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SYMPOSIUM REVIEW

TRPM2 protects against tissue damage following oxidative stress and ischaemia–reperfusion

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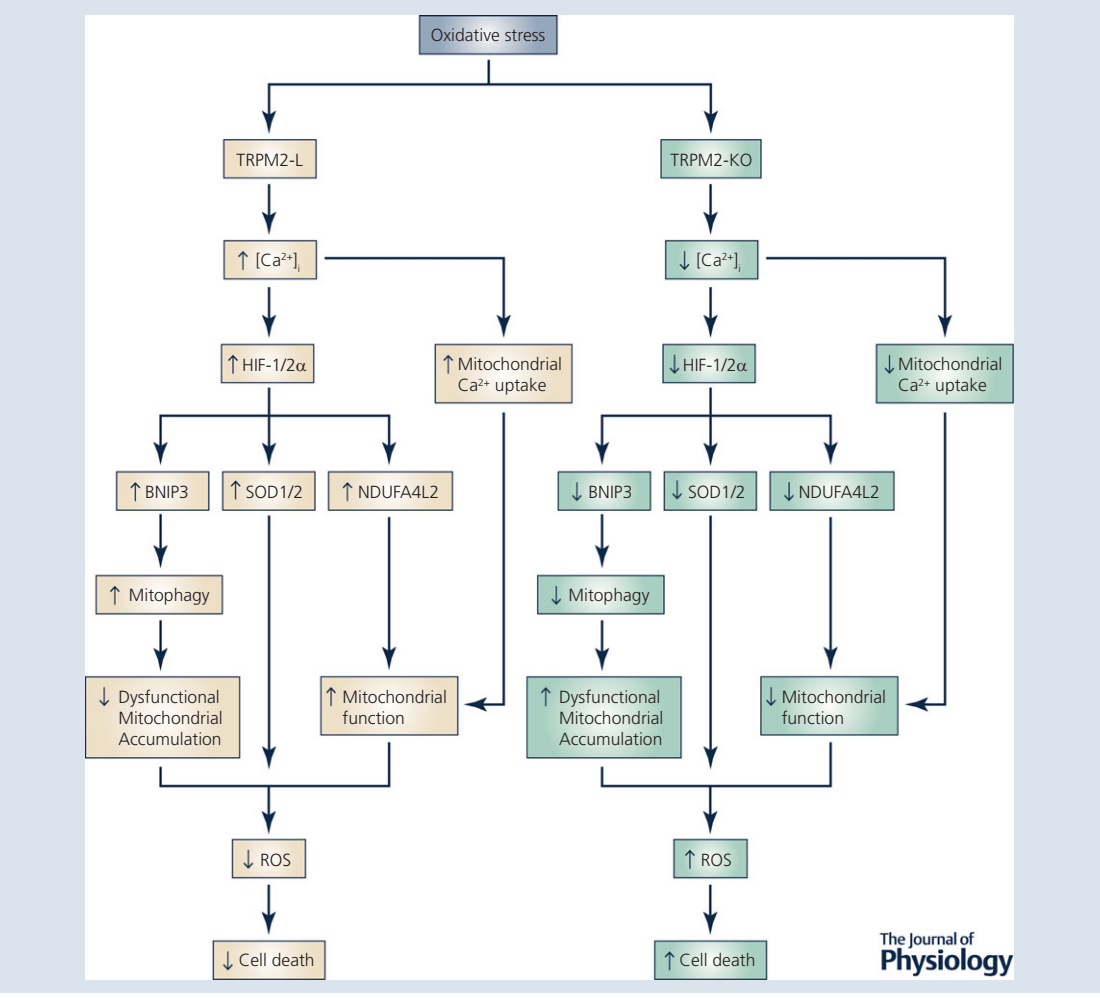

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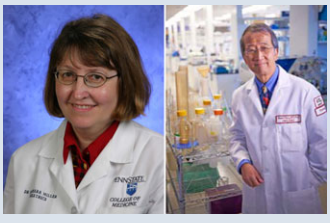

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Abstract TRPM channels are a subgroup of the transient receptor potential (TRP) channel superfamily whose members have important roles in cell proliferation and survival. TRPM2, the second subfamily member to be cloned, is expressed in many tissues including brain, heart, vasculature and haematopoietic cells. TRPM2 is activated by oxidative stress and several other extracellular signals including tumour necrosis factor α (TNF-α) and amyloid β-peptide, which increase production of ADP-ribose (ADPR). ADPR binds to the TRPM2 C-terminal NUDT9-H domain, activating the channel. Early studies support the paradigm that TRPM2 activation induces cell death by sustained Ca^{2+} influx or by enhancing cytokine production, aggravating inflammation and tissue injury. However, more recent data show that for a number of physiological processes, TRPM2 is protective. TRPM2 protects lungs from endotoxin-induced injury by reducing reactive oxygen species (ROS) production by phagocytes. It protects hearts from oxidative damage after ischaemia–reperfusion or hypoxia–reoxygenation by maintaining better mitochondrial bioenergetics and by decreasing ROS. Sustained Ca^{2+} entry through TRPM2 is required to maintain cellular bioenergetics and protect against hypoxia–reoxygenation injury. TRPM2 also protects neuroblastoma from moderate oxidative stress by decreasing ROS through increased levels of forkhead box transcription factor 3a (FOXO3a) and a downstream effector, superoxide dismutase 2. TRPM2 is important for tumour growth and cell survival through modulation of hypoxia-inducible transcription factor expression, mitochondrial function and mitophagy. These findings in cardiac ischaemia and in neuroblastoma suggest that TRPM2 has a basic role in sustaining mitochondrial function and in cell survival that applies to a number of physiological systems and pathophysiological processes including ischaemia–reperfusion injury.

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Abstract figure legend Although activation of the ion channel TRPM2 can induce cell death in some circumstances, TRPM2 can also preserve cell viability and protect against tissue damage following oxidative stress and ischaemia–reperfusion. TRPM2 dependent Ca^{2+} entry can modulate HIF-1/2α expression. One mechanism through which this may occur is through enhancement of calcineurin activity through TRPM2-dependent Ca^{2+} entry, which may increase HIF-1/2α stability. HIF-1/2α enhances expression of a number of target genes including those involved in energy metabolism, antioxidant expression and mitophagy. Ca^{2+} entry through TRPM2 may also directly influence mitochondrial Ca^{2+} uptake. Together, the impact on mitochondrial function results in reduced ROS production and reduced cell death. In contrast, in the TRPM2 KO, Ca^{2+} influx is reduced after oxidative stress and HIF-1/2α expression is decreased, as are proteins downstream of HIF-1/2α including BNIP3, SOD1/2, and NDUFA4L2. In addition, mitochondrial Ca^{2+} uptake is reduced, which may contribute to dysfunctional mitochondria along with decreased NDUFA4L2, and reduced mitochondrial bioenergetics. Decreased BNIP3, which results in reduced mitophagy, contributes to an accumulation of dysfunctional mitochondria and along with decreased SOD1/2 antioxidant activity, increased ROS. The cell has reduced tolerance to a further rise in ROS, for example following ischaemia or doxorubicin, leading to reduced cell survival and increased cell death in the absence of TRPM2.

Abreviations ADPR, ADP-ribose; cKO, cardiac-specific knock-out; +dP/dt, first time derivative of the left ventricular pressure rise; FOXO3a, forkhead box transcription factor 3a; gKO, global knock-out; GFP, green fluorescent protein; HIF-1α, hypoxia inducible factor-1α; H/R, hypoxia–reoxygenation; I/R, ischaemia/reperfusion; mPTP, mitochondrial permeability transition pore; NRVM, neonatal rat ventricular myocyte; OCR, oxygen consumption rate; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF-α, tumour necrosis factor α; TRP, transient receptor potential; WT, wild-type.

Introduction

Transient receptor potential (TRP) channels are members of a superfamily of monovalent and divalent cation-permeable ion channels with six transmembrane domains (Ramsey *et al.* 2006; Nilius *et al.* 2007; Chen *et al.* 2014). The TRPM subfamily is named after the first member to be described, TRPM1 (M, melastatin), which is a putative tumour suppressor protein (Duncan *et al.* 1998). Other TRPM channel family members also have important roles in cell proliferation and cell survival including TRPM2 (Hara *et al.* 2002; Miller & Zhang, 2011), TRPM4 (Abriel *et al.* 2012; Simard *et al.* 2012), TRPM5 (Prawitt *et al.* 2000), TRPM7 (Aarts *et al.* 2003; Aarts & Tymianski, 2005; Guilbert *et al.* 2009) and TRPM8 (Lehen'kyi & Prevarskaya, 2011). The second member of the TRPM family to be cloned, the gene *TRPM2* is located on human chromosome 21q22.3, has 32 exons

and encodes a protein of 1503 amino acids (predicted molecular mass ∼170 kDa) (Nagamine *et al.* 1998). The mouse *TRPM2* gene is highly homologous, with 34 exons encoding a protein of 1507 amino acids (Uemura *et al.* 2005).

TRPM2 channels are widely expressed in many cell types including brain, heart and haematopoietic, vascular, smooth muscle and endothelial cells and are permeable to Ca^{2+}, Na^{+} and K^{+} (Perraud *et al.* 2003; Miller & Zhang, 2011). TRPM2 has been known since its identification to play an essential role in oxidative stress, but understanding its physiological role has become more complex with recent *in vivo* studies as well as *in vitro* mechanistic data. This review will focus on new data elucidating the role of TRPM2 in ischaemia–reperfusion (I/R) injury and the new concept that calcium entry via TRPM2 is protective rather than injurious to some tissues, including heart, when subjected to oxidative stress.

TRPM2 regulation

Extracellular signals which activate TRPM2 include oxidative stress, tumour necrosis factor α (TNF-α), amyloid β-peptide and concanavalin A (Hara *et al.* 2002; Wehage *et al.* 2002; Fonfria *et al.* 2005; Gasser *et al.* 2006; Chen *et al.* 2013). Stimulation with these extracellular signals results in production of ADPR, which binds to the TRPM2 C-terminal NUDT9-H domain (Perraud *et al.* 2001), activating the channel (Kolisek *et al.* 2005; Perraud *et al.* 2005; Gasser *et al.* 2006; Toth & Csanady, 2010). Cyclic adenosine diphosphoribose can potentiate the effects of ADPR at low concentrations and gates TRPM2 by itself. ADPR is produced primarily by mitochondria (Perraud *et al.* 2005) or by activation of poly(ADPR)polymerase (Fonfria *et al.* 2004; Buelow *et al.* 2008). TRPM2 is also positively regulated by intracellular Ca^{2+} and calmodulin (McHugh *et al.* 2003; Tong *et al.* 2006; Du *et al.* 2009*a*). Interaction of ADPR with TRPM2 supports initial Ca^{2+} entry through TRPM2. The subsequent increase in Ca^{2+}-bound calmodulin enhances calmodulin binding to an IQ motif in the N terminus of TRPM2, providing positive feedback for TRPM2 activation and increased Ca^{2+} influx (Tong *et al.* 2006). TRPM2 with mutant ADPR binding sites can be directly activated by an increase in $[Ca^{2+}]_i$, and TRPM2 may be activated in a wide range of physiological situations through this mechanism (Du *et al.* 2009*b*). TRPM2 has also been reported to be temperature sensitive (Togashi *et al.* 2006) and inhibited by acidification (Du *et al.* 2009*b*; Starkus *et al.* 2010), thereby providing a mechanism for limiting Ca^{2+} entry during ischaemia.

In addition to full length TRPM2 (TRPM2-L), physiological splice variants include TRPM2-S (short) (Zhang *et al.* 2003), TRPM2-ΔN, TRPM2-ΔC (Wehage *et al.* 2002) and TRPM2-TE (Orfanelli *et al.* 2008). TRPM2, like other TRP channels, is a tetramer, and association of splice variants with TRPM2-L may modulate TRPM2 function. Indeed, TRPM2-S, which lacks the entire C terminus and the putative Ca^{2+} pore, has been shown to inhibit Ca^{2+} influx through TRPM2-L and functions as a dominant negative (Zhang *et al.* 2003). Little is known about the mechanisms by which alternative splicing to alter isoform expression is regulated *in vivo*, or the physiological functions and interactions of TRPM2 isoforms, other than that they influence and can disrupt full length channel function.

TRPM2 electrophysiology in adult cardiac cells

In the heart, intracellular ADPR elicited a large inward and outward current in wild-type (WT) but not in TRPM2 global knock-out (gKO) myocytes (Miller *et al.* 2014; Hoffman *et al.* 2015). ADPR-activated currents displayed the characteristic TRPM2 linear current–voltage relationship with E_{rev} close to 0 mV (Perraud *et al.* 2001; Miller *et al.* 2014). Flufenamic acid abolished the current elicited by ADPR in WT myocytes, consistent with the notion that the ADPR-activated current was mediated by TRPM2. In WT myocytes, TRPM2 currents did not inactivate, consistent with observations in HEK293 cells stably expressing WT TRPM2 (Perraud *et al.* 2001). The ratio of calcium conductance to sodium conductance was 0.56 ± 0.02 (Miller *et al.* 2014) and 0.65 ± 0.08 (Hoffman *et al.* 2015) in adult cardiac myocytes and transiently transfected HEK293 cells, respectively.

Role of TRPM2 in inflamation and ischaemia–reperfusion injury

A number of TRP channels have been shown to mediate oxidative-stress induced injury (Miller & Zhang, 2011), but in this review we will focus on TRPM2. Early studies on the function of the TRPM2 channel support the paradigm that activation of TRPM2 induced cell death by sustained increase in $[Ca^{2+}]_i$ (Hara *et al.* 2002; Kaneko *et al.* 2006; Hecquet *et al.* 2008) or by enhanced cytokine production, which was associated with increased inflammation and tissue injury (Knowles *et al.* 2011; Knowles *et al.* 2013). In a model of human inflammatory bowel disease (dextran sulfate sodium-induced colitis in mice), TRPM2-mediated Ca^{2+} influx stimulated chemokine (CXCL2) production in monocytes, resulting in recruitment of neutrophils to the site of inflammation, and was also associated with tissue damage (Yamamoto *et al.* 2008). However, more recent reports suggest a different paradigm, that TRPM2 can be protective rather than deleterious in pathophysiological situations. *In vitro*, TRPM2-L protected neuroblastoma cells from low to moderate oxidative stress through increased expression of forkhead box transcription factor 3a (FOXO3a) and

superoxide dismutase (SOD)2, reducing reactive oxygen species (ROS) levels, whereas cells expressing the dominant negative TRPM2-S isoform had reduced FOXO3a and SOD2 and enhanced ROS with increased susceptibility to oxidative stress (Miller *et al.* 2013). This is similar to studies on pyramidal neurons subjected to oxidant injury in which inhibition of TRPM2 enhanced cellular damage (Bai & Lipski, 2010), although this finding is controversial (Kaneko *et al.* 2006). The different results in the more recent *in vitro* studies may be accounted for by the lower, more physiological levels of oxidative stress (H_2O_2 dose), or potentially by the use of different cell types with different endogenous oxidant production and antioxidant defences. Recent *in vivo* studies confirmed these findings that TRPM2 activation in physiological or pathophysiological conditions can reduce tissue injury. In WT mice subjected to intraperitoneal injection of endotoxin, survial was 5 times higher than in gKO mice (Di *et al.* 2012). This was due to cation entry via TRPM2 channels, resulting in plasma membrane depolarization and decreased NOX-mediated ROS production in WT phagocytes, thereby preserving viability. In humans, a TRPM2 mutant (P1018L) was identified in a subtype of Guamanian amyotropic lateral sclerosis and Parkinsonism dementia patients (Hermosura *et al.* 2008). Whereas WT TRPM2 channel did not inactivate, the P1018L mutant inactivated after channel opening by ADPR (Xia *et al.* 2008), effectively limiting Ca^{2+} entry and suggested that WT TRPM2 was necessary for normal neuronal function. In this review, the role of TRPM2 in oxidative stress-induced injury and ROS production is largely derived from our experimental results of two models: cardiac I/R injury in WT and gKO and cardiac-specific knock-out (cKO) mice and in a neuroblastoma xenograft model treated with doxorubicin.

Role of TRPM2 in cardiac ischaemia–reperfusion injury

In the heart, ROS are produced physiologically during respiration by the mitochondrial electron transport chain, and increased ROS are observed after stimulation of myocytes with β-adrenergic agonists and in pathological conditions including I/R and doxorubicin exposure. ROS play a major role in myocyte injury through protein oxidation, lipid peroxidation and mutagenesis. During hypoxia–reoxygenation (H/R, simulated I/R *in vitro*), a significant increase in intracellular ADPR occurs in cardiac myocytes, which may activate TRPM2 channels and leads to elevation in $[Ca^{2+}]_i$. We studied WT and TRPM2-gKO mice to determine the impact of abrogation of TRPM2-dependent Ca^{2+} influx on I/R injury. Using confocal microscopy, we first determined that TRPM2 channels were expressed in the sarcolemma and transverse tubules of adult cardiac myocytes (Miller *et al.* 2013). Exposure of myocytes to H_2O_2 resulted in a large increase in $[Ca^{2+}]_i$, which was significantly higher in WT compared with gKO myocytes; the $[Ca^{2+}]_i$ increase was blocked by clotrimazole and was dependent on extracellular Ca^{2+}, demonstrating that cardiac TRPM2 channels were functional. H_2O_2 did not increase $[Na^+]_i$ in WT cardiac myocytes (Hoffman *et al.* 2015). At baseline, there were no differences in body weights, left venticular masses, heart rates, *in vivo* haemodynamics, fractional shortening, or response to isoproterenol stimulation between WT and gKO hearts (Miller *et al.* 2013). I/R injury was induced by occluding the left anterior descending coronary artery for 30 min followed by release, and cardiac function was evaluated 2–3 days post surgery. TRPM2 deficiency was associated with aggravation of *in vivo* cardiac contractile dysfunction, as demonstrated by significantly lower fractional shortening and first time derivative of the left ventricular pressure rise ($+dP/dt$). Similarly, worse cardiac contractility was observed in gKO hearts treated with doxorubicin (another oxidative cardiac injury model) compared to similarly treated WT hearts (Hoffman *et al.* 2015). To simulate I/R *in vitro*, freshly isolated myocytes from WT or gKO hearts were exposed *in vitro* to 30 min of hypoxia followed by 2 h of reoxygenation. Whereas there were no differences in ROS levels between WT and gKO myocytes incubated under normoxic conditions, after H/R ROS levels in gKO myocytes were significantly higher than in WT myocytes. SOD1 and SOD2 were significantly lower in I/R in KO hearts compared with WT-I/R hearts. Upstream regulators of SOD expression including hypoxia inducible factor-1α (HIF-1α), FOXO1 and FOXO3a were also reduced in KO-I/R hearts, suggesting that reduced Ca^{2+} influx in gKO myocytes may have a role in reduction of HIF-1α, FOXOs and SODs (Liu *et al.* 2007; Chen *et al.* 2014), with the reduction in antioxidants leading to increased ROS. In addition, NOX4 was increased in KO-I/R hearts. We could not distinguish whether increased ROS scavenging capacity or reduced ROS generation was the dominant method by which TRPM2 protected heart from I/R injury.

Mice with cardiac-specific knock-out (cKO) of *TRPM2* were generated to differentiate whether the benefical effect of TRPM2 channels on cardiac contractility post-I/R was due to the better capability of WT myocytes to withstand oxidative stress, or to the reduced inflammatory response secondary to reduced ROS production by infiltrating WT monocytes and macrophages (Hoffman *et al.* 2015). At 2 months of age, 64% of the floxed *TRPM2* gene was deleted in cKO hearts as evaluated by qPCR and 79% of the floxed *TRPM2* gene was deleted in cKO myocytes as determined by TRPM2 current measurements. Similar to gKO hearts, cKO hearts subjected to I/R exhibited significantly lower $+dP/dt$ and maximal systolic pressure when compared to WT-I/R hearts. This important observation strongly argues that it was the cardiac TRPM2 channels, rather than phagocytic TRPM2 channels (by

inhibiting NOX-mediated ROS production and thereby reducing inflammatory response) (Di *et al.* 2012), which mediated protection against I/R injury. In this context, it is relevant to note that the weight of evidence argues against a pivotal role of neutrophils as a causative factor in most forms of I/R injury in the heart and brain (Baxter, 2002). Finally, the role of TRPM2 in angiogenesis and ischaemic neovascularization in protection against cardiac I/R injury (Mittal *et al.* 2015) is likely to be small in view of our results obtained with cKO hearts.

A recent study using an independent gKO mouse (C57BL/6 background) reported very different results (Hiroi *et al.* 2012). Specifically, after 45 min of ischaemia followed by 24 h of reperfusion *in vivo*, neutrophil infiltration was less, infarct size was smaller and $+dP/dt$ was higher in gKO compared to WT hearts. The authors speculated that increased neutrophil adhesion to endothelial cells mediated by TRPM2 channels caused increased damage post-I/R. The proposed mechanism, however, is not compatible with the results from a recent study that found that Ca^{2+} entry via activated TRPM2 channels decreased NOX-mediated ROS production in phagocytes (Di *et al.* 2012), thereby lessening the inflammatory response. The proposed mechanism would also have predicted similar post-I/R myocardial dysfunction in WT and cKO mice since TRPM2 channels were present in bone marrow cells in cKO animals. This prediction is not supported by our observation that myocardial function was worse in cKO mice post-I/R (Hoffman *et al.* 2015). The reasons for the different results between our studies (Miller *et al.* 2013; Miller *et al.* 2014; Hoffman *et al.* 2015) and those of Hiroi *et al.* (2012) may include the following: (1) TRPM2 was deleted by targeting different exons; (2) 45 *vs.* 30 min of ischaemia resulted in much larger infarcts (45% *vs.* 27% of area-at-risk); (3) cardiac function was examined at 24 h *vs.* 72 h of reperfusion; (4) different anaesthetics (phenobarbital *vs.* isoflurane); (5) different surgical techniques used in measuring *in vivo* haemodynamics (opening the chest followed by left ventricular puncture *vs.* catheterizing the right carotid artery in a closed-chest preparation); and (6) increased heat dissipation in open-chest mice compared with closed-chest mice. Although the use of the cardioprotective volatile anaesthetic isoflurane during our surgical procedure may be a confounding factor (Roberge *et al.* 2014), our current results with cKO hearts, together with the positive rescue experiments by *TRPM2* but not by the loss-of-function E960D mutant in gKO myocytes (Hoffman *et al.* 2015), strongly argue in favour of beneficial, rather than detrimental, effects of TRPM2 on cardiac bioenergetics and function, under both basal and stressed conditions. In addition, our observations on cKO hearts suggest the role played by phagocytic TRPM2 channels on cardiac I/R injury is likely to be small.

Another study utilizing neonatal rat ventricular myocytes (NRVMs) reported that TRPM2 exacerbated H_2O_2 injury (Yang *et al.* 2006). In this scenario, H_2O_2 (100 μM) activated TRPM2 through increased ADPR/NAD^+ formation, leading to Ca^{2+} and Na^+ overload in mitochondria, myocyte apoptosis through mitochondrial membrane disruption, cytochrome *c* release, and caspase-3-dependent chromatin condensation and fragmentation. It should be noted that NRVMs exhibit a very different phenotype from adult cardiac myocytes. For example, in culture NRVMs proliferate while mature adult cardiac cells do not divide. Another difference is that NRVMs spontaneously beat in culture whereas healthy adult left ventricular myocytes are quiescent. More relevant to our discussion is that while activation of TRPM2 by H_2O_2 resulted in increases in both $[Ca^{2+}]_i$ and $[Na^+]_i$ in NRVMs (Yang *et al.* 2006), in adult cardiac myocytes, H_2O_2 increased $[Ca^{2+}]_i$ (Miller *et al.* 2013) but not $[Na^+]_i$ (Hoffman *et al.* 2015). An explanation for the discrepancy may relate to the fact that NRVMs expressed α1- and α3-isoforms (Lucchesi & Sweadner, 1991) whereas adult mouse cardiac myocytes expressed α1- and α2-isoforms of Na^+–K^+-ATPase (Berry *et al.* 2007). When expressed in Sf-9 insect cells, the α3-isoform exhibited > 2-fold less affinity for Na^+ when compared to the α2-isoform (Blanco & Mercer, 1998) and may account for lower Na^+ transport rate. In addition, adenovirus-mediated overexpression of the α2-isoform in adult rat cardiac myocytes led to greater affinity for Na^+ and higher Na^+–K^+-ATPase activity compared to control myocytes expressing β-galactosidase (Correll *et al.* 2014). Finally, NRVMs do not possess the highly organized t-tubules in which the α2-isoform is preferentially localized in adult mouse cardiac myocytes (Berry *et al.* 2007). These fundamental differences in physiology may account for different results observed in NRVMs *vs.* adult cardiac myocytes.

Ca^{2+} influx via TRPM2 is essential for maintenance of cardiac bioenergetics and protection against H/R Injury

To examine if Ca^{2+} influx through TRPM2 was required for maintenance of bioenergetics, WT *TRPM2*, *TRPM2* loss-of-function mutant (E960D) (Xia *et al.* 2008), inactivating mutant P1018L (Hermosura *et al.* 2008), or control green fluorescent protein (GFP) were expressed in gKO hearts by adenovirus-mediated gene transfer (Hoffman *et al.* 2015) (Fig. 1). gKO-GFP heart slices had significantly higher ROS levels and lower oxygen consumption rate (OCR) compared to WT-GFP heart slices. WT *TRPM2* but not loss-of-function E960D mutant decreased ROS and increased OCR when expressed in gKO hearts. These results indicate that TRPM2-mediated

Ca^{2+} influx was necessary for normal bioenergetics maintenance.

To futher evaluate if TRPM2-mediated Ca^{2+} entry was necessary for protection against H/R injury, we expressed GFP, WT *TRPM2*, E960D and P1018L mutants in gKO myocytes followed by H/R (Hoffman *et al.* 2015). After H/R, hearts with WT *TRPM2* but not the loss-of-function E960D or inactivating P1018L mutants had significantly lowered mitochondrial superoxide levels in gKO myocytes, and this occurred only in the presence of extracellular Ca^{2+}. *TRPM2* but not E960D or P1018L also restored OCR. This indicates that sustained Ca^{2+} entry was necessary for TRPM2-mediated reduction of ROS and for protection against H/R injury.

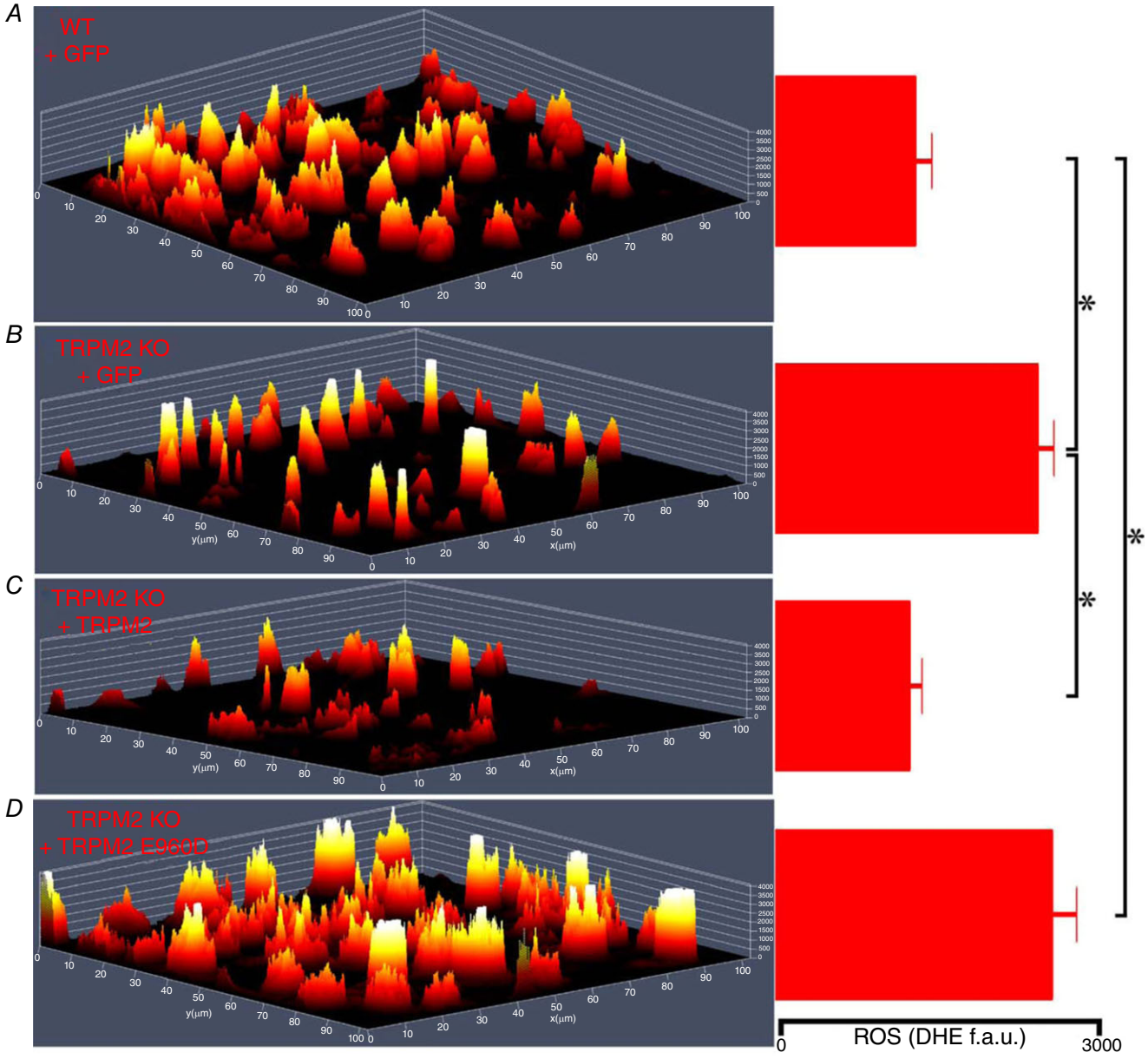

Figure 1. Ca^{2+} entry through TRPM2 is required to lower intracellular ROS in gKO myocytes
Hearts from WT and global TRPM2 KO mice were injected with adenovirus expressing either Adv-GFP, Adv-*TRPM2*, or Adv-*TRPM2*-E960D. Heart slices were generated from areas of left ventricle exhibiting GFP fluorescence after 5 days. Multiphoton confocal images of dihydroethidium (DHE)-stained heart slices were obtained and a 2.5-dimensional heatmap plot of mean DHE intensity is shown for WT+GFP (*A*), KO+GFP (*B*), KO+*TRPM2* (*C*), and KO+*TRPM2* E960D (*D*). Quantification of DHE fluorescence in fluorescence arbitrary units (f.a.u.) is shown on the right. DHE fluorescence was quantified from at least 3 slices from each heart. $^*P < 0.05$.

TRPM2, Ca^{2+} entry and I/R injury

Early studies showed diastolic $[Ca^{2+}]_i$ increases progressively (to 3–4 μM) during ATP depletion resulting in contracture (Murphy *et al.* 1994). Ca^{2+} overload is proposed to lead to mitochondrial permeability transition pore (mPTP) opening and irreversible cell death. How then can TRPM2-mediated Ca^{2+} entry during I/R be beneficial? Recent data suggest that in isolated cardiac myocytes subjected to I/R, Ca^{2+} overload is the *consequence* of bioenergetic failure after mPTP opening rather than its *cause* (Lemasters *et al.* 2009). In addition, in isolated perfused mouse hearts subjected to H/R, the mitochondrial-targeted SOD mimetic MitoQ dramatically reduces Ca^{2+} wave-associated mPTP opening (Davidson *et al.* 2012). Our data indicate that TRPM2-mediated Ca^{2+} entry supports mitochondrial function and reduces mitochondrial ROS under both basal and H/R conditions (Hoffman *et al.* 2015). Maintenance of bioenergetics by TRPM2 may prevent mPTP opening and protect hearts from oxidative injury. Another possibility is that TRPM2-mediated Ca^{2+} entry activates Ca^{2+}-dependent signalling pathways (e.g. Pyk2, RACK1) that result in reduction in ROS production and enhance pro-survival signals. Finally, absence of TRPM2 results in a tenuous bioenergetics condition and sensitizes KO myocytes to further oxidative stress brought about by I/R or Doxo treatment (Miller *et al.* 2013; Hoffman *et al.* 2015). This second 'hit' leads to much worse bioenergetics (compared to WT) and higher ROS levels, resulting in impaired contractile performance.

TRPM2 and renal I/R injury

The ability of TRPM2 to protect against I/R injury did not extend to the kidney; rather, gKO exacerbated renal I/R injury (Gao *et al.* 2014). In the kidney, I/R was proposed to activate TRPM2, which physically interacted with Rac1, which in turn recruited NOX subunits to the membrane and increased ROS production. It is worth noting that while TRPM2 was expressed in the sarcolemma of the heart (Miller *et al.* 2013), in renal tubular epithelial cells TRPM2 was widely distributed in the cytoplasm and intracellular organelles but not on the plasma membrane (Gao *et al.* 2014). The role of Ca^{2+} permeation via activated TRPM2 in enhancing renal I/R injury is therefore not clear. Another factor contributing to different findings between heart and kidney may be the different levels of endogenous oxidants and antioxidants present in different cell types. Different levels of H_2O_2 affect different p53-regulated gene expression patterns. We hypothesize that whereas exposure of TRPM2-expressing cells to low levels of H_2O_2 may result in low levels of cation entry and activation of pathways including increased SOD2 which protect viability, exposure of TRPM2-expressing cells to high levels of H_2O_2 results in significantly higher Ca^{2+} entry and leads to cell death. In the former case, inhibition of TRPM2 function is detrimental, whereas in the latter case it can preserve viability. Our experimental conditions in studies of heart and cardiac myocytes involved mild to moderate oxidative stress in which Ca^{2+} influx through TRPM2 would not be overwhelming but rather benefical, activating pathways which reduce ROS production and preserve viability.

TRPM2 enhances cell survival in cancer

TRPM2 is highly expressed in a number of malignancies including melanoma (Orfanelli *et al.* 2008), breast cancer (unpublished observations, Miller lab), and neuroblastoma (Chen *et al.* 2014). Neuroblastoma cells that expressed dominant negative TRPM2-S had increased levels of ROS and increased sensitivity to doxorubicin (Chen *et al.* 2013). We demonstrated that TRPM2-L protected neuroblastoma SH-SY5Y cells from moderate oxidative stress through increased levels of FOXO3a and SOD2. In contrast, cells expressing the dominant negative TRPM2-S had reduced FOXO3a and SOD levels, reduced Ca^{2+} influx in response to oxidative stress, and enhanced ROS, leading to reduced cell viability after exposure to a low or moderate level of H_2O_2. *In vivo*, growth of tumours expressing TRPM2-S was also significantly reduced compared to tumours expressing TRPM2-L, particularly following treatment with doxorubicin. These findings were confirmed in a second neuroblastoma cell line, SK-N-AS, and with the breast cancer cell line MCF-7 (unpublished observations, Miller lab). Expression of HIF-1/2α was significantly decreased in TRPM2-S-expressing tumour cells, as was expression of downstream target proteins regulated by HIF-1/2α including proteins involved in glycolysis (lactate dehydrogenase), oxidant stress (FOXO3a), angiogenesis (VEGF), mitophagy, mitochondrial function (BNIP3 and NDUFA4L2), and mitochondrial electron transport chain activity (cytochrome oxidase 4.1/4.2 in complex IV) (Chen *et al.* 2014). Inhibition of TRPM2-L by several approaches significantly increased sensitivity of cells to doxorubicin, and the reduced survival of TRPM2-S expressing cells was rescued by gain of HIF-1α or -2α function. These data show that TRPM2 activity and its modulation of HIF-1/2α and its downstream effectors is important for cell viability and survival following doxorubicin treatment. Similar to the results observed with cardiac myocytes, TRPM2 played an important role in regulation of cellular ROS levels and cell survival in neuroblastoma and enhanced viability following doxorubicin.

TRPM2 in regulation of cellular bioenergetics

In order to identify the mechanisms through which TRPM2 protects heart from I/R injury, we analysed the

proteomes of the left ventricles from WT-I/R and gKO-I/R mice to identify the major cellular mechanism which afford protection (Miller *et al.* 2014). Global label-free proteomics analysis using GeLC-MS/MS (in-gel tryptic digestion followed by liquid chromatography-tandem mass spectroscopy) technology revealed that the largest differences in canonical pathways between gKO-I/R and WT-I/R hearts were in mitochondrial function and the TCA cycle. In mitochondria, Complexes I, III and IV were down-regulated, whereas Complexes II and V were upregulated in gKO-I/R compared with WT-I/R hearts. We then determined that both mitochondrial membrane potential (Ψ_M) and mitochondrial Ca^{2+} uptake were lower in gKO compared with WT myocytes post-H/R. Reduced mitochondrial Ca^{2+} uptake in gKO myocytes was due to reduction in both the driving force Ψ_M and mitochondrial Ca^{2+} uniporter activity. OCR was lower in gKO myocytes, and ATP levels were markedly lower in gKO compared to WT myocytes. These studies demonstrated that loss of TRPM2-mediated Ca^{2+} entry resulted in impaired cardiac mitochondrial bioenergetics. MitoSOX Red staining of heart slices demonstrated that the elevated ROS in KO heart was largely mitochondrial superoxide.

Similar results were observed in the neuroblastoma cell line SH-SY5Y expressing the dominant negative TRPM2-S. In cells expressing TRPM2-S, Ψ_M and mitochondrial Ca^{2+} uptake, basal and maximal oxygen consumption, and ATP production were reduced compared to cells expressing only TRPM2-L, mitochondrial ROS was increased, and mitochondria were dysmorphic (Chen *et al.* 2014). In cells expressing TRPM2-S, the elevated ROS derived from mitochondria may be due to the lower levels of HIF-1α, FOXO3a, SOD2, BNIP3 and NDUFA4L2, resulting in aberrant mitochondrial bioenergetics. Ca^{2+} has been shown to affect HIF-1α translation and stability, and through this mechanism, Ca^{2+} influx through TRPM2 could affect a number of effectors downstream of HIF-1/2α involved in cell survival. A critical role for low level mitochondrial Ca^{2+} uptake in regulation of bioenergetics has been recently demonstrated (Cardenas *et al.* 2010; Wallace, 2012), and reduced Ca^{2+} entry may directly affect cellular bioenergetics.

Conclusions

The findings that TRPM2 was critically important in protecting cells from cardiac I/R injury as well as in enhancing tumour cell survival after doxorubicin suggest that TRPM2 has a basic role in cell survival and mitochondrial function that may apply to a number of physiological systems. Our data demonstrate that Ca^{2+} influx through TRPM2 modulated cell viability, and we hypothesize that this may occur through mechanisms involving Ca^{2+} modulation of HIF-1/2α expression. Ca^{2+} influx through TRPM2 enhances calcineurin activity and HIF stability (Liu *et al.* 2007). HIF-1/2α enhances cell viability through regulation of a number of target genes including those involved in angiogenesis, glycolysis, energy metabolism, redox homestasis and mitophagy. In the *TRPM2* KO or when TRPM2 function is inhibited, HIF-1/2α expression is decreased, as is expression of downstream mitochondrial proteins including NDUFA4L2, and together with decreased mitochondrial Ca^{2+} uptake, this results in compromised mitochondria. Decreased HIF-1/2α also results in reduced BNIP3, which contributes to reduced mitophagy, leading to an accumulation of dysfunctional mitochondria and increased ROS (Abstract Figure). With reduced SOD2, ROS levels are elevated and the cell has reduced tolerance to a further rise in ROS, for example from ischaemia–reperfusion or doxorubicin, leading to reduced cell survival and increased cell death in the absence of TRPM2. The work summarized here shows that TRPM2 channels protect cardiac myocytes from ischaemia–reperfusion injury and tumour cells from doxorubicin toxicity, and demonstrates that the mechanisms involve preservation of mitochondrial bioenergetics and modulation of ROS.

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Additional information

Competing interests

None of the authors have a conflict of interest.

Author contributions

All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.