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TNF-α-mediated caspase-8 activation induces ROS production and TRPM2 activation in adult ventricular myocytes

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Aims TRPM2 is a Ca^{2+}-permeable cationic channel of the transient receptor potential (TRP) superfamily that is linked to apoptotic signalling. Its involvement in cardiac pathophysiology is unknown. The aim of this study was to determine whether the pro-apoptotic cytokine tumour necrosis factor-α (TNF-α) induces a TRPM2-like current in murine ventricular cardiomyocytes.

Methods and results Adult isolated cardiomyocytes from C57BL/6 mice were exposed to TNF-α (10 ng/mL). Western blotting showed TRPM2 expression, which was not changed after TNF-α incubation. Using patch clamp in whole-cell configuration, a non-specific cation current was recorded after exposure to TNF-α (I_{TNF}), which reached maximal steady-state amplitude after 3 h incubation. I_{TNF} was inhibited by the caspase-8 inhibitor z-IETD-fmk, the antioxidant *N*-acetylcysteine, and the TRPM2 inhibitors clotrimazole, *N*-(*P*-amylcinnamoyl) anthranilic acid and flufenamic acid (FFA). TRPM2 has previously been shown to be activated by ADP-ribose, which is produced by poly(ADP-ribose) polymerase 1 (PARP-1). TNF-α exposure resulted in increased poly-ADP-ribosylation of proteins and the PARP-1 inhibitor 3-aminobenzamide inhibited I_{TNF}. TNF-α exposure increased the mitochondrial production of reactive oxygen species (ROS; measured with the fluorescent indicator MitoSOX Red), and this increase was blocked by the caspase-8 inhibitor z-IETD-fmk. Clotrimazole and TRPM2 inhibitory antibody decreased TNF-α-induced cardiomyocyte death.

Conclusion These results demonstrate that TNF-α induces a TRPM2 current in adult ventricular cardiomyocytes. TNF-α induces caspase-8 activation leading to ROS production, PARP-1 activation, and ADP-ribose production. TNF-induced TRPM2 activation may contribute to cardiomyocyte cell death.

Keywords TNF-α • TRPM2 • Ischaemia–reperfusion • Cell death • Cardiac

1. Introduction

Elevated plasma levels of tumour necrosis factor-α (TNF-α) are associated with a broad range of cardiac diseases, such as reperfusion injury, myocardial infarction, and congestive heart failure progression.[1] TNF-α activates two distinct receptors: TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2). Cumulative evidence indicates that TNF-α/TNFR1 signalling induces deleterious effects such as mitochondrial dysfunction, altered Ca^{2+} handling, and cell death.[2] In acute ischaemia, TNF-α induces caspase-8 activation, which alters mitochondrial function, increases production of reactive oxygen species (ROS), and induces sarcoplasmic reticulum (SR) Ca^{2+} leak.[3] Accordingly, caspase-8 inhibition reduces reperfusion injury.[3,4]

The transient receptor potential (TRP) superfamily is subdivided into seven groups: TRPC1–7 (canonical), TRPV1–6 (vanilloid), TRPM1–8 (melastatin), TRPP1–3 (polycystine), TRPML (mucolipine), TRPA (ankyrine), and TRPN (NOMPC, not present in mammalians). Within

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the TRPM family, TRPM2 is a Ca^{2+}-permeable channel, widely expressed in mammalian tissues (including brain, blood vessels, spleen, heart, liver, and lungs), that is activated by oxidative stress.[5] Its activation confers susceptibility to cell death.[5] For instance, hydrogen peroxide (H_2O_2)-induced apoptotic cell death involves TRPM2 activation in cultured rat cardiomyocytes.[6] H_2O_2 induces TRPM2 activation via activation of the intracellular second messenger adenosine diphosphoribose (ADP-ribose).[7] ADP-ribose is primarily generated by poly(ADP-ribose) polymerase 1 (PARP-1), which is a nuclear enzyme involved in DNA repair.[8] TRPM2 can also be activated by nicotinamide adenine dinucleotide (NAD^+) and cyclic ADP-ribose (cADP-ribose).[9,10]

The aim of this study was to test the functional linkage between TNF-α and TRPM2 in adult ventricular cardiomyocytes. Our results indicate that TNF-α activates a TRPM2 current via caspase-8 activation and also increases ROS production and PARP-1 activation. Furthermore, TRPM2 channel inhibition protects against TNF-α-induced ventricular cell death.

2. Methods

2.1 Animal model and cell isolation

Adult (3–5 months) C57BL/6 mice were killed by rapid neck disarticulation and the hearts were excised. Single cardiomyocytes were isolated from the ventricles following the protocols developed by the Alliance for Cellular Signalling (AfCS Procedure Protocol ID PP00000 125).[11] Experiments conform to the Directive 2010/63/EU of the European Parliament and are approved by The Animal Care and Use Committee Languedoc-Roussillon. This protocol is recorded under the following reference: CEEA-LR-12079.

2.1.1 Materials

1-Oleoyl-2-acetyl-*sn*-glycerol (OAG), 2-aminoethoxydiphenyl borate (2-APB), 3-aminobenzamide (3-AB), SK&F96365, *N*-acetyl cysteine (NAC), z-IETD-fmk (caspase-8 inhibitor), clotrimazole, *N*-(*P*-amylcinnamoyl) anthranilic acid (ACA), flufenamic acid (FFA), and H_2O_2 were from Sigma. TNF-α was from Calbiochem. Inhibitory TRPM2 antibody[12] was from Bethyl Labs (A300-414A). All compounds were prepared as stock solutions in appropriate solvents. On the day of the experiment, stock solutions were diluted to the desired final concentration in the incubation medium and the same volume of solvent was added to the control medium. The final concentration of DMSO was $<0.1\%$.

2.2 Measurement of non-selective cationic currents

Cardiomyocytes were placed in a perfusion chamber on the stage of a Zeiss Axio Observer A.1 inverted microscope and continuously perfused with standard Tyrode solution of the following composition (in mM): NaCl 135, KCl 4, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 2, glucose 10, pH adjusted to 7.4 with NaOH. Whole-cell patch-clamp experiments were performed at room temperature (~24°C) using an Axopatch 200B amplifier (Axon Instruments, Burlingham, CA, USA). Patch pipettes had a resistance of 2–3 MΩ. Currents were normalized to the cell membrane capacitance and presented as current densities (pA/pF). Series resistances were electronically compensated before voltage-clamp recordings. Leak current was not compensated, and all cells exhibiting a leak current larger than 100 pA were excluded. The patch-pipette solution contained (in mM): 120 CsCl, 6.8 $MgCl_2$, 5 Na_2ATP, 5 sodium creatine phosphate, 0.4 Na_2GTP, 11 EGTA, 4.7 $CaCl_2$ (120 nM free [Ca^{2+}]), and 20 HEPES; pH was adjusted with CsOH to 7.2. In some experiments, CsCl was equimolarly replaced by Cs-aspartate. To record a non-specific cationic current induced by TNF-α, H_2O_2, or OAG, the bathing solution was switched to a modified Tyrode solution as previously described,[11] where Na^+ was replaced by Li^+ to inhibit the Na^+–Ca^{2+} exchanger; 200 μM ouabain was added to inhibit Na^+–K^+ ATPase; 1 mM $BaCl_2$ was added to block residual K^+ and background conductance. In the *N*-methyl-D-glucamin (NMDG) solution, Li^+ was replaced with an equimolar amount of NMDG (pH adjusted to 7.4 with HCl). Quasi steady-state *I–V* relations of the non-specific cationic current were obtained by applying a descending voltage ramp that covered the physiological range of membrane potentials. The holding potential was maintained at −80 mV and the voltage ramp decreased from +50 to −120 mV with a slope of 0.057 V/s[11] and was repeated at 0.03 Hz, which inactivates both rapid Na^+ currents and L-type Ca^{2+} currents. TNF-α-, H_2O_2-, and OAG-mediated non-specific cationic currents were determined as the difference between steady-state currents recorded after and before application of drugs.

2.3 Measurements of mitochondrial ROS production

MitoSOX Red was used to measure mitochondrial ROS production.[13] Isolated cardiomyocytes were loaded with MitoSOX Red (5 μM) in Tyrode for 15 min at room temperature, followed by washout. Confocal images were obtained by excitation at 488 nm and measuring the emitted light at 585 nm. MitoSOX Red fluorescence was measured in five different areas in each cell, and the signal was normalized to that at the start of the experiment.

2.4 Western blot

Freshly isolated cardiomyocytes were homogenized directly into lysis buffer (20 mM HEPES pH 7.4, 40 mM KCl, 1 mM dithiothreitol, 0.3% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, 1 μg/mL pepstatin, and 1 mM EDTA). Lysates were centrifugated at 6000 × *g* for 5 min at 4°C. Proteins were quantified with DC Protein Assay (Biorad). Twenty-five micrograms (for poly-ADP-ribose) or 50 μg (for TRPM2) of the total protein were loaded on 4–20% gradient gel of acrylamide. Proteins were transferred onto the nitrocellulose membrane (0.2 μm) (GE Healthcare). The membranes were blocked (blocking buffer from Thermo Scientific) and then incubated with primary antibody at 4°C overnight: anti-TRPM2 (1:300; Novus Biologicals, NB110-81601), anti-poly-ADP-ribosylated protein (PAR; 1:1000; Trevigen, 4335-MC-100-AC), or anti-GAPDH (1:60 000; Abcam, ab8245). The membranes were then incubated for 1 h in the dark with secondary antibodies: anti-rabbit (1:30 000; LI-COR) for TRPM2 and anti-mouse (1:30 000; LI-COR) for PAR and GAPDH. After the final washes, the membranes were scanned with an Odyssey infra-red imager (LI-COR Biosciences).

2.5 Simultaneous measurement of cell viability and intracellular Ca^{2+}

Experimental procedure was adapted from ref.[6] Briefly, freshly isolated cardiomyocytes were loaded for 15 min at RT with fluo-4 AM (5 μmol/L, Molecular Probes). Cells were then placed in a standard Tyrode solution containing 2.5 μg/mL of propidium iodide (PI), which stained dead cells in red upon membrane permeabilization. Changes in fluo-4 fluorescence and PI staining were simultaneously recorded using time-lapse confocal microscopy over 4 h. To allow comparison between control and TNF-α conditions, acquisition was performed for each cell isolation simultaneously on two LSM510 Meta Zeiss confocal microscopes equipped with either a ×10 or ×25 objective (NA: 1.3). Measurements were performed in a frame mode (1.54 s/images) every 20 s. An excitation wavelength of 488 nm was used, and emitted light was collected through a 505-nm long pass filter. The laser intensity used was 1–3% of the maximum and had no noticeable deleterious effect on the fluorescence signal. To enable comparisons between cells, the change in fluorescence (*F*) was divided by the fluorescence detected immediately before TNF-α application (10 ng/mL) (F_0). Spontaneous Ca^{2+} spikes were counted as such in all viable cells when $F/F_0 > 1.5$.

TNF-α (10 ng/mL) was applied after 30 min of acquisition to ensure steady state. In some experiments, cells were placed in a standard Tyrode solution containing inhibitory TRPM2 antibody[12] (1 μg/mL) or clotrimazole (10 μM). Ca^{2+} spike frequency (events/min) was calculated during the following 3 h or until induction of membrane permeabilization. The percentage of dead cells was determined by the ratio between red cells and total cells.

2.6 Statistics

Data are presented as mean ± SEM. Statistically significant differences were assessed with Student's *t*-test (for paired or unpaired samples) or when three or more groups were compared, one-way analysis of variance (ANOVA) with a Newman–Keuls *post hoc* test. A *P*-value of <0.05 was considered significant.

3. Results

3.1 TNF-α induces a non-specific cationic current via caspase-8 activation and ROS production

Adult freshly isolated ventricular cardiomyocytes incubated with TNF-α (10 ng/mL) developed a non-specific cationic current (I_{TNF}) recorded under whole-cell patch-clamp techniques that reached

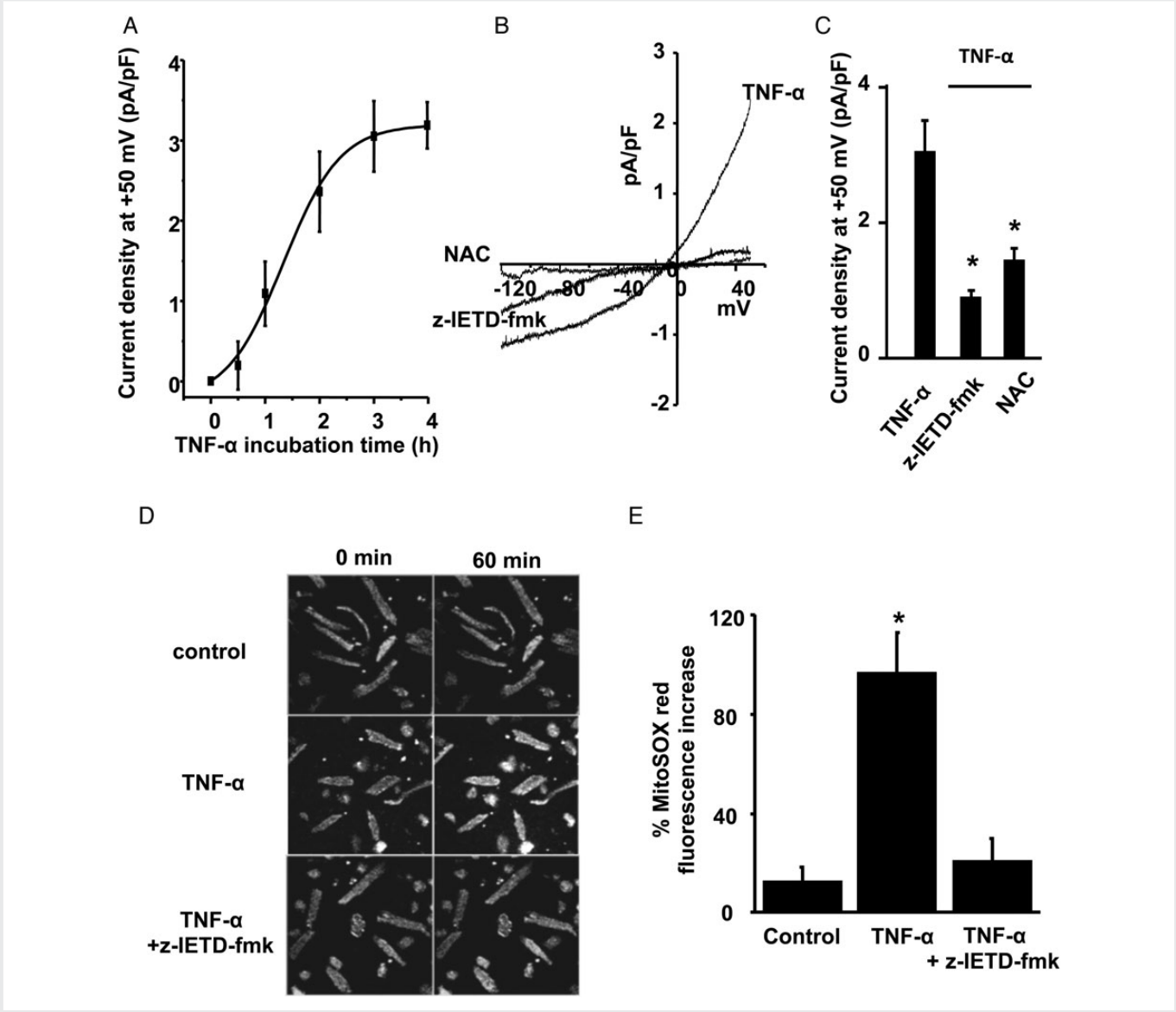

Figure 1 TNF-α induces a non-specific cationic current via caspase-8 activation and ROS production. (*A*) Non-specific cationic current density recorded at +50 mV at different TNF-α incubation times. TNF-α induced a progressive activation of a non-specific cationic current, which reaches its maximum after 4 h ($t = 0$, $n = 8$ cells, four mice; $t = 30$ min, $n = 8$ cells, five mice; $t = 1$ h, $n = 8$ cells, five mice; $t = 2$ h, $n = 8$ cells, five mice; $t = 3$ h, $n = 10$ cells, five mice; $t = 4$ h, $n = 6$ cells, three mice). (*B* and *C*) Current induced by 3 h of TNF-α incubation is abolished in the presence of caspase-8 inhibitor (z-IETD-fmk) or antioxidant (NAC) (TNF-α, $n = 10$ cells, five mice; z-IETD-fmk, $n = 6$ cells, three mice; NAC, $n = 7$, three mice; $^*P < 0.05$ compared with TNF-α conditions). (*D* and *E*) Mitochondrial ROS production after TNF-α exposure. TNF-α induced a ROS production within 60 min and caspase-8 inhibition by z-IETD-fmk abolished this ROS production ($^*P < 0.05$ compared with control conditions, $n > 15$ cells, three mice).

maximal steady-state amplitude after 3 h incubation (*Figure 1A*). I_{TNF} presents an outward rectification *I–V* relationship with a reversal potential close to 0 ($E_{rev} = -7.8 \pm 1.0$ mV) ($n = 10$) (*Figure 1*B). One of the early events in the TNF-α/TNFR1 signalling cascade is the activation of caspase-8 through the death-inducing signalling complex (DISC). We therefore co-incubated cells with TNF-α and z-IETD-fmk (10 μM), a specific caspase-8 inhibitor. z-IETD-fmk was applied 20 min prior to TNF-α application. When incubated with z-IETD-fmk, I_{TNF} was markedly decreased after 3 h of TNF-α application (*Figure 1B* and *C*). I_{TNF} was also decreased when cells were co-incubated with the non-specific antioxidant NAC (20 mM) (*Figure 1B* and *C*). These results indicated that I_{TNF} activation by TNF-α requires ROS production through caspase-8 activation, as demonstrated by the progressive increase in MitoSOX Red signal within 1 h of TNF-α application (*Figure 1D* and *E*). When caspase-8 was inhibited, TNF-α did not affect MitoSOX Red fluorescence compared with control (*Figure 1C* and *D*).

3.2 TNF-α does not activate a TRPC3 current

As TRPC3 expression may increase with pro-inflammatory cytokines, such as TNF-α,[14] we determined whether I_{TNF} is related to TRPC3. Anti-TRPC3 antibody can block cationic currents when applied on the inside of the plasma membrane.[11] We therefore measured I_{TNF} after

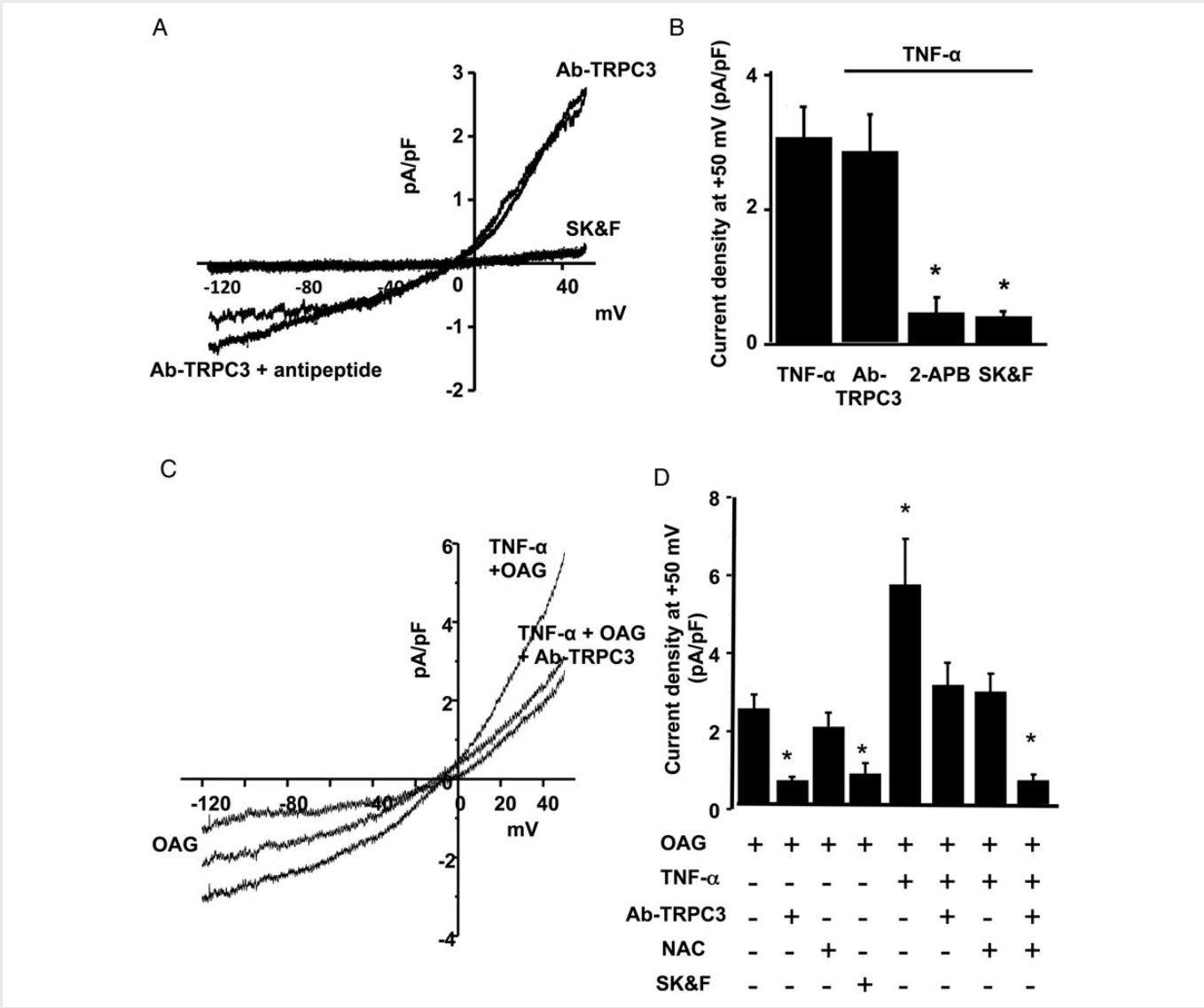

Figure 2 TNF-α does not activate a TRPC3 current. (*A* and *B*) Patch-clamp recordings from cardiomyocytes incubated with TNF-α in the presence of several blockers. TRPC3 antibody (Ab-TRPC3) did not affect TNF-α-induced current, but TRP inhibitors, 2-APB and SK&F96365, blocked this current (TNF-α, $n = 10$ cells, five mice; Ab-TRPC3, $n = 6$ cells, two mice; 2-APB, $n = 6$ cells, three mice; SK&F96365, $n = 6$ cells, three mice; $^*P < 0.05$ compared with TNF-α). (*C* and *D*) Patch-clamp recordings from cardiomyocytes incubated with OAG, a DAG analogue known as a TRPC3 activator. OAG induced a TRPC3-like current inhibited by TRPC3 antibody (Ab-TRPC3), but not by the antioxidant NAC. In the presence of OAG, TNF-α induced an additive current (OAG, $n = 10$ cells, four mice; Ab-TRPC3, $n = 9$ cells, three mice; NAC, $n = 6$ cells, three mice; SK&F96365, $n = 6$ cells, three mice; OAG + TNF, $n = 8$ cells, four mice; OAG + TNF + NAC, $n = 6$ cells, three mice; OAG + TNF + Ab-TRPC3, $n = 7$ cells, three mice; OAG + TNF + Ab-TRPC3 + NAC, $n = 6$ cells, two mice; $^*P < 0.05$ compared with OAG conditions).

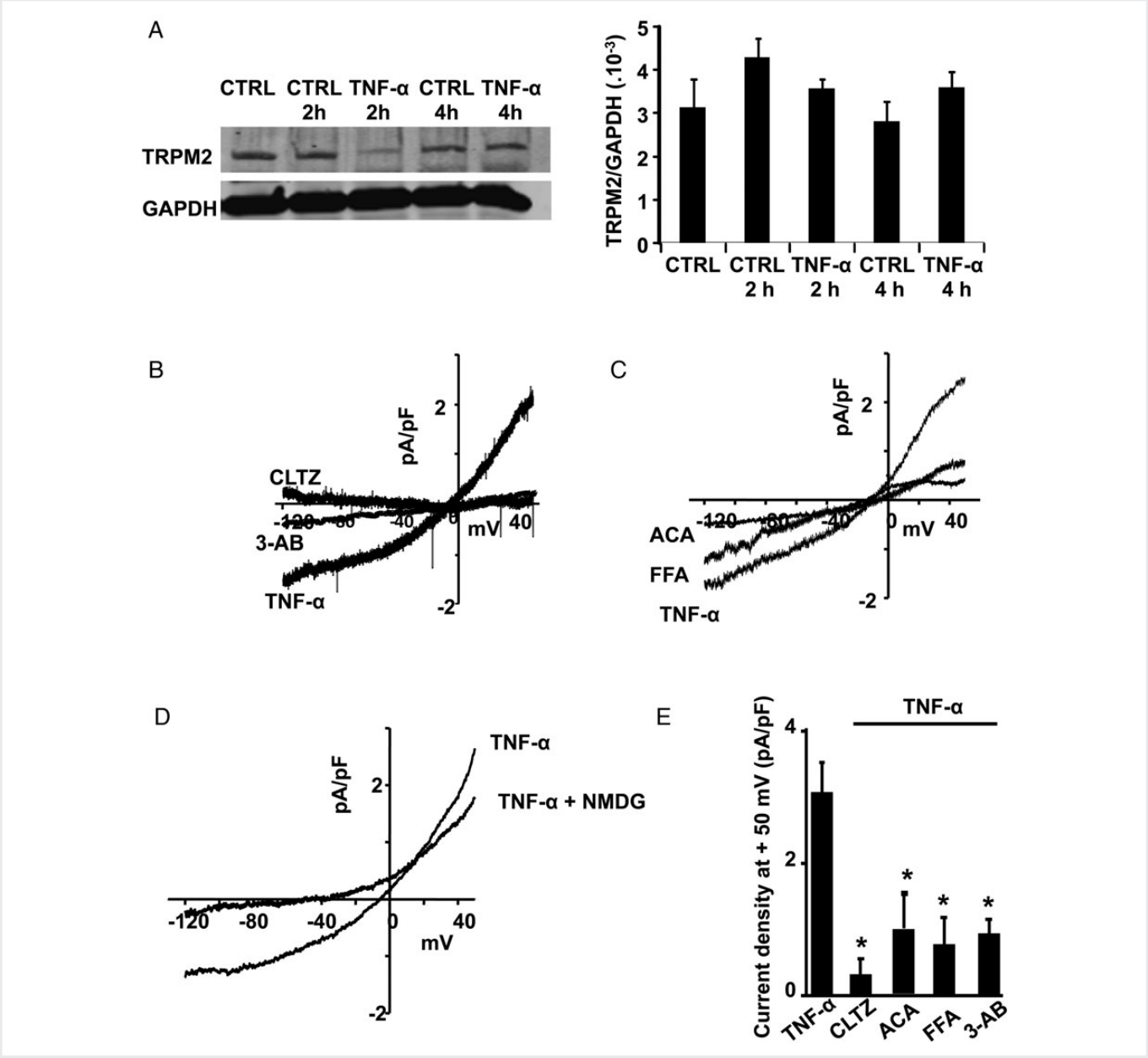

Figure 3 TNF-α activates a TRPM2 current. (*A*) Expression of TRPM2 protein in cardiomyocytes in control conditions and after TNF-α exposure. TRPM2 is expressed in mouse adult ventricular cardiomyocytes and TNF-α did not affect its expression level after 2 and 4 h of incubation ($n = 5$ mice). TRPM2 were normalized to GAPDH expression revealed on the same western blot. Each samples correspond to adjacent wells. (*B* and *C*) Patch-clamp recordings in the presence of TRPM2 inhibitors or the PARP inhibitor. (*D*) TNF-α-induced currents recorded with Cs-aspartate replacing CsCl in the intra-pipette medium in Li^+ or NMDG-based external medium ($n = 6$, three mice). (*E*) Mean values of current density at + 50 mV of cells incubated with TNF-α in the presence of TRPM2 inhibitors (clotrimazole, CLTZ, $n = 10$ cells, four mice; ACA, $n = 6$ cells, three mice; FFA, $n = 6$ cells, three mice; 3-AB, $n = 8$ cells, four mice). All these inhibitors abolished the current induced by TNF-α (*$P < 0.05$ compared with TNF-α).

3 h of TNF-α treatment with anti-TRPC3 antibodies (+/− an excess of TRPC3 blocking peptide) present in the patch pipette. I_{TNF} was not affected by addition of anti-TRPC3 antibodies to the patch-pipette solution (*Figure 2A* and *B*). Next, we tested different pharmacological tools frequently used to inhibit TRP channel activity such as 2-APB (30 μM) and SK&F96365 (10 μM). Both inhibitors significantly inhibited I_{TNF} (*Figure 2A* and *B*). As previously reported, a diacylglycerol analogue, OAG, activates a TRPC3 current (I_{OAG}) in adult ventricular cardiomyocytes.[11] Therefore, we tested whether TNF-α would also affect TRPC3 properties. OAG alone (30 μM) gradually activated an outwardly rectifying current within minutes with a reversal potential of -8.2 ± 2.4 mV ($n = 10$) (*Figure 2C* and *D*). I_{OAG} was inhibited by anti-TRPC3 and SK&F96365 in the patch pipette (*Figure 2D*). However, NAC incubation, which inhibits I_{TNF}, did not significantly affect I_{OAG} (*Figure 2D*), suggesting that I_{OAG} activation is not mediated by ROS production. In addition, when I_{TNF} reached its maximum, OAG was added and this resulted in an increased current density that reached its maximal amplitude within 15 min (*Figure 2C* and *D*). When anti-TRPC3 was present in the patch pipette, OAG did not significantly affect I_{TNF} (*Figure 2C* and *D*). When I_{TNF} was inhibited by NAC, I_{OAG} was still

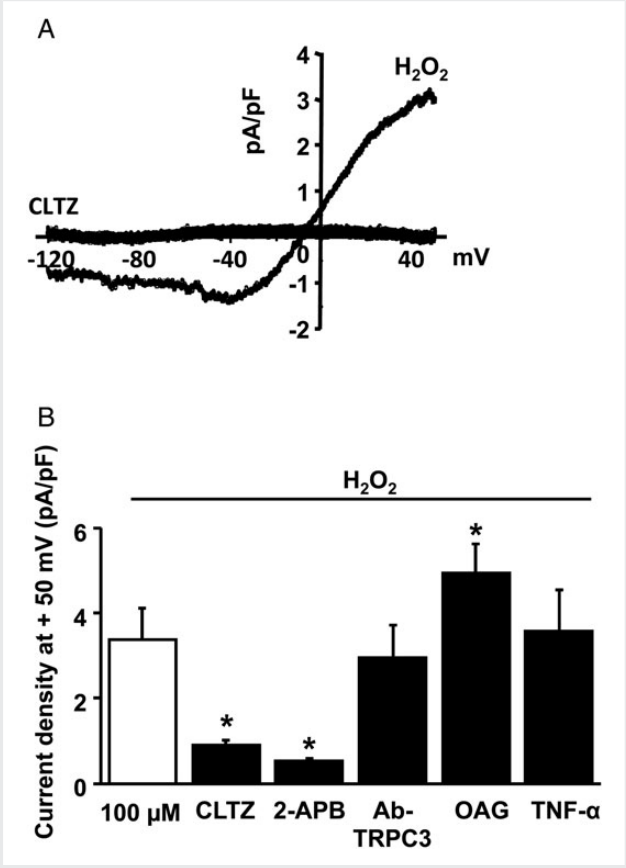

Figure 4 H_2O_2 activates a non-specific cationic current. (*A*) *I/V* curve obtained by patch-clamp recordings on cells incubated with H_2O_2. H_2O_2 activated a non-specific cationic current blocked by clotrimazole (H_2O_2, $n = 10$ cells, four mice; CLTZ, $n = 8$ cells, four mice). (*B*) Mean values of currents density at + 50 mV of cells incubated with H_2O_2 in the presence of several compounds (2-APB, $n = 6$ cells, three mice; NAC, $n = 6$ cells, three mice; OAG, $n = 6$ cells, three mice; Ab-TRPC3, $n = 6$ cells, three mice; TNF, $n = 6$ cells, two mice). TRPC3 antibody (Ab-TRPC3) did not affect the current induced by H_2O_2. Although OAG activates an additive current, TNF-α did not induce an additional current in the presence of H_2O_2 (*$P < 0.05$ compared with H_2O_2 conditions).

present and comparable with I_{OAG} recorded in the absence of TNF-α (*Figure 2D*). Finally, in the presence of NAC in the medium and anti-TRPC3 in the patch pipette, we were unable to activate either I_{TNF} or I_{OAG} (*Figure 2D*). All together, these results indicate that TNF-α does not activate TRPC3 current.

3.3 TNF-α activates a TRPM2 current

Western blot analyses showed that TRPM2 is expressed in adult ventricular cardiomyocytes and TNF-α exposure did not affect TRPM2 expression level (*Figure 3A*). We then tested whether TRPM2 mediates I_{TNF} by applying clotrimazole (10 µM), a TRPM2 inhibitor.[15] When clotrimazole was applied acutely (<5 min), I_{TNF} induced by 3 h of TNF-α treatment was almost abolished (*Figure 3B* and *E*). Similarly, acute application of ACA (20 µM) as well FFA (100 µM), two anthranilic acid derivates known to inhibit TRPM2,[16] significantly decreased I_{TNF} (*Figure 3C* and *D*). The nuclear enzyme PARP-1 produces ADP-ribose, which can activate TRPM2 by binding to its C-terminal.[17] Application of the PARP-1 inhibitor 3-AB markedly decreased I_{TNF} (*Figure 3B* and *E*). To exclude participation of a Cl^- conductance, intra-pipette CsCl was equimolarly replaced by Cs-aspartate. *I–V* curves were comparable and the reversal potential was not shifted ($E_{rev} = -8.4 \pm 1.2$ mV, $n = 6$; *Figure 3D*). In addition, when Li^+ was replaced by NMDG, the inward component of I_{TNF} was reduced and the reversal potential was shifted to the left ($E_{rev} = -58 \pm 6$ mV, $n = 6$; *Figure 3D*).

To confirm TRPM2 activation, we tested the effect of H_2O_2, a known activator of TRPM2. We applied H_2O_2 (100 µM) on the extracellular side, which induced a non-specific cationic current ($I_{H_2O_2}$) within 10 min with an outward rectifying *I–V* curve and with a reversal potential close to 0 ($E_{rev} = -5.2 \pm 1.0$ mV, $n = 10$) (*Figure 4A*). As for I_{TNF}, $I_{H_2O_2}$ was inhibited by clotrimazole (*Figure 4B*) and 2-APB, but not by anti-TRPC3 (*Figure 4B*). In addition, OAG was able to activate an additive non-specific cationic current in the presence of H_2O_2, whereas H_2O_2 did not affect cardiomyocytes incubated for 3 h with TNF-α (*Figure 4B*). These results indicate that I_{TNF} as well as $I_{H_2O_2}$ are carried by a TRPM2 channel.

3.4 PARP-1 activation is responsible for TRPM2 activation

PARP-1 inhibition by 3-AB blocked the TRPM2-like current (*Figure 3B* and *D*), which indicates that the current was activated by ADP-ribose. TRPM2 can also be activated by cADP-ribose[18] and we performed patch-clamp experiments with cADP-ribose (200 µM) in the patch pipette and observed a progressively developing non-specific cationic current with a linear *I–V* relationship and $E_{rev} = -9.2 \pm 1.0$ mV, $n = 6$. The cADP-ribose-induced current was inhibited by clotrimazole (*Figure 5A* and *B*). Most notably, the antioxidant NAC and the caspase-8 inhibitor z-IETD-fmk did not affect the cADP-ribose-induced current (*Figure 5B*).

To confirm PARP-1 activation during TNF-α exposure, we measured poly-ADP-ribosylated cardiomyocyte proteins induced by TNF-α incubation. After 4 h of TNF-α exposure, western blot analysis showed a significant increase in PAR compared with control (*Figure 5C*). To sum up, our results indicate that TNF-α-induced TRPM2 activation can occur via PARP-1 activation and increased ADP-ribose formation.

3.5 TRPM2 inhibition protects against ventricular cell death

Next, we assessed whether TRPM2 activation is involved in TNF-α-induced cardiomyocyte death. To determine whether TNF-α-induced TRPM2 activation precedes and is causally linked to myocyte cell death, we monitored intracellular Ca^{2+} in fluo-4 loaded cells placed in medium containing PI, which stained nuclei after membrane permeabilization. Upon TNF-α exposure, Ca^{2+} spike frequency progressively increases and systematically precedes membrane permeabilization (*Figure 6A* and *B*). Clotrimazole, as well as the TRPM2 blocking antibody,[12] significantly reduced Ca^{2+} spike occurrence and subsequent TNF-α-induced cell death (*Figure 6C–E*), supporting the hypothesis that TRPM2 activation is involved in TNF-α-induced cardiomyocyte death.

4. Discussion

The results of the present study fit with a model where: (i) TNF-α induces a non-specific cationic current (I_{TNF}) through ROS production induced by caspase-8 activation in mouse left ventricular cardiomyocytes; (ii) I_{TNF} is associated with activation of TRPM2; and (iii) clotrimazole and TRPM2 inhibitory antibody are protective against TNF-induced cardiomyocyte cell death.

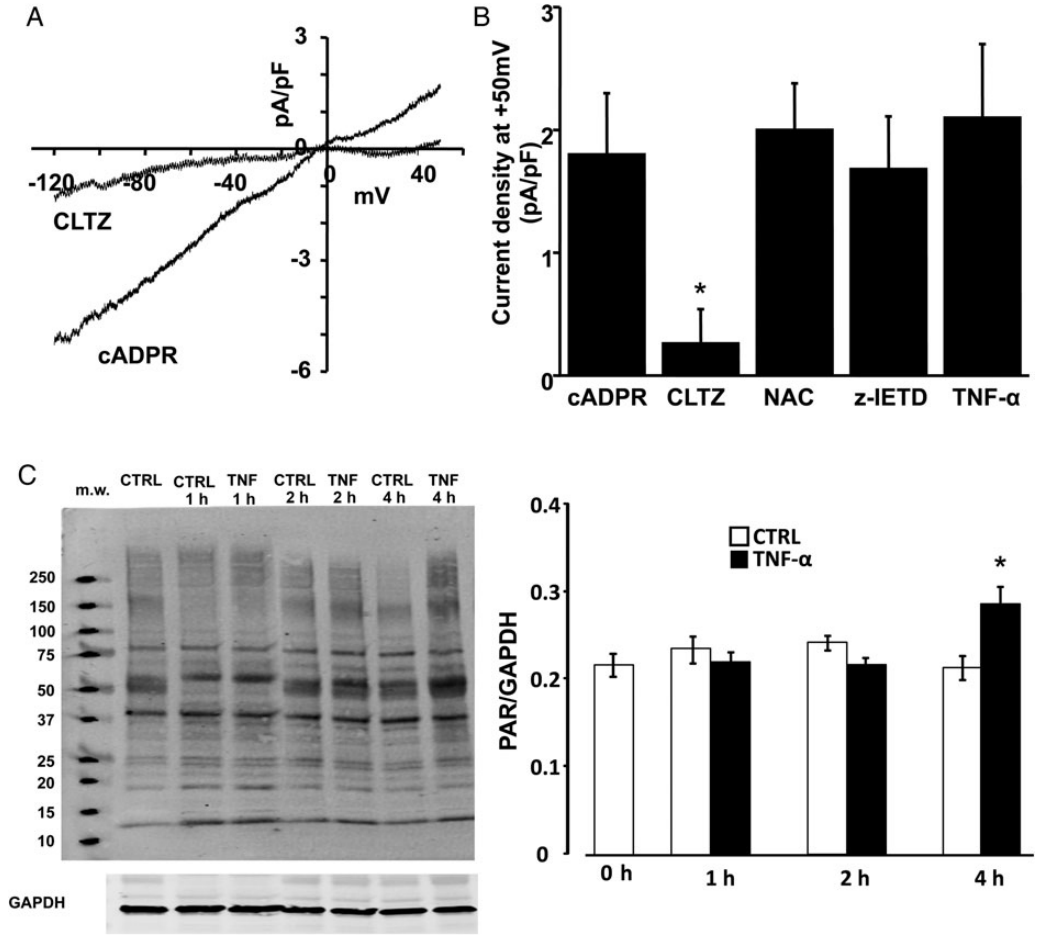

Figure 5 cADPR activates TRPM2 current in adult cardiomyocytes. (*A*) Representative *I/V* curve of a cADPR-induced non-specific cationic current. The current was inhibited by external application of clotrimazole (10 μM). (*B*) Mean values of the maximal current activated by intracellular application of cADPR. Although the TRPM2 inhibitor clotrimazole inhibits the current, the caspase-8 inhibitor (z-IETD) or the antioxidant NAC is ineffective. Similarly, TNF-α did not induce an additional current in the presence of cADPR ($n = 6$ cells, three mice, in each condition; $^*P < 0.05$ compared with cADPR conditions). (*C*) Western blot analysis indicating that TNF-α increases cardiomyocytes poly(ADP-ribosyl)ation compared with control conditions. Freshly isolated cardiomyocytes from C57BL/6 mouse hearts were incubated from 1 to 4 h with TNF-α or maintained during the same period in control conditions. Poly(ADP-ribose) signal between 15 and 250 kDa was quantitated by densitometry and normalized to GAPDH content revealed on the same western blot. Each samples correspond to adjacent wells. Each lane represents cell lysate isolated and prepared from an individual mouse heart ($^*P < 0.05$ compared with control conditions; $n = 5$ hearts in each conditions).

Several studies have reported that mitochondria are the principal source of ROS production in the TNF-α pathway.[19] The binding of TNF-α to TNFR1 causes an intracellular cascade initiated by the recruitment of the adapter protein Fas-associated protein with death domain to form a complex termed DISC.[20] The formation of this complex allows the recruitment of procaspase-8. Owing to DISC, procaspase-8 is autocleaved and activated. Bid, a pro-apoptotic protein, is cleaved by active caspase-8 and translocates to the mitochondria where it induces loss of mitochondrial transmembrane potential (ψ_m).[3] An increase of ROS generation occurs and is likely to be the result of a feedback increase of respiratory chain activity due to the loss of ψm and ATP production. Thus, caspase-8 inhibition prevents mitochondrial ROS production (*Figure 1A*).[3] Elevation of ROS production leads to PARP-1 activation, which contributes to TNF-α-induced necrotic cell death.[21] PARP-1 detects and binds DNA breaks induced by ROS.[21] PARP-1 catalyses the cleavage of NAD^+ into nicotinamide and ADPR, and PARP-1 activation is associated with inhibition of ATP synthesis and compromised energy metabolism.[22]

In parallel, oxidative stress-induced PARP-1 activation has been shown to activate TRPM2 in several cell types.[23] ADPR binds to and activates TRPM2 channels allowing Ca^{2+} to enter into the cells. TRPM2 activation increased with ADPR concentration.[9] Its activation is modulated by nicotinic acid adenine dinucleotide phosphate (NAADP), H_2O_2, and/or Ca^{2+}.[5,24–26] TRPM2 exhibits a moderate outward rectification due to a steady-state inactivation at negative potentials.[9,17,24] Intracellular Ca^{2+} level facilitates TRPM2 activation through an increase in ADPR sensitivity.[24,25] At low $[Ca^{2+}]_i$, TRPM2 inactivation at negative potential is more pronounced.[17,25,27] Intracellular application of cADPR induces TRPM2 gating either directly or following secondary processes.[10,26] cADPR is a precursor of ADPR formation and also a potent activator of ryanodine receptors.[28] The synergy of a large increase in intracellular Ca^{2+} in the vicinity of the channel with an increase in ADPR and cADPR may

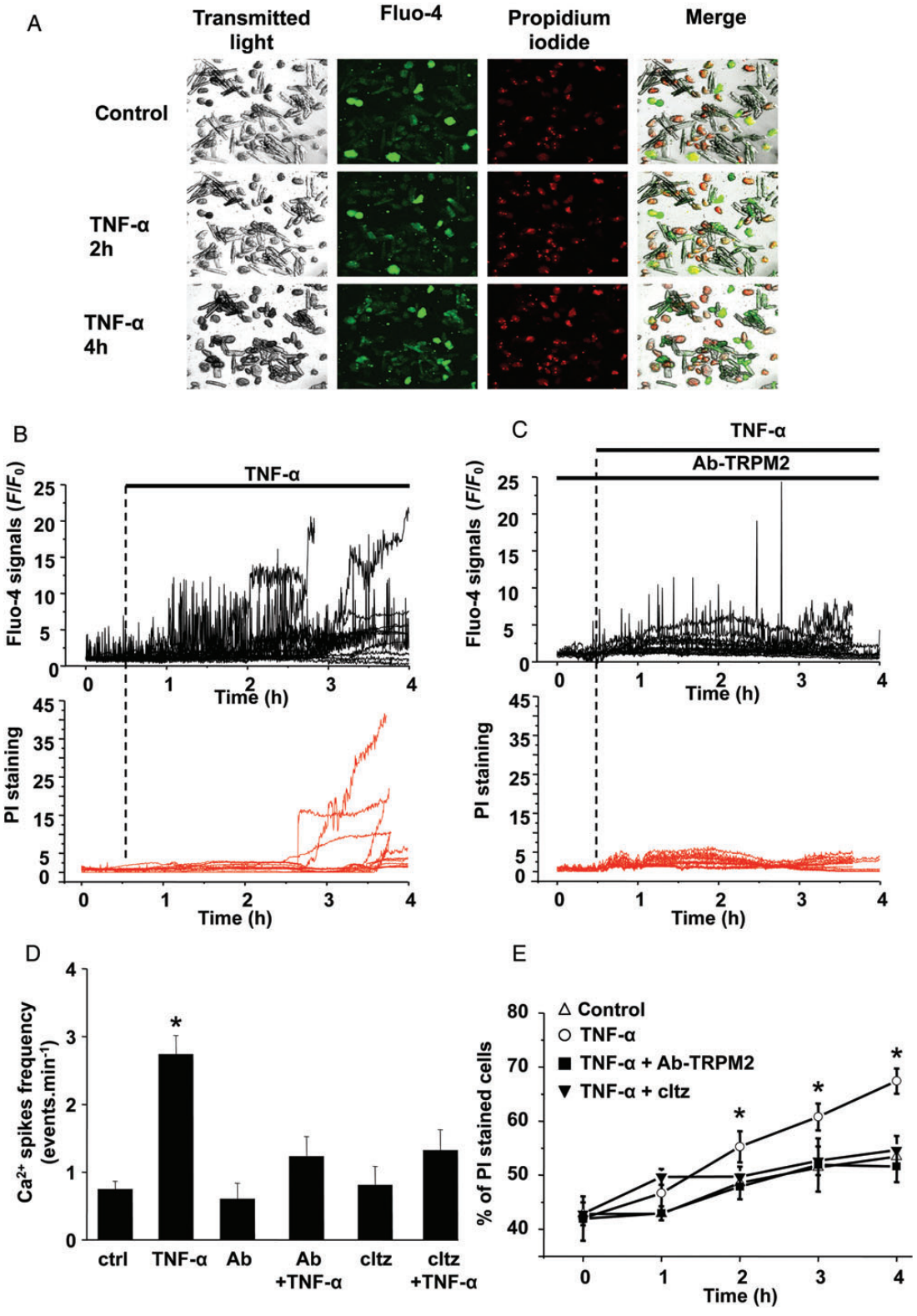

Figure 6 Role of TRPM2 in cell death. (*A*) Intracellular Ca^{2+} and cell death were simultaneously assessed using fluo-4 and PI staining, respectively, in freshly isolated cardiomyocytes from C57BL/6 mouse hearts, during 4 h of TNF-α exposure (10 ng/mL). (*B* and *C*) Typical examples of simultaneous time-lapse recordings of TNF-α-induced intracellular Ca^{2+} changes and PI influx (membrane permeabilization) in freshly ventricular adult cardiomyocytes isolated from the same heart incubated or not with the blocking TRPM2 antibody ($n = 20$ cardiomyocytes in both conditions). TNF-α progressively increases Ca^{2+} spike frequency prior PI staining. TRPM2 antibody reduced Ca^{2+} spike occurrence and PI staining. (*D*) Mean Ca^{2+} spike frequency recorded in viable cardiomyocytes during 3 h of TNF-α exposure (ctrl, $n = 35$, two mice; TNF, $n = 68$, four mice; Ab-TRPM2, $n = 30$, two mice; Ab-TRPM2 + TNF; $n = 60$, four mice; Cltz, $n = 34$, two mice; TNF + Cltz, $n = 44$; two mice) (*$P < 0.05$ compared with control conditions). (*E*) Mean values of PI-stained cardiomyocytes were normalized to the total number of cells visualized in transmitted light. TNF-α incubation increased the % of PI-stained cells progressively. In the presence of clotrimazole (CLTZ) or TRPM2 blocking antibody, TNF-α-induced cell death is prevented (*$P < 0.05$ compared with control conditions).

account for the reduced inactivation of the TRPM2,[10] and thus the linear *I–V* relationship with cADPR.[17]

External application of H_2O_2 activates TRPM2 within minutes.[5,7] Activation mode under external application of H_2O_2 is associated with ADPR formation as well as direct activation of TRPM2.[5,9,17] In addition, H_2O_2 may activate TRPM2 independently of ADPR production.[29] H_2O_2 is also capable of increasing mitochondrial ROS production, which would amplify its gating effects on TRPM2.[7,19]

Although TRPM2-induced sustained Ca^{2+} entry is a key component of oxidative stress and cell death,[5] expression and/or function of several members of the TRP channel family are sensitive to the redox status and oxidative stress. Redox regulation of TRP channels has been reported to be direct (TRPC3, TRPC5, TRPM4, TRPM6, TRPA1, and TRPV1) or indirect (TRPM2, TRPM7, and TRPC6) and appears to be critical in pathophysiological regulation of Ca^{2+} signalling pathways.[30] Among the TRP channel family, TRPM2 is the most extensively studied regarding redox regulation of cell death.

TRPM2 knockdown limits Ca^{2+} rise under oxidative stress and/or TNF-α and prevents subsequent cell death.[5] Accordingly, TNF-α and ROS activate a non-selective cationic current in adult cardiomyocytes (*Figures 1* and *3* and Jabr and Cole[31]), which is presumably carried by TRPM2. TNF-α induced a non-selective cationic current that appears to occur via caspase-8 and PARP-1 activation.

TRPM2 structure and function have widely been studied in several cell types such as immune cells and pancreatic beta cells, and in neurodegenerative diseases.[18] Although TRPM2 involvement in cardiac function is largely unknown, its role in ischaemic heart disease has recently emerged.[32,33] Rapid restoration of coronary blood flow of an ischaemic heart is the main strategy to improve outcomes. However, reperfusion is accompanied by cell damage, arrhythmias, myocardial stunning, and no re-flow phenomena due to vascular defects.[34] Oxidative stress and inflammatory processes are ubiquitously observed during the early phase of reperfusion and promote intracellular Ca^{2+} overload, which contributes to reperfusion injuries.[35] Elevation of intracellular Ca^{2+} level results from both trans-sarcolemmal Ca^{2+} entry and altered SR Ca^{2+} cycling. Recently, a reduction of reperfusion injury in TRPM2 knockout mice has been reported, presumably through a decrease in inflammatory processes and neutrophil infiltration.[32] Accordingly, it is possible that the inflammatory response that occurs during the first hour of reperfusion is blunted in the cardiomyocytes level when TRPM2 is inhibited. In addition, reduced endothelial permeability and cardiomyocytes cell death may have cumulative cardioprotective effects during acute ischaemia[12] (*Figure 6*). Surprisingly, global TRPM2 knockout mice did not protect against reperfusion injuries.[33,36] Although the use of the cardioprotective volatile anaesthetic isoflurane during the surgery procedure[33,37] might be a confounding factor, global TRPM2 knockout resulted in decreased mitochondrial function and enhanced mitochondrial ROS production.[36] This is in agreement with the results of the present study where TRPM2 function and mitochondrial ROS production are closely connected and appear to play a critical role in cell survival. Further studies are necessary to clarify the role of TRPM2 activation in cardiac reperfusion injury. Unfortunately, the present lack of specific TRPM2 inhibitors and/or activators constitutes a major obstacle in this context.

Indeed, pharmacology of TRPM2 is poorly understood, but some organic substances such as antifungal imidazole, clotrimazole, and econazole are known to inhibit the channel.[15] Clotrimazole has been shown to prevent cell death induced by TNF-α *in vitro* in several cell types as well as in neonatal and adult cardiomyocytes[6] (*Figure 6*). In addition, econazole significantly decreases the infarct size.[32] Although clotrimazole affects, in addition to TRPM2, a wide range of signalling pathways, clotrimazole and TRPM2 inhibition have also been reported to be protective in liver ischaemia/reperfusion injury and stroke, cardiac arrest-induced global cerebral ischaemia, and focal brain ischaemia.[38–41] Other studies have also shown that PARP-1 inhibition might be cardioprotective;[8] but here, we show that the protective effect of PARP-1 inhibition may involve TRPM2 inhibition.

In summary, we demonstrate that TNF-α, a pro-inflammatory cytokine that increases during ischaemia–reperfusion, induces a TRPM2-like current involving PARP-1 activation through caspase-8 activation and mitochondrial ROS production. TRPM2 inhibition prevents ventricular cell death and demonstrates that TRPM2 channel is functional in native adult cardiomyocytes. Thus, specifically targeting TRPM2 might provide a new therapeutical strategy to combat ischaemia–reperfusion injuries.

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