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Overexpression of TRPC3 increases apoptosis but not necrosis in response to ischemia/reperfusion in adult mouse cardiomyocytes

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Abstract

An increase in cytosolic Ca^{2+} via a capacitative calcium entry mediated pathway, attributed to members of the transient receptor potential (TRP) superfamily, TRPC1 and TRPC3, have been reported to play an important role in regulating cardiomyocyte hypertrophy. Increased cytosolic Ca^{2+} also plays a critical role in mediating cell death in response to ischemia/reperfusion (I/R). Therefore, we tested the hypothesis that overexpression of TRPC3 in cardiomyocytes will increase sensitivity to I/R injury. Adult cardiomyocytes isolated from wild-type (WT) mice and from mice over expressing TRPC3 in the heart were subject to 90 min ischemia and 3hr reperfusion. After I/R, viability was $51 \pm 1\%$ in WT and $42 \pm 5\%$ in TG ($p < 0.05$). Apoptosis assessed by Annexin-V was significantly increased in TRPC3 group compared to WT ($32 \pm 1\%$ vs. $21 \pm 3\%$; $p < 0.05$); however, there was no significant difference in necrosis between groups. Treatment of TRPC3 cells with the CCE inhibitor SKF96365 ($0.5 \mu\text{M}$), significantly improved cellular viability ($54 \pm 4\%$) and decreased apoptosis ($15 \pm 4\%$); in contrast, the L-type Ca^{2+} channel inhibitor verapamil ($10 \mu\text{M}$) had no effect. Calpain-mediated cleavage of α -fodrin was increased ~3-fold in the TG group following I/R compared to WT ($p < 0.05$); this was significantly attenuated by SKF96365. The calpain inhibitor, PD150606 ($25 \mu\text{M}$) attenuated the increase in both α -fodrin cleavage and apoptosis in the TRPC3 group. Increased TRPC3 expression also increased sensitivity to Ca^{2+} overload stress, but did not affect the response to TNF- α induced apoptosis. These results suggest that CCE mediated via TRPC may play a role in cardiomyocyte apoptosis following I/R due, at least in part, by increased calpain activation.

Keywords

Capacitative calcium entry (CCE); calpain; Tumor necrosis factor (TNF)-alpha

INTRODUCTION

In cardiomyocytes calcium plays a central role not only in regulating excitation contraction coupling (3), but also in modulating the response to range of stress stimuli (6). An increase in cytosolic Ca^{2+} plays a critical role in initiating cardiomyocyte apoptosis and necrosis that occurs in response to ischemia/reperfusion (4,6). Although a number of Ca^{2+} entry pathways, including the $\text{Na}^+/\text{Ca}^{2+}$ exchanger and the L-type Ca^{2+} channel have been implicated in

mediating cardiomyocyte Ca^{2+} overload (4,6,32) there is little consensus as to which pathways are critical in mediating this process. We have identified a capacitative calcium entry (CCE) or store-operated calcium (SOC) entry pathway in neonatal and adult rat cardiomyocytes (12,13) that not only influences the response to hypertrophic stimuli but may also play a role in mediating Ca^{2+} overload in the heart (20). CCE or SOC, which was first described in nonexcitable cells (34), refers to the influx of Ca^{2+} through plasma membrane calcium channels, activated in response to depletion of calcium in the endo/sarcoplasmic reticulum (ER/SR). We have demonstrated that in cardiomyocytes Ca^{2+} entry via CCE-mediated pathway is clearly distinct from both L-type voltage-gated channels and the reverse mode of $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger (12,13).

The specific proteins responsible for facilitating CCE have yet to be fully characterized; however, there is growing evidence that members of the transient receptor potential (TRP) protein superfamily of proteins may be involved in regulating CCE (14). Mammalian TRP channels are divided in six subfamilies and the TRPC (canonical) family in particular has been implicated in contributing to cellular Ca^{2+} homeostasis (9,14,23,24,43). There are seven members of the TRPC family (TRPC1-7), 6 of which (TRPC1-6) have been shown, at least by RT-PCR, to be present in the heart (9). Structurally TRP channels consist of six transmembrane domains containing a putative Ca^{2+} pore region between the fifth and sixth domains; TRPC channels have ankyrin repeat domains in their N-terminus, which may mediate protein-protein interactions (23). Prototypical activation of TRPC channels occurs in response to agonist stimulation resulting in the generation of diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). Subsequent activation of the ER/SR IP_3 receptor (IP_3R) can mediate depletion of Ca^{2+} from internal stores thereby triggering Ca^{2+} entry across the sarcolemma via TRPCs. Independent of store depletion TRPC activity can be regulated by an increase in DAG; alternatively, IP_3R bound to IP_3 may activate TRPC via direct interaction (23).

As noted above, until relatively recently CCE was considered to be primarily a feature of non-excitable cells and consequently, the majority of data supporting a role for TRPCs in mediating CCE is also from non-excitable cells. However, a number of recent studies have provided evidence that TRPCs may also be functional in the heart (18,28,30). Ohba et al., demonstrated that hypertrophic stimuli increased TRPC1 expression both in the intact heart and in isolated cardiomyocytes (30). They also showed that in isolated cardiomyocytes the increase in TRPC1 expression was associated with increased CCE. The functional significance of TRPC1 was confirmed by using siRNA techniques to prevent agonist induced increase in TRPC1 expression, which attenuated both the hypertrophic response and CCE (30). Conversely, Nakayama et al. demonstrated that overexpression of TRPC3 in cardiomyocytes increased CCE and this was associated with increased cardiac hypertrophy in response to neuroendocrine agonists or pressure overload stimulation (28). Kuwahara et al., (18) also reported that cardiac specific overexpression of TRPC6 increased the propensity for cardiac hypertrophy and heart failure; however, they did not determine whether this was associated with an alteration in CCE.

Therefore, in light of the growing evidence linking TRPC proteins to CCE in cardiomyocytes, combined with our finding that CCE inhibition attenuated cardiac injury in response to calcium overload (20), we tested the hypothesis that increased expression of TRPC3 in cardiomyocytes would result in increased injury due to ischemia/reperfusion. We found that TRPC3 overexpression increased apoptosis and calpain mediated proteolysis resulting from ischemia/reperfusion injury and also increased sensitivity to Ca^{2+} overload; however, TRPC3 had no effect on the response to $\text{TNF-}\alpha$ induced apoptosis. These data strongly suggest that CCE mediated via TRPC may contribute to Ca^{2+} -induced cardiomyocyte apoptosis resulting from ischemia/reperfusion.

MATERIALS AND METHODS

Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

Animals

All animal experiments were approved by the University of Alabama at Birmingham (UAB) Institutional Animal Care and Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals published by National Institute of Health (NIH publication no. 85-23, 1996). Mice overexpressing TRPC3 in the heart, were a kind gift from Dr. Jeffrey D. Molkentin (University of Cincinnati), and have been characterized in detail elsewhere (28); the mice used in this study were derived from line 23 described in the original study (28). All animals for this study were bred at UAB and were genotyped prior to use. Normal, non-transgenic (WT) littermates were used as controls.

Cardiomyocytes Isolation

Male mice, 2-4 months of age (25g - 40g) were heparinized (5000 U/kg, i.p.) 20 min prior to being anesthetized with ketamine (100 mg/kg i.p.). Hearts were rapidly excised and arrested in ice-cold, Ca^{2+} -free perfusion buffer, consisting of (in mM) NaCl (113), KCl (4.7), KH_2PO_4 (0.6), Na_2HPO_4 (0.6), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (1.2), Phenol red (0.032), NaHCO_3 (12), KHCO_3 (10), HEPES (10), Taurine (30), 2,3-Butanedione monoxime (10), Glucose (5.5), pH 7.46. The aorta was cannulated, and the heart was perfused retrogradely with Ca^{2+} -free perfusion buffer at a constant flow of 3 ml/min at 37°C, for 4 min, followed by the same perfusion buffer containing 12.5 μM CaCl_2 and 0.4 mg/ml collagenase type 2 (Worthington). After 15-25 min perfusion with collagenase containing buffer, the heart appeared swollen, pale, and flaccid at which time the ventricles were removed and finely minced. Dispersed myocytes were filtered through a 100 μm mesh and allowed to sediment by gravity for 10 min. The supernatant was removed and centrifuged for 1 min at 180 X g. The pellet was resuspended and combined with the original sedimented myocytes in perfusion buffer containing 5% bovine calf serum and 12.5 μM CaCl_2 . The calcium concentration was increased gradually from 12.5 μM to 1 mM in 5 steps over approximately 20 min. Freshly isolated cardiomyocytes were kept in 2% CO_2 incubator at 37°C. All experiments were performed at least 1 hour after myocyte isolation.

Experimental protocols

Modified cell pelleting model of ischemia—We used a cell-pelleting model to assess hypoxia- and reoxygenation-induced cell death as described in detail by Yamawaki et al. (39). Briefly, an aliquot of cardiomyocytes suspended in MEM (0.5 ml) was placed into a microcentrifuge tube and centrifuged at 80 × g for 60 s. After centrifugation 0.2 ml of the supernatant was removed and replaced by 0.2 mls of mineral oil, which was layered on top of the cell pellet to prevent diffusion of oxygen into the sample. The cell pellet was maintained at 37°C for 90 min at which time cells were either assessed for apoptosis and necrosis as described below or reoxygenated for 3 hours by re-suspending in fresh MEM. In all experiments additional aliquots of cardiomyocytes were incubated under time-controlled normoxic conditions.

Calcium-induced cell death—To determine the effects of TRPC3 expression on the response to calcium-induced cell death, cardiomyocytes were placed on coverslips, and preincubated with 5 μM Thapsigargin for 5 min followed by addition of 2.5 mM extracellular calcium. The cells were imaged on an Olympus I × 70 inverted microscope through a X40

Uplan APO objective. Rounded cells (defined as cells when the ratio between the length and width was < 2) were considered irreversibly injured and dead (5,26).

TNF- α induced apoptosis—Cardiomyocytes were incubated with 10 ng/ml TNF- α in serum-free MEM media (2). Apoptosis was assessed by Annexin V-propidium iodide staining 2h or 18h after treatment with TNF- α .

Assessment of cell viability, apoptosis and necrosis

Several different methods were used to assess cell viability, apoptosis and necrosis.

Cell Morphology—Healthy viable adult cardiomyocytes typically exhibit rod-shaped morphology whereas non-viable cells are usually rounded in nature; therefore the percentage of rod shaped cells was used as an indicator of cell viability.

Annexin V and propidium iodide staining—Myocytes were stained with fluorescein isothiocyanate (FITC), annexin V, and propidium iodide, as per the manufacturer's directions (Vybrant Apoptosis Assay Kit 2, Molecular Probes). The cells were visualized under a fluorescence microscope and were counted regardless of morphology.

Cells not binding FITC-annexin V and excluding propidium iodide were classified as annexin V-negative (29,33) and were deemed viable. Myocytes that bound FITC-annexin V [excitation wavelength (λ_{ex}) = 488 nm and emission wavelength (λ_{em}) = 520 nm] but excluded propidium iodide (λ_{ex} = 540 nm and λ_{em} = 630 nm) were deemed apoptotic. Myocytes permeant to propidium iodide (regardless of whether or not they bound FITC-annexin V) were deemed necrotic (29,33). For quantification, 300 randomly distributed cells were counted in each experiment (29) and the percentage of total number of apoptotic and necrotic cells determined.

Lactate dehydrogenase (LDH) release—As previously described (7), necrosis was assessed by determining the release of lactate dehydrogenase (LDH) in the medium using an LDH assay kit (Sigma). Briefly, the percentage of LDH release was calculated by the ratio of the released LDH into the media to the total LDH (release plus cellular content).

DNA Fragmentation—The Cell Death Detection ELISA plus kit (Roche Molecular Biochemicals, Mannheim, Germany) was used as another indicator of apoptosis. In this assay internucleosomal DNA fragmentation was quantitatively assayed by antibody-mediated capture and detection of cytoplasmic mononucleosome- and oligonucleosome-associated histone-DNA complexes. Briefly, after centrifugation (200 g), cardiomyocytes (1×10^4 cells in each well) resuspended in 200 μ L of the lysis buffer supplied by the manufacturer, and incubated for 30 minutes at room temperature. After pelleting nuclei (200 g, 10 minutes), 20 μ L of the supernatant (cytoplasmic fraction) was used in the enzyme-linked immunosorbent assay (ELISA) following the manufacturer's standard protocol. Following, incubation with peroxidase substrate for 5 minutes, absorbance at 405 nm and 490 nm (reference wavelength) was determined with a microplate reader (Bio-Tec Instruments, Winooski, VT). Signals in wells containing the substrate only were subtracted as background (19).

Western Blot Analysis

Proteins were separated by electrophoresis on 6% or 8% SDS gel, transferred onto a polyvinylidenedifluoride (PVDF) membrane, and immunoblotted with antibodies against β -actin (1:20000, Abcam), α -fodrin (1:2000, Millipore), TRPC1 and TRPC3 (1:200, Alomone Labs). The immunoblots were developed with chemiluminescence (Pierce) and the signal was recorded on X-ray film. Densitometry analysis was performed on the entire lane of each sample using Labworks Analysis Software (UVP).

Data Analysis

All data are presented as means $\pm$ SEM from 3-6 separate experiments, where each experiment represents cells isolated from a single heart. Statistical analysis was performed by either an unpaired t-tests or one-way analysis of variance (ANOVA) followed by Dunnett's Multiple Comparisons Test as appropriate. Statistically significant differences between groups were defined as $p < 0.05$ and are indicated in the legends to the figures.

RESULTS

Expression of TRPC1 and TRPC3

Immunoblot analysis demonstrated that TRPC3 protein levels were significantly increased in hearts from TRPC3 mice compared to wild-type mice (Fig. 1A); however, there was no change in the expression of levels of TRPC1, the other predominant TRPC isoform present in the mouse heart (Fig. 1B). This is consistent with the original study (28), which showed that increased TRPC3 expression was not associated with any changes in other TRPC proteins.

TRPC3 overexpression leads to increased apoptosis in response to ischemia/reperfusion

Adult cardiomyocytes isolated from WT and TRPC3 mice prior to ischemia exhibited predominantly rod-shaped morphology ($> 80\%$; Fig. 2A, 3A). At the end of ischemia and ischemia/reperfusion the number of rod-shaped cells in both groups significantly decreased compared to both baseline conditions (Fig 2A, Fig 3A) and time-controlled normoxic controls. At the end of ischemia/reperfusion, the % rod shaped cells were significantly reduced in the TRPC3 group compared to WT ($42 \pm 5\%$ vs. $51 \pm 1\%$, $p < 0.06$; Fig 3A). Prior to and at the end of ischemia there were no significant differences in apoptosis, as assessed by annexin V positive, propidium iodide negative stained cells, between WT and TRPC3 groups; however, at the end of ischemia/reperfusion apoptosis was approximately 50% higher in the TRPC3 group ($32 \pm 1\%$ vs. $21 \pm 3\%$, $p < 0.05$; Fig 2B, 3B) compared to WT. Necrosis as indicated by propidium iodide positive cells was not different between groups at any time point (Fig 2B, 3C). In time-controlled normoxic incubations for the I/R protocol, both WT and TRPC3 groups compared to baseline there were small, but non-significant increases in apoptosis (from ~ 3.5 to 4.5%) and necrosis (from ~ 15 - 18%); however, there were no differences between WT and TRPC3 groups at any time-point in during time-controlled normoxic incubations. The apparent decrease in necrosis from the end of ischemia to the end of I/R (Fig 2C) is presumably a consequence of a loss of cells early during reperfusion; however, since total cell number was not assessed at each time point this cannot be confirmed. It is also possible, although we believe unlikely, that the higher necrosis in the ischemia only cells was an artifact associated of the preparation of these cells for annexin V and propidium iodide staining.

Apoptosis was also assessed at the end of ischemia/reperfusion by quantifying internucleosomal DNA fragmentation. Consistent with the annexin V assays apoptosis was significantly increased TRPC3 group compared to WT controls ($4.3 \pm 0.2\%$ vs. $2.7 \pm 0.3\%$, $p < 0.05$; $n = 6$). As an alternative index of necrosis, LDH released during ischemia and reperfusion was measured in both groups and quantified as a percentage of total LDH. Consistent with the propidium iodide results (Fig 3C) there was no significant difference between TRPC3 and WT groups ($27 \pm 1\%$ vs. $26 \pm 3\%$; $n = 6$).

Effect of L-type channel inhibitor and CCE inhibitor on TRPC3-induced apoptosis

Treatment of TRPC3 cells with $0.5 \mu\text{M}$ SKF96365, an inhibitor of CCE, significantly improved cellular viability following ischemia/reperfusion ($54 \pm 4\%$ vs. $42 \pm 5\%$, $p < 0.05$; $n = 6$), to a level similar to that seen in WT cells following ischemia/reperfusion (Fig. 3A). SKF96365 also significantly decreased apoptosis in TRPC3 cells ($15 \pm 4\%$ vs. $32 \pm 1.4\%$, $p < 0.05$; $n =$

6) to levels similar to that seen in WT cells (Fig. 4). Similar to SKF96365, PD150606, an inhibitor of calpain, also attenuated apoptosis in the TRPC3 group (Fig 4). However, the L-type Ca^{2+} channel inhibitor verapamil (10 μM) had no significant effect on either viability (data not shown) or apoptosis in TRPC3 cells (Fig. 4). The untreated WT and TRPC3 data in Fig. 4 are from the same experiments as those shown in Fig 3B; however, it should be noted that the experiments in Fig. 4 were performed simultaneously with, and on cells from the same isolations as those used in Fig 3, thereby permitting direct comparisons.

TRPC3-induced apoptosis associated with increased calpain activation

One pathway by which increased cytosolic Ca^{2+} contributes to apoptosis is by activation of the calcium sensitive protease calpain, leading to proteolysis of structural proteins (10,11). The cytoskeletal protein α -fodrin is a well-characterized substrate for calpain, and increased calpain activity has been shown to lead to the formation of 140/150kD cleavage fragment of α -fodrin (37,40,41). In Fig 5A it can be seen that in the TRPC3 group there is significant increase in α -fodrin cleavage following ischemia/reperfusion compared to WT group ($p < 0.05$). The increase in α -fodrin cleavage in the TRPC3 cells was attenuated by the CCE inhibitor SKF96365 and the calpain inhibitor PD150606 (Fig 5B).

TRPC3 overexpression has an increased sensitivity to high extracellular calcium induced cell death

Cell death induced by ischemia/reperfusion is a multi-factorial process involving metabolic inhibition, intracellular acidosis as well as increased cytosolic calcium. Therefore, we determine whether increased TRPC3 expression would increase the sensitivity to cell death induced solely by Ca^{2+} -overload. Cells were treated with 5 μM thapsigargin in the absence of extracellular calcium for 5 mins followed by addition of 2.5 mM extracellular Ca^{2+} and the ratio of cell length/width followed as a function of time. In Fig 6 it can be seen that in the WT group, cells maintained a rod shaped viable form for at least 6 minutes and complete rounding of the cells took occurred between 7 and 9 minutes following addition of extracellular calcium. In contrast TRPC3 cells were completely rounded after only 6 minutes ($p < 0.05$).

TRPC3 overexpression does not contribute TNF- α induced apoptosis

To determine whether TRPC3 overexpression increased sensitivity to other apoptotic stimuli, cardiomyocytes isolated from WT and TRPC3 mice were incubated with 10 ng/ml TNF- α in serum-free media for 2h and 18h. Apoptosis, assessed by Annexin V-propidium iodide staining, increased in both groups following TNF- α treatment; however, in contrast to ischemia/reperfusion, there was no difference in apoptosis between the two groups (Fig. 7). In time-controlled normoxic incubations there were no differences in apoptosis or % rod shaped cells between WT or TRPC3 groups at any time point (data not shown). There was also no increase in either parameter at 2 or 18 hours compared to baseline in either WT or TRPC3 groups (data not shown).

DISCUSSION

First described in non-excitable cells, CCE has now been shown to co-exist with L-type channels in smooth and skeletal muscle cells (17,35) and in both neonatal and adult cardiomyocytes (12,13,31). The TRPC protein family have been prime candidates for CCE channel proteins (14) and TRPC1, 3, 4, 5 and 6 have all been identified in rat and mouse hearts (14,28,30). Here we demonstrate for the first time that TRPC3 overexpression increased the sensitivity of cardiomyocytes to apoptosis following ischemia/reperfusion, which was due, at least in part, by an increase in calpain-mediated proteolysis. We also demonstrated that increased TRPC3 expression sensitizes cardiomyocytes to Ca^{2+} overload induced cell death, but did not alter the response to TNF- α mediated apoptosis. This is consistent with our report

that CCE inhibition markedly attenuated Ca^{2+} -overload injury in the intact heart (20) and suggests that in addition to playing a role in regulating cardiomyocyte hypertrophy, TRPC-mediated CCE also contributes to cardiomyocyte apoptosis. These data provide further support for both TRPC and CCE in regulating the response of cardiomyocytes to a range of pathophysiological stimuli.

In vitro studies showed that CCE played a critical role in mediating the hypertrophic response to IP_3 -generating agonists such as angiotensin and phenylephrine in neonatal cardiomyocytes (11). Hunton et al. (12) also demonstrated that the increase in cytosolic Ca^{2+} induced by these agonists was clearly independent of Ca^{2+} -entry mediated via L-type Ca^{2+} channels and the reverse mode of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. Further evidence, demonstrating a physiological role for CCE in the heart was provided by Ohba *et al.* who showed that TRPC1 expression increased in the intact heart following pressure overload and in isolated cardiomyocytes in response to hypertrophic stimuli; the latter was associated with increased CCE (30). Consistent with the study by Ohba *et al.*, overexpression of TRPC3 in the mouse heart enhanced CCE at the cardiomyocyte level and was associated with increased hypertrophy in response to either pressure overload or neuroendocrine agonists in vivo (28).

Although a number of Ca^{2+} entry pathways have been implicated in mediating cardiomyocyte Ca^{2+} overload following ischemia reperfusion, including the $\text{Na}^+/\text{Ca}^{2+}$ exchanger and the L-type Ca^{2+} channel (4,8,32), there is still considerable controversy as to which pathways are critical in mediating this process. In cardiomyocytes, angiotensin- and phenylephrine-induced CCE is inhibited by glucosamine and SKF96365 (12,27); glucosamine also protected the isolated perfused heart from calcium-overload induced by the calcium paradox (20). We have also shown that glucosamine improves recovery following ischemia/reperfusion including lowering end-diastolic pressure (20) and decreasing Ca^{2+} -mediated proteolysis, consistent with attenuation of ischemia/reperfusion induced increase in cytosolic Ca^{2+} . Given that glucosamine inhibited CCE in isolated cardiomyocytes (12,27), these studies raised the possibility that Ca^{2+} entry mediated via CCE pathways may contribute to injury resulting from ischemia/reperfusion. Consistent with this notion we found that following simulated ischemia and reperfusion cardiomyocytes overexpressing TRPC3 had increased levels of apoptosis assessed by both annexin V staining (Fig 3) and DNA fragmentation, as well as decreased viability as indicated by percentage of rod-shaped cells. Interestingly, however, TRPC3 overexpression did not contribute to increased necrosis as indicated by either LDH release or propidium iodide/annexin V staining (Fig 3).

Additional evidence for a role of TRPCs in regulating cell death has been described in other cell types. For example, Marasa *et al.*, reported that increased TRPC1 expression sensitized intestinal epithelial cells to apoptosis as a result of increased Ca^{2+} influx (21). It is also worth noting that oxidative stress has been shown to activate TRPC3 and TRPC4 in endothelial cells (23) and another submember of TRP channels, TRPM, has been reported to contribute to oxidative stress induced cell death (23). However, in contrast to these reports suggesting that TRPCs contribute to increased cell death, Jia *et al.*, showed that TRPC3 and TRPC6 played a role in promoting neuronal survival in response to serum deprivation (16). Taken together with our results, these studies, support the notion that TRPCs play a role in regulating cell survival; however, whether they contribute to cell death or survival may be both cell and stress specific.

Further supporting a role of Ca^{2+} via CCE in contributing to the increase in apoptosis in the TRPC3 group, we also found that the CCE inhibitor SKF96365 attenuated the increase in apoptosis associated with TRPC3 overexpression, whereas the L-type Ca^{2+} channel inhibitor verapamil had no such effect (Fig. 4). Elevated intracellular Ca^{2+} levels activate numerous Ca^{2+} -regulated enzymes including protein kinases, protein phosphatases, phospholipases, NO synthases, Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) and the cysteine protease

calpain (42). There is increasing evidence that calpain plays a major role in post-ischemic injury (15,36), in part because of proteolysis of structural proteins (10,11) including the cytoskeletal protein α -fodrin (37,40,41). We found that in WT cardiomyocytes ischemia/reperfusion did not significantly increase cleavage of α -fodrin; in contrast, TRPC3 overexpression was associated with ~3-fold increase in α -fodrin cleavage consistent with increased calpain activity (Fig 5A). The calpain inhibitor PD150606, attenuated both α -fodrin cleavage and apoptosis in cardiomyocytes overexpressing TRPC3 (Fig. 4,5B); furthermore, consistent with its effect on apoptosis, SKF96365 markedly attenuated ischemia/reperfusion-induced increase in α -fodrin cleavage in the TRPC3 group (Fig 5B). Taken together these data support the concept that in cardiomyocytes overexpressing TRPC3 Ca^{2+} entry via CCE contributes to increased apoptosis, at least in part by calpain activation; however, while there is considerable data demonstrating that α -fodrin cleavage is calpain-specific (37,40,41), a direct measure of calpain activity would have further substantiated this conclusion. Nakayama et al., (28), have shown that TRPC3 overexpression was also associated with increased calcineurin activity; consequently, it is possible that calcineurin inhibition may also attenuate the increased apoptosis seen in the TRPC3 group. It should be noted that because we did not evaluate the effects of SKF96365 or PD150606 in WT cells we cannot comment about the potential role of CCE in contributing to cardiomyocyte apoptosis where TRPC3 levels are not increased. However, in preliminary experiments in the normal intact perfused heart, SKF96365 markedly attenuated tissue injury resulting from the calcium paradox (Marchase et al., unpublished data), suggesting that CCE may contribute to calcium mediated cell death in the normal intact heart.

Ischemia/reperfusion is a multifactorial process that includes intracellular acidosis and energy depletion, which may contribute to both necrosis and apoptosis either directly or via altered Ca^{2+} homeostasis, mediated via Ca^{2+} entry pathways such as the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. Therefore, we asked whether TRPC3 overexpression also increased sensitivity to a specific Ca^{2+} overload stress. Cells were first exposed to thapsigargin (5 μM) in the absence of extracellular Ca^{2+} followed by the addition of 2.5 mM extracellular Ca^{2+} , this is a protocol that has been used previously to increase CCE (12,27). We found that in the absence of extracellular Ca^{2+} cardiomyocytes maintained a normal rod-shaped morphology; however, the addition of extracellular Ca^{2+} resulted in a loss of viability as indicated by rounding of the cells, which was markedly accelerated in cells with increased TRPC3 expression. In contrast, TRPC3 overexpression had no effect on the response of cardiomyocytes to $\text{TNF-}\alpha$, which induces apoptosis via a Ca^{2+} independent pathway by means of the TNF type-1 receptor and Fas activation (22) (Fig 7). Thus, the increased sensitivity of TRPC3 to ischemia/reperfusion and Ca^{2+} overload was not a consequence of a general decreased tolerance to stress. However, $\text{TNF-}\alpha$ has been shown to increase TRPC3 expression in smooth muscle cells (38). Since, we did not determine whether $\text{TNF-}\alpha$ affected TRPC3 levels here, we cannot rule out the possibility that a preferential increase in TRPC3 expression in $\text{TNF-}\alpha$ treated WT cells could mask potential differences in the response of WT and TRPC3 cardiomyocytes to $\text{TNF-}\alpha$.

The role of CCE and TRPC in mediating cardiomyocyte function remains somewhat controversial, in part due to the fact that the predominant models describing Ca^{2+} handling in the heart are focused on understanding the regulation of excitation-contraction coupling (3). The importance of Ca^{2+} as a second messenger regulating a diverse array of cellular functions, including activation of gene transcription and cell growth, initiation of apoptosis and necrosis (6), raises the fundamental question as to how the cardiomyocyte distinguishes between the fluctuations in Ca^{2+} that occur in response contraction and relaxation from calcium signals (25). One characteristic of CCE is that it leads to a low-amplitude, sustained elevation in cytosolic Ca^{2+} , which could be distinguished from the high frequency and high amplitude oscillations in Ca^{2+} associated with contraction and relaxation. A number of studies have demonstrated a role for CCE mediated via TRP channel in mediating the responses to hypertrophic stimuli (12,28,30); here, we provide evidence to that calcium entry via TRPC

may also contribute to Ca^{2+} -mediated apoptosis. Clearly, there are limitations in extrapolating studies on isolated cardiomyocytes to the intact heart; furthermore, the fact that TRPC3 overexpression is associated with increased apoptosis does not necessarily imply that Ca^{2+} entry via TRP channels contributes to apoptosis in the normal heart. However, hypertrophic agonists such as angiotensin and phenylephrine, which have been shown to stimulate CCE, are also known to induce apoptosis. Furthermore, cardiac hypertrophy, which increases TRPC1 expression levels in the normal intact adult heart (30) is also associated with decreased tolerance to ischemic injury (1).

In this study we have focused entirely on isolated cardiomyocytes and thus any extrapolation with regard to the role of TRPCs in mediating cell death in the intact heart must be made with caution. In the intact heart, tissue injury following I/R is a result not only of metabolic and ionic events, but also a consequence of mechanical stress that occurs due to muscle contraction mediated in part by cell-to-cell connections. Such events are clearly absent in studies of cultured adult cardiomyocytes, which do not contract spontaneously and where there are no cellular connections. Conversely, cardiomyocyte isolation is itself an appreciable stress and this could potentially have a greater effect on cells overexpressing TRPC3 than on WT cells. It is conceivable that this could make TRPC3 cells more susceptible to subsequent I/R injury; however, it is likely that this would be manifest by increased basal cell death or a significantly greater increase in necrosis or apoptosis in time-controlled normoxic incubations, neither of which were seen here. Clearly, studies in the isolated perfused heart or *in vivo* would have demonstrated a more definitive role for TRPC3 in mediating I/R reperfusion in the intact heart. Another limitation of this study is the potential for chronic changes in cardiomyocytes overexpressing TRPC3 such as hypertrophy or increased calcineurin activity (28) that could also increase sensitivity to I/R injury independent of acute CCE-mediated increase in cytosolic Ca^{2+} . Future studies using acute adenoviral transfection approaches or conditional overexpression models would help resolve this issue. The use of siRNA to decrease TRPC expression in normal cells would also provide valuable insight into the contribution of TRPCs to cardiomyocyte injury.

In conclusion, we have shown that increased TRPC3 expression contributes to increased apoptosis but not necrosis in cardiomyocytes subjected to ischemia/reperfusion. This increase in apoptosis was associated with increased calpain-mediated proteolysis that was attenuated by the calpain inhibitor PD150606 and the CCE inhibitor SKF96365. However, while increased TRPC3 expression increased sensitivity to Ca^{2+} stress, it did not increase the sensitivity to TNF- α induced apoptosis, demonstrating that TRPC3 overexpression did not result in general decreased tolerance to stress. These results provide further evidence for a role of TRP channels in mediating cardiomyocyte function and suggest that TRPC mediated CCE may contribute to increased tolerance to injury seen in cardiac hypertrophy.

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Figure 1.

A) TRPC3 and B) TRPC1 protein expression in the hearts from wild-type (WT) and TRPC3 transgenic mice. Upper panels are representative immunoblots and the lower panels are mean densitometric data from 6 individual experiments normalized to β-actin. # = $p < 0.05$.

Figure 2.

A) Bright field phase contrast images of cardiomyocytes from wild-type (WT) and TRPC3 transgenic mice at baseline, at the end of 90 min ischemia and after 90 min ischemia and 3 hours reperfusion; B) cardiomyocytes from wild-type (WT) and TRPC3 transgenic mice after 90 min ischemia and 3 hours reperfusion showing Annexin V and propidium iodide staining. Arrows in merged image indicate necrotic cells, staining positive for both Annexin V and propidium iodide.

Figure 3.

A) Cell viability assessed by % rod shaped cells; B) % apoptotic cells indicated by Annexin V positive and propidium iodide negative staining; C) % necrotic cells indicated by Annexin V and propidium iodide positive staining in cardiomyocytes from wild-type (WT) and TRPC3 transgenic mice at baseline, at the end of 90 min ischemia and after 90 min ischemia and 3 hours reperfusion (I/R). Data presented as mean $\pm$ SEM of six individual experiments (i.e., six separate mouse cardiomyocytes isolations; with at least 300 cells counted per experiment under each condition). # = $p < 0.05$ vs. WT I/R.

Figure 4.

Apoptosis after 90 min ischemia and 3 hours reperfusion in cardiomyocytes from wild-type (WT) and TRPC3 transgenic mice and in TRPC3 cardiomyocytes treated with the L-type calcium channel inhibitor verapamil (VER, 10 μ M), the CCE inhibitor SKF96365 (SKF, 0.5 μ M) and the calpain inhibitor PD150606 (PD, 25 μ M). The untreated WT and TRPC3 data are from the same experiments as those shown in Fig 3B; however the experiments with VER, SKF and PD were performed simultaneously with, and on cells from the same isolations as those in Fig 3. Data presented as mean $\pm$ SEM of six individual experiments, i.e., six separate mouse cardiomyocytes isolations; with at least 300 cells counted per experiment under each condition. # = $p < 0.05$ vs. TRPC3.

Figure 5.

A) Total and cleaved α -fodrin (145/150kD) in cardiomyocytes from wild-type (WT) and TRPC3 transgenic mice under normoxic conditions (NX) and following 90 min ischemia and 3 hours reperfusion (I/R); B) total and cleaved α -fodrin (145/150kD) following 90 min ischemia and 3 hours reperfusion (I/R) in untreated cardiomyocytes from TRPC3 transgenic mice and in TRPC3 cardiomyocytes treated with the CCE inhibitor SKF96365 (SKF, 0.5 μ M) and the calpain inhibitor PD150606 (PD, 25 μ M). Upper panels are representative immunoblots and the lower panels are mean densitometric data from 3 individual experiments. # = $p < 0.05$ vs. TRPC3 NX and WT I/R; * = $p < 0.05$ vs. I/R.

Figure 6.

Cardiomyocytes from wild-type (WT) and TRPC3 transgenic mice were treated with 5 μ M thapsigargin for 5 mins in the absence of extracellular Ca^{2+} and were then exposed to 2.5 mM extracellular calcium. Upper panels show a typical time course of myocyte rounding following addition of 2.5 mM Ca^{2+} in WT and TRPC3 cardiomyocytes. Data are mean $\pm$ SEM from 15 cells of at least 3 individual experiments. # = $p < 0.05$ vs. TRPC3 6min.

Figure 7.

After 1h of isolation, cardiomyocytes from WT and TRPC3 transgenic mice were treated with 10 ng/ml TNF- α for 2h or 18h, viability and apoptosis were measured by Annexin V-propidium iodide staining. Data presented as mean $\pm$ SEM of six individual experiments, i.e., six separate mouse cardiomyocytes isolations; with at least 300 cells counted per experiment under each condition. * = $p < 0.05$ vs. baseline.