and ADPR and cADPR do not cross the membrane freely.

In studies exploring mechanisms of activation of TRPM2, inhibitors of poly(ADP-ribose) polymerase (PARP) were observed to block H_2O_2-mediated increases in $[Ca^{2+}]_i$ as well as mitochondrial dysfunction in HEK 293 cells expressing TRPM2 and in CRI-GI insulinoma cells (Fonfria et al., 2004; Perraud et al., 2005). Ca^{2+} influx was still evoked by ADPR, suggesting that PARP inhibitors did not directly block TRPM2 channel activity. PARP inhibitors also protected rat striatal cells from H_2O_2 and amyloid β-peptide-induced cell death (Fonfria et al., 2005). These studies suggest that oxidative stress-induced activation of TRPM2 requires PARP activity. One mechanism through which this may occur is the following: H_2O_2 treatment may result in PARP activation, possibly through peroxide damage to DNA. PARP enzymes catalyze the breakdown of NAD into nicotinamide and ADPR, which can in turn activate TRPM2. Conversely, activation of TRPM2 has been shown to result in cleavage and inactivation of PARP (Zhang et al., 2006). These studies suggest a possible feedback loop in which TRPM2 is activated by PARP, but TRPM2 activation in turn results in PARP cleavage and inactivation, protecting cells from death. However, because PARP inhibitors are not completely specific and the concentrations required to inhibit TRPM2 activation by oxidative stress are above that required for inhibition of PARP-1 *in vitro*, ambiguity about the role of PARP in TRPM2 activation remains. A model of activation of TRPM2 following oxidative stress and downstream effectors mediating cell death is shown in Figure 4.

Once Ca^{2+} influx is activated, the rise in $[Ca^{2+}]_i$ may contribute to oxidative stress-induced cell death through a number of different pathways which have been reviewed in detail elsewhere (Chandra et al., 2000; Schild et al., 2001; Ermak & Davies, 2002; Orrenius, Zhivotovsky & Nicotera, 2003; Jo et al., 2004; Langley & Ratan, 2004). These include caspase cleavage and PARP inactivation (Zhang et al., 2006), mitochondrial release of cytochrome c, activation of tyrosine kinases and phosphatases, and binding of

transcription factors, including NF-κB, to their target genes. Macrophages and neutrophils express TRPM2, and elevation of $[Ca^{2+}]_i$ may be an important part of the respiratory burst and regulate other aspects of the inflammatory response including cytokine production (Heiner et al., 2003a, 2003b; Perraud, Knowles & Schmitz, 2004).

Inhibition of TRPM2 function may be an important and broadly applicable *in vivo* approach to protect cells from death following oxidant stress and other adverse stimuli. This strategy could protect a diverse range of tissues including brain and heart from death following ischemia/reperfusion, and protect other organs including bone marrow (TNFα), pancrease (diabetes), and brain (Alzheimer's, Parkinson's diseases) from injury associated with tissue-specific insults (Fonfria et al., 2005; McNulty & Fonfria, 2005; Zhang et al., 2006). Selective TRPM2 inhibitors have not been identified but are a subject of intensive research. Down-regulation of TRPM2 by other strategies including antisense may also protect cells from ischemic or toxic death. Much more needs to be learned about how TRPM2 protein levels are regulated and how the channel is activated, because other mechanisms that affect TRPM2 expression or activation could also be targeted to preserve cell viability.

TRPM7

TRPM7, also called TRP-PLIK, ChaK1, and LTRPC7, functions as an ion channel but also has a C-terminal Ser/Thr kinase domain with homology to the eEF2 α-kinase family (Nadler et al., 2001; Runnels, Yue & Clapham, 2001). TRPM7 is widely expressed, and electrophysiological analyses of currents in TRPM7-expressing cells have revealed both non-selective conductance of Na^+ and Ca^{2+} (Runnels et al., 2001) and a selectivity towards divalent cations (Nadler et al., 2001). TRPM7 is permeable to Mg^{2+}, a rare feature among known ion channels. TRPM7 currents are inhibited by Mg^{2+} and Zn^{2+}, blocking monovalent cation flow, and are activated by low Mg·ATP levels (Nadler et al., 2001; Kozak & Cahalan, 2003).

TRPM6 and TRPM7 are the two ion channels involved in regulation of intracellular Mg^{2+}, and both have an intrinsic kinase domain. Patients with autosomal dominant familial hypomagnesemia carry mutations in the TRPM6 gene (Schlingmann et al., 2002; Walder et al., 2002). The roles of TRPM6 and TRPM7 in regulation of intracellular magnesium levels have recently been reviewed (Perraud et al., 2004; McNulty & Fonfria, 2005; Schmitz & Perraud, 2005). Targeted deletion of TRPM7 in DT-40 B cells is lethal. These cells exhibited Mg^{2+} deficiency, growth arrest, and died within 24 hours unless they were rescued by exposure to increased levels of extracellular Mg^{2+} (Nadler et al., 2001). The decrease in intracellular magnesium in TRPM7-deficient cells suggests that one role of TRPM7 is to act as a Mg^{2+}-sensing or uptake pathway controlling cellular Mg^{2+} homeostasis (Montell, 2003; Schmitz et al., 2003). RNA interference was used to down-regulate TRPM7 in vascular smooth muscle cells, and this resulted in a significant decrease in basal $[Mg^{2+}]_i$ but did not affect $[Ca^{2+}]_i$ (He et al., 2005). In TRPM7-deficient vascular smooth muscle cells, ^{3}[H]-thymidine and ^{3}[H]-leucine incorporation were decreased following angiotensin II stimulation, indicating decreased DNA and protein synthesis and a role of TRPM7 in cell growth. Down-modulation of TRPM7 in retinoblastoma cells further demonstrated that TRPM7 is involved in cell proliferation and cell cycle progression (Hanano et al., 2004).

Cation conductance through TRPM7 is activated by reactive oxygen/nitrogen species, resulting in anoxic neuronal death. Overexpression of TRPM7 in HEK 293 cells results in cell swelling, detachment, and death by 48–72 hours (Nadler et al., 2001; Aarts et al., 2003). These studies, along with the depletion studies described above, demonstrate that precise regulation of TRPM7 channel expression is required for cell survival. H_2O_2 markedly enhanced inward TRPM7 currents (Aarts et al., 2003). Suppression of TRPM7 expression in primary cortical neurons with RNA interference blocked TRPM7 currents, anoxic Ca^{2+} influx, and reactive oxygen-species production, protecting cells from anoxia and demonstrating a role for endogenous TRPM7 in anoxic cell death (Aarts et al., 2003). Suppressing TRPM7 allowed the extended survival of anoxic neurons following prolonged oxygen glucose deprivation. However, siRNA targeted to TRPM7 also reduced TRPM2 levels. These data suggest that expression of TRPM2 and TRPM7 is interdependent and make it difficult to clearly distinguish the roles of TRPM7 and TRPM2 in anoxic injury in these cells. The interaction of TRPM2 and TRPM7 in modulation of cell survival and death, and the ability of these two channels to heterodimerize is currently being investigated.

Although TRPM7 is involved in anoxia, and reduction of TRPM7 levels or blockade of TRPM7 might appear to be a method of reducing cell death, depletion of TRPM7 in cell lines is lethal (Nadler et al., 2001) and precise regulation of intracellular expression may be required. While a conventional TRPM7 knockout mouse is unlikely to be viable, tissue-specific or inducible TRPM7 knockouts could be very informative about in vivo activity. The importance of TRPM2 and TRPM7 in oxidative stress is shown by numerous experiments reviewed here, and one or both of these channels may be pharmaceutical targets in the near future.

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