Transient Receptor Potential Ankyrin 1 Activation within the Cardiac Myocyte Limits Ischemia–reperfusion Injury in Rodents

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ABSTRACT

Background: Recent evidence suggests that cross talk exists between cellular pathways important for pain signaling and ischemia–reperfusion injury. Here, the authors address whether the transient receptor potential ankyrin 1 (TRPA1) channel, important in pain signaling, is present in cardiac myocytes and regulates cardiac ischemia–reperfusion injury.

Methods: For biochemical analysis of TRPA1, techniques including quantitative polymerase chain reaction, Western blot, and immunofluorescence were used. To determine how TRPA1 mediates cellular injury, the authors used an *in vivo* model of rat cardiac ischemia–reperfusion injury and adult rat–isolated cardiac myocytes subjected to hypoxia–reoxygenation.

Results: The authors' biochemical analysis indicates that TRPA1 is within the cardiac myocytes. Further, using a rat *in vivo* model of cardiac injury, the TRPA1 activators ASP 7663 and optovin reduce myocardial injury (45 ± 5%* and 44 ± 8%,* respectively, *vs.* control, 66 ± 6% infarct size/area at risk; n = 6 per group; mean ± SD; *P < 0.001). TRPA1 inhibition also blocked the infarct size–sparing effects of morphine. In isolated cardiac myocytes, the TRPA1 activators ASP 7663 and optovin reduce cardiac myocyte cell death when given during reoxygenation (20 ± 3%* and 22 ± 4%* *vs.* 36 ± 3%; percentage of dead cells per field, n = 6 per group; mean ± SD; *P < 0.05). For a rat *in vivo* model of cardiac injury, the infarct size–sparing effect of TRPA1 activators also occurs during reperfusion.

Conclusions: The authors' data suggest that TRPA1 is present within the cardiac myocytes and is important in regulating myocardial reperfusion injury. The presence of TRPA1 within the cardiac myocytes may potentially explain why certain pain relievers that can block TRPA1 activation, such as cyclooxygenase-2 inhibitors or some nonsteroidal antiinflammatory drugs, could be associated with cardiovascular risk. **(Anesthesiology 2016; 125:1171-80)**

MORE than 200 million opioid prescriptions are written annually in the United States to treat pain.[1] These drugs have serious unwanted side effects including abuse, dependence, and addiction. As a result, an opioid epidemic within the United States is shifting medical practice toward prescribing nonopioid analgesics or opioid adjuvants to reduce opioid use intraoperatively, postoperatively, and in the clinic.[2,3] Further, developing novel analgesics to replace opioids within medical practice is a continued focus for scientists.

For these reasons, understanding how the pathways of pain signaling may contribute to protection of organs from ischemia–reperfusion injury is important since cellular cross talk exists between these two pathways.[3] Nonnarcotic analgesics may also have a deleterious effect by blocking the endogenous mechanisms that reduce the ischemia–reperfusion injury of organs. For example, inhibition of cyclooxygenase-2 (COX-2) experimentally blocks the natural ability of the heart to protect against ischemia–reperfusion injury.[4,5] Clinically, in addition to COX-2 inhibitors, the cardiac safety of some nonsteroidal antiinflammatory drugs (NSAIDs) has also been recently questioned.[2,3]

What We Already Know about This Topic

- Transient receptor potential ankyrin 1 (TRPA1) receptors function as pain receptors within the nervous system.
- TRPA1 receptor antagonists are novel targets for nonopioid pain management. However, a physiologic role for TRPA1 receptors during myocardial ischemia–reperfusion injury has not been investigated.

What This Article Tells Us That Is New

- The authors report that the TRPA1 receptors exist within the cardiac myocyte, and TRPA1 activation protects the heart from myocardial ischemia–reperfusion injury.
- The results reported have the potential to impact novel targets for cardiac protection and have significant implications regarding the safety of TRPA1 antagonists being developed as pain medications.

Since the heart unlike other organs also possesses neuroendocrine qualities, pain receptors from the transient

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receptor potential family could potentially exist within the cardiac myocytes. In particular, transient receptor potential ankyrin 1 (TRPA1) inhibitors are being explored and developed as an alternative to opioids for pain control.[6] Further, the TRPA1 receptor is also modulated by different pain relievers including NSAIDs, COX-2 inhibitors, and acetaminophen.[7–9]

The TRPA1 receptor, besides regulating pain, also serves multiple functions within the cell.[10,11] In particular, TRPA1 functions as a sensor that is activated by reactive aldehydes and is modulated when intracellular changes in oxygen levels occur.[12,13] Both factors are important in regard to organ ischemia–reperfusion injury. In particular, the production of reactive aldehydes as a result of the breakdown of lipid membranes is considered a critical mediator of cellular injury.[13]

However, little is known about whether the TRPA1 receptor exists in the cardiac myocyte and if TRPA1 contributes to regulating injury during cardiac ischemia–reperfusion. If TRPA1 is present in the cardiac myocyte, this could explain why some nonopioid analgesics block natural pathway(s) of protection from ischemia–reperfusion injury. This would also be important to understand when developing drugs targeting TRPA1 to provide analgesia. Here, we address the question whether TRPA1 is present in the cardiac myocyte and further if this receptor is important in mediating ischemia–reperfusion injury.

Materials and Methods

Procedures and protocols were approved by the Animal Care and Use Committee at Stanford University, Stanford, California. All animal studies conformed to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (eighth edition, 2011). Eight- to 10-week-old male Sprague–Dawley rats (Charles River, USA) were used for the studies outlined.

Pharmacologic Agents

The TRPA1 receptor activators, ASP 7663 (ASP; 3 mg/kg *in vivo* and 3 μM *in vitro*) and optovin (1 mg/kg *in vivo* and 1 μM *in vitro*), in addition to the TRPA1 inhibitors, TCS 5861528 (TCS; 1 mg/kg *in vivo* and 1 mM *in vitro*) and AP 18 (AP; 1 mg/kg *in vivo* and 1 mM *in vitro*), were dissolved in dimethyl sulfoxide (DMSO). Doses were chosen for TRPA1 activators and inhibitors based on data for these compounds provided in the manufacturer insert (Tocris, United Kingdom). Morphine (0.3 mg/kg IV bolus) was dissolved in saline, and the dose was determined from our previous studies.[14,15]

Biochemical Studies

Biochemical studies consisted of quantitative polymerase chain reaction (qPCR), Western blot, and immunofluorescence.

For qPCR, adult rat cardiac myocytes, left ventricle heart tissue, and H9C2 cell samples were stored in RNA later (Ambion, USA) at −80°C and homogenized in 1 ml TRI reagent (Molecular Research Center, USA). Total RNA was isolated from tissue and cell homogenates using RNeasy Mini Kit 50 (Qiagen, Germany). Complementary DNA (cDNA) synthesis was performed with the high capacity RNA-to-cDNA kit (Applied Biosystems, USA). qPCR was performed using TaqMan Gene Expression Assays (Applied Biosystems). The 20 μl reactions contained 10 μl TaqMan Fast Universal PCR Master Mix (2×; Thermo Fischer Scientific, USA), 1 μl of the specific TaqMan assay, 1 μl cDNA, and 8 μl water. Cycling parameters were 30-s initial setup at 95°C, followed by 40 cycles at 95°C for 3 s and 30 s at 61°C (ABI 7500 Fast; Applied Biosystems). Primers used were as follows: TRPA1 forward: GCTTCTGCAAGACATCAGCG, TRPA1 reverse: CCTCTCCATCTGGCAGCAAA, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward: CTCAGTTGCTGAGGAGTCCC, and GAPDH reverse: ATTCGAGAGAAGGGAGGGCT.

For Western blot, adult cardiac myocytes, left ventricle tissue, and H9C2 cells were used. The left ventricles of male Sprague–Dawley rats were excised, finely minced with scissors, and homogenized with mannitol–sucrose lysis buffer (210 mM mannitol, 70 mM sucrose, 5 mM 3-*N*-morpholino propanesulfonic acid, 1 mM EDTA with pH 7.4, protease/phosphatase inhibitors, and 1% Triton-X [Sigma, USA]). Homogenates were centrifuged at 800*g* for 5 min to remove cellular debris, and the supernatant was kept as the total fraction. Adult cardiac myocytes and H9C2 cells were also lysed in mannitol–sucrose buffer. For the three types of samples used (adult cardiac myocytes, left ventricle heart tissue, and H9C2 cells), total protein content was determined by using Bradford assay with 35 μg of each homogenate run on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels. Membrane proteins were transferred to a polyvinylidene difluoride membrane and probed overnight at 4°C for specific antibodies to TRPA1 (1:500 dilution in 5% milk-tris-buffered saline with tween-20; Novus, USA) and GAPDH (1:1,000 dilution in 5% milk-tris-buffered saline with tween-20; Sigma). The next day, membranes were washed and incubated in secondary anti-rabbit antibody for 2 h (1:1,000 for TRPA1 and 1:3,000 for GAPDH in 5% milk; ThermoScientific, USA). Membranes were developed in enhanced chemiluminescent reagent, and images were acquired by using an Azure Biosystems c300 (Azure Biosystems, USA).

For immunofluorescence, isolated cardiomyocytes were plated on precoated laminin (Invitrogen, USA) glass coverslips (2 μg/cm^2) for 24 h and fixed with 2% buffered paraformaldehyde for 10 min at room temperature. The fixed cells were washed three times at 5-min intervals with phosphate-buffered saline (PBS). The cells were blocked and permeabilized with blocking buffer (40 mM HEPES, 3% dry milk, PBS, and 0.1% Triton-X 100) for 30 min at room temperature. Cells were incubated with TRPA1 primary antibody

(1:250 dilution) in blocking buffer for 1 h at room temperature. Excess antibody was removed by washing samples with PBS three times at 5-min intervals. The cells were then incubated with donkey anti-rabbit Alexa Fluor 488 (1:500 dilution; Invitrogen) in blocking buffer for 2 h at room temperature. To remove excess secondary antibody, the cells were washed six times at 5-min intervals. The nucleus was stained with dye 4′,6-diamidino-2-phenylindole (1 μg/ml) diluted in PBS for 20 min. Cells were then washed for 10 min with PBS and mounted in ProLong gold reagent antifade reagent (Invitrogen) for microscopic imaging.

In Vivo *Myocardial Infarction Rodent Model*

The model has been described in a number of publications.[1,14,15] After obtaining body weight, rats were anesthetized with Inactin (thiobutabarbital, 100 mg/kg intraperitoneal; Sigma). A tracheotomy was performed in addition to cannulation of the carotid artery and internal jugular vein to measure blood pressure and to administer drugs, respectively. Rats were placed on a ventilator (30 to 40 breaths/min; tidal volume, 8 ml/kg) and adjusted to maintain a normal pH (7.35 to 7.45) and end-tidal carbon dioxide (35 to 45 mmHg) by using a blood gas machine (Radiometer ABL-80; Radiometer America, USA). Body temperature was monitored with a rectal thermometer (Thermalert TH-5; Physiotemp Instruments, USA) and maintained at 36° to 38°C by using heating pads and heat lamps. The heart was exposed by an incision in the fourth intercostal space, the pericardium was excised, and a suture was placed around the left anterior descending coronary artery (6-0 prolene; Ethicon, USA). After surgical manipulation and adjustment of the ventilator settings based on blood gas analysis, rodents were allowed to stabilize for 30 min before initiation of the experimental protocol.

The experimental protocol had 14 treatment groups, which are described in detail throughout the manuscript. Based on our previous studies where a power analysis with $\alpha = 0.05$ and 80% power to detect at least a 15% difference, a minimum of six experiments are required.[14] Rodents were randomized to experimental treatment groups, and all rats were subjected to 30 min of left anterior descending coronary artery occlusion followed by 2 h of reperfusion. After reperfusion, the left anterior descending coronary artery was again occluded, and the heart was negatively stained for the area at risk by injection of patent blue dye (Sigma) given through the internal jugular vein. The heart was then excised, both atria and the right ventricle were removed, and the left ventricle was cut into five equal slices to create cross sections from apex to base. The slices were separated into normal zone and area at risk, both followed by incubation in 1% triphenyl tetrazolium chloride to measure the viability of myocardial tissue. Viable tissue stained red, while nonviable tissue remained unstained or white. Infarct size as a percentage of area at risk was determined gravimetrically. Heart rate, blood pressure, and rate pressure product were monitored and calculated throughout the experimental protocol using PowerLab monitoring system (MLS060/8 PowerLab 4/35; AD Instruments, USA). We defined rate pressure product (RPP) as the product of heart rate and systolic blood pressure.

In Vitro *Cardiac Myocytes Hypoxia and Reoxygenation Injury Model*

Adult rat primary cardiac myocytes were isolated from male Sprague–Dawley rat hearts by enzymatic dissociation as previously described.[16] The isolated cardiac myocytes were plated in 4% fetal bovine serum (Gemini Bioproducts, USA) and medium 199 (Gibco-Thermo Fischer Scientific, USA) on laminin coated plates (2 $\mu g/cm^2$) for 2 h. The plating medium was changed to serum-free medium (1% bovine serum albumin medium 199) to remove nonmyocytes. Cardiomyocytes were incubated at 37°C in 5% CO_2 for 24 h before experiments in 24-well plates with a seeding density of 5×10^4 per milliliter.

After 24 h, serum-free media 199 was changed the morning of the study 1 h before hypoxia and remained on the cells until completion of the study. Experiments were divided into two subsets: a normoxic control group and a hypoxia–reoxygenation group. In the hypoxia–reoxygenation groups, cardiac myocytes were subjected to DMSO, a TRPA1 activator (optovin, 1 μM or ASP, 3 μM), or a TRPA1 inhibitor (TCS, 1 μM; AP, 1 μM) immediately before reoxygenation. For the normoxic control group, cardiac myocytes were also treated with DMSO, a TRPA1 activator, or a TRPA1 inhibitor. Hypoxia was induced by placing the plates into an anaerobic gas pouch (GasPak EZ Gas Generating Pouch Systems; BD Biosciences, USA) for 2 h. The pouch creates an anaerobic environment where the oxygen level within the pouch is less than 0.1%.[17] The cells were then removed from the anaerobic gas pouches and reoxygenated within the cell culture incubator for an additional 4 h.

To determine cell death in this model, trypan blue exclusion and lactate dehydrogenase (LDH) release were used in separate biologic replicates. For the trypan blue experiments after 4 h of reoxygenation, trypan blue was added to adult cardiac myocytes at a final concentration of 0.04%. To distinguish viable cells from dead cells, two digital images for each experiment were acquired using a camera (Nikon Coolpix 8800; Nikon, USA) attached to an adapter (MM99 adapter S/N: 1925; Martin Microscope Company, USA) connected to the microscope (Motic AE21; China). All images were taken within 3 min of trypan blue application to minimize the variability associated with changes in the ratio of stained/unstained cells over time. Cell death was determined by a person blinded to the experimental groups that were provided the digital images. The number of trypan blue–positive cells were counted and further expressed as a percentage of the total cells in the image. Approximately 300 cells were counted per well from two images. A total of six experiments per group were performed from two biologic

replicates. The LDH release was measured as previously described and quantified as the ratio of LDH release after 4 h of reoxygenation to total LDH.[18]

Statistical Analysis

All data were shown as mean ± SD. For analysis of *in vivo* and cardiac myocyte models of ischemia–reperfusion or ischemia–reoxygenation, a one-way ANOVA followed by Bonferroni correction for multiplicity was used in order to compare each group to the control group. A two-way ANOVA was used to determine significance for hemodynamic parameters. For differences between two groups, a two tailed Student's *t* test was performed. Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software Inc., USA). $P < 0.05$ was considered statistically significant.

Results

A total of 107 rats were used for the study. Twenty-one rats were used to isolate adult cardiac myocytes to determine the TRPA1 expression, conduct ischemia–reoxygenation experiments, and obtain heart left ventricle. One prep was excluded due to complications with cannulating the aorta for cardiac myocyte isolation. Further, 86 rats were used for the *in vivo* experiments for completion of 84 successful experiments. Two rats were excluded for this portion of the study with one rat due to intractable ventricular fibrillation during reperfusion (ASP in the reperfusion group) and another rat secondary to a small area at risk per left ventricle (AP plus morphine group). No differences in the percentage of the area at risk per left ventricle were noted for any of the groups (table 1). Further, no differences in hemodynamics including heart rate, blood pressure, and rate pressure product occurred between any of the treatment groups (table 1). Within the group where DMSO was given during reperfusion, significant differences were noted in the heart rate, mean arterial pressure, and rate pressure product at 2 h of reperfusion when compared to baseline values (table 1).

To initially address whether TRPA1 is present in the heart, we performed qPCR and Western blot on the cardiac myocytes, the left ventricle–derived stable cell line, H9C2 cells, and heart homogenates from the left ventricle. Both PCR and protein expression detected TRPA1 in the cardiac myocytes (fig. 1, A and B). Further, by immunofluorescence, isolated adult cardiac myocytes also displayed TRPA1 expression (fig. 1C). These findings were further supported by detecting TRPA1 in the H9C2 cell line and homogenate of left ventricle (Supplemental Digital Content 1, qPCR showing the presence of TRPA1 in the H9C2 cell line and homogenate of left ventricle, and Supplemental Digital Content 2, which shows Western blot validation, http://links.lww.com/ALN/B321).

TRPA1 activators, including ASP 7663 and optovin, are selective for TRPA1 (fig. 2A). We gave these agents before ischemia in an *in vivo* rodent model of heart attack injury (fig. 2B). ASP 7663 and optovin reduced myocardial damage when compared to the vehicle DMSO (ASP 7663, 45 ± 5%* and optovin, 44 ± 8% *vs.* DMSO, 66 ± 6%; percentage of infarct size/area of risk; n = 6 per group; mean ± SD; *$P < 0.001$ *vs.* DMSO; fig. 2, C and D). Together, these data suggest that TRPA1 is present in the cardiac myocyte and TRPA1 activators can modulate injury from a heart attack.

TRPA1 is also selectively modulated by the reactive aldehyde, cinnamaldehyde (Supplemental Digital Content 3A, which shows the chemical structure of cinnamaldehyde, http://links.lww.com/ALN/B321). Unlike the other TRPA1 activators tested that are not reactive aldehydes (ASP 7663 and optovin), cinnamaldehyde given at 0.01 or 0.1 mg/kg increased blood pressure dose-dependently immediately on

Table 1. Number of Animals Used, Area at Risk Per Left Ventricle (%), and Hemodynamic Values Measured

			Baseline			15-min Ischemia			2-h Reperfusion		
Groups	n	AAR/LV%	HR	MAP	RPP	HR	MAP	RPP	HR	MAP	RPP
DMSO	6	41 ± 9	398 ± 20	98 ± 12	47 ± 5	407 ± 37	89 ± 21	42 ± 7	397 ± 27	80 ± 11	43 ± 8
Optovin	6	40 ± 6	419 ± 32	104 ± 21	54 ± 9	437 ± 22	102 ± 22	52 ± 10	425 ± 13	79 ± 14	43 ± 7
ASP	6	41 ± 9	422 ± 41	114 ± 11	60 ± 5	428 ± 33	105 ± 28	52 ± 13	415 ± 25	89 ± 12	49 ± 6
MOR	6	37 ± 5	410 ± 35	109 ± 14	53 ± 9	407 ± 37	104 ± 14	49 ± 9	374 ± 19	73 ± 10	37 ± 6
TCS + MOR	6	42 ± 3	420 ± 38	104 ± 12	53 ± 9	407 ± 35	92 ± 26	44 ± 11	398 ± 44	73 ± 8	42 ± 8
AP + MOR	6	40 ± 9	423 ± 14	107 ± 14	54 ± 5	378 ± 28	107 ± 31	48 ± 15	388 ± 29	77 ± 11	40 ± 8
TCS	6	36 ± 4	427 ± 30	111 ± 16	57 ± 8	424 ± 25	109 ± 27	54 ± 10	404 ± 23	83 ± 15	47 ± 7
AP	6	40 ± 8	425 ± 32	103 ± 22	53 ± 13	421 ± 34	100 ± 43	50 ± 22	397 ± 25	75 ± 14	41 ± 10
DMSO at rep	6	39 ± 3	421 ± 33	105 ± 11	50 ± 8	447 ± 36	96 ± 15	48 ± 9	370 ± 28*	73 ± 18*	34 ± 9*
Optovin at rep	6	40 ± 8	413 ± 43	108 ± 16	54 ± 8	434 ± 36	100 ± 24	54 ± 13	388 ± 31	84 ± 20	42 ± 9
ASP at rep	6	45 ± 7	421 ± 12	104 ± 8	52 ± 4	423 ± 22	94 ± 25	49 ± 14	395 ± 33	81 ± 15	42 ± 8

Described are the groups, number of animals per group (n), AAR/LV, and hemodynamics acquired for the *in vivo* studies. HR, MAP, and RPP defined as the product of HR and systolic blood pressure were assessed at baseline, during ischemia, and at 2 h of reperfusion. RPP was calculated as the product of HR and systolic blood pressure. Data are presented as mean ± SD (n = 6). No significant differences were found between groups.

*$P < 0.05$ *vs.* baseline.

AAR/LV = area at risk per left ventricle percent; AP = AP18; ASP = ASP 7663; DMSO = dimethyl sulfoxide; HR = heart rate; MAP = mean arterial pressure; MOR = morphine; n = number of animals per group; rep = reperfusion; RPP = rate pressure product; TCS = TCS 5861528.

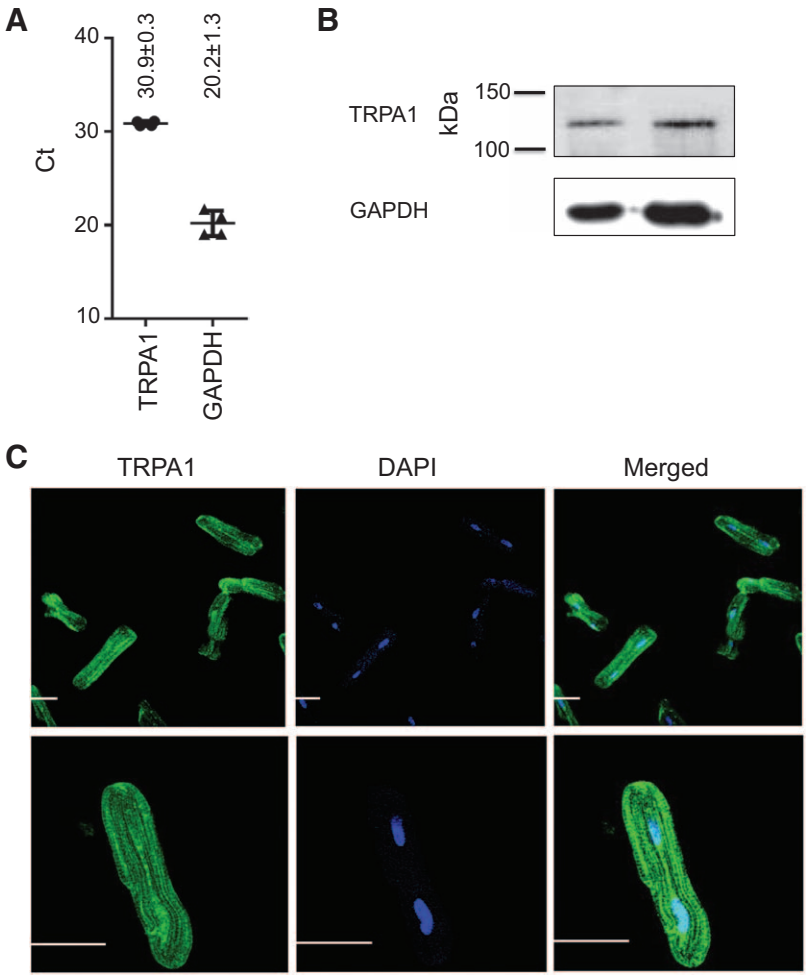

Fig. 1. Biochemical evidence transient receptor potential ankyrin 1 (TRPA1) is present in cardiac myocytes. (*A*) Quantitative PCR of cardiac myocytes with four biologic replicates. (*B*) Western blot of cardiac myocytes representative of four biologic replicates. (*C*) Immunofluorescence for TRPA1 in cardiac myocytes and nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI). *Top*, Multiple cardiomyocytes per field (×20). *Bottom*, A single cardiomyocyte under higher magnification (×63). *White bar* = 30 μm. Ct = cycle threshold; GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

administration (cinnamaldehyde, 0.01 mg/kg: 124 ± 10* mmHg; cinnamaldehyde, 0.1 mg/kg: 134 ± 12* mmHg compared to DMSO, 92 ± 2 mmHg; 5 min after administration; n = 6 per group; *P < 0.01 *vs.* DMSO; Supplemental Digital Content 3B, which shows mean arterial blood pressures after cinnamaldehyde administration, http://links.lww.com/ALN/B321). Further, cinnamaldehyde did not affect myocardial infarct size (cinnamaldehyde, 0.01 mg/kg: 61 ± 3%; cinnamaldehyde, 0.1 mg/kg: 61 ± 5% *vs.* control, 62 ± 3%; n = 6 per group; Supplemental Digital Content 3, C and D, which shows infarct size data and representative images of infarct size, http://links.lww.com/ALN/B321). For these studies, no differences were noted between groups for the area at risk per left ventricle percentage. In addition, no changes were noted between groups for the recorded hemodynamics at baseline, 15 min of ischemia, and at 2 h of reperfusion (Supplemental Digital Content 4, which shows a table of hemodynamics for the cinnamaldehyde portion of the study, http://links.lww.com/ALN/B321).

Transient receptor potential channel family members are also known to colocalize with opioid receptors in the nervous system, and opioids activate TRPA1.[19] Thus, we further questioned whether morphine decreases myocardial injury by TRPA1 activation. Selective inhibitors of TRPA1 include AP 18 and TCS 5861528 (fig. 3A). We tested whether these TRPA1 inhibitors affect the ability of morphine to reduce myocardial injury in an *in vivo* rodent heart attack injury model (fig. 3B). When either TRPA1 inhibitor was given before morphine, the ability of morphine to decrease heart damage was blocked (morphine, 44 ± 5%*; TCS plus morphine, 62 ± 5%#; AP plus morphine, 65 ± 6%# *vs.* DMSO, 66 ± 6%; *P < 0.001 *vs.* all groups; #P < 0.001 *vs.* morphine; fig. 3, C and D). No effect on myocardial infarct size was noted when either TRPA1 inhibitor was alone given (fig. 3, C and D).

Since the data we obtained were from an *in vivo* rodent model, the effect seen could potentially be due to modulation of the nervous system rather than a direct effect on

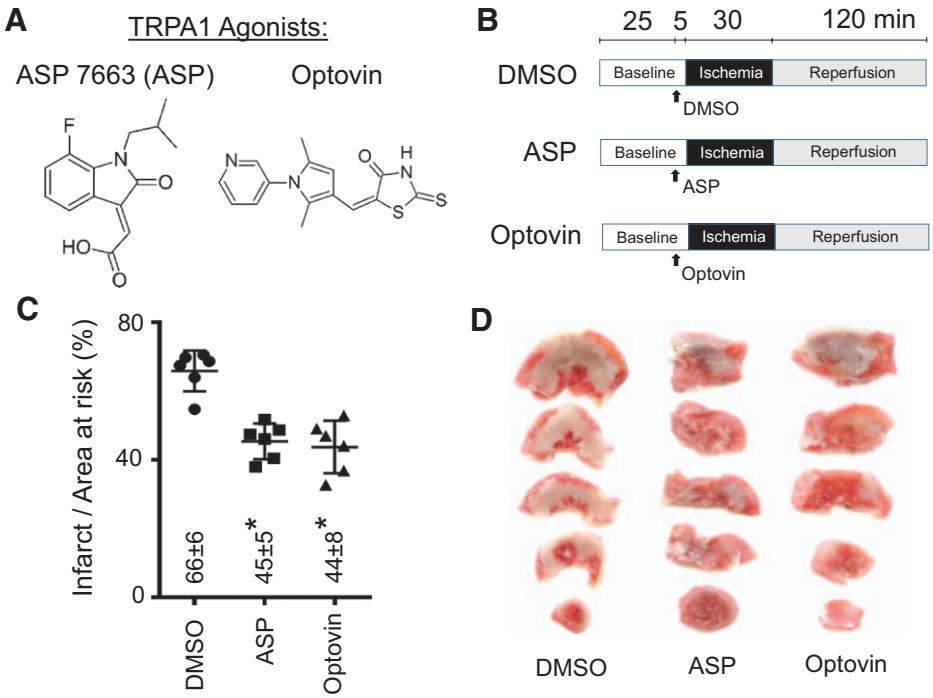

Fig. 2. Transient receptor potential ankyrin 1 (TRPA1) activators reduce myocardial infarct size. (*A*) Chemical structure of TRPA1 activators. (*B*) Experimental protocol for myocardial ischemia–reperfusion studies. Rats were given a TRPA1 activator (ASP [ASP7663] or optovin) or vehicle (dimethyl sulfoxide [DMSO]) 5 min before 30 min of left anterior descending coronary artery ligation to cause ischemia followed by 2 h of reperfusion. (*C*) Infarct size per area at risk percentage for each experimental group. Data points represent individual biologic results for each experiment in addition to values presented as mean ± SD (n = 6); **P* < 0.001 *versus* DMSO group. (*D*) Representative images of left ventricle area at risk. Infarcted areas are unstained and remain white, while viable tissue is stained red by triphenyl tetrazolium chloride.

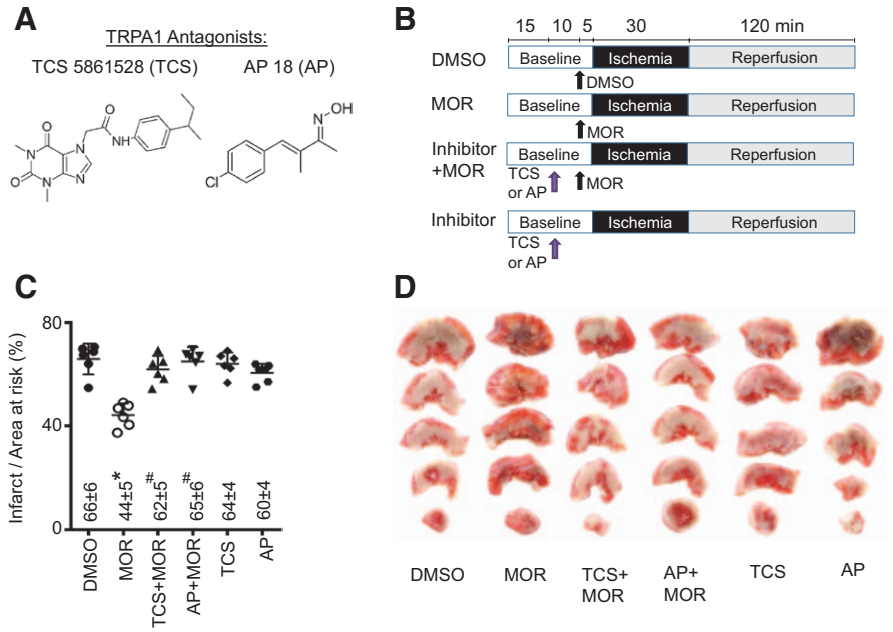

Fig. 3. Opioid-induced reduction of myocardial infarct size is mediated by transient receptor potential ankyrin 1 (TRPA1). (*A*) Chemical structure of two TRPA1 inhibitors (TCS [TCS 5861528] and AP [AP18]). (*B*) Experimental protocol for myocardial ischemia–reperfusion studies. Dimethyl sulfoxide (DMSO) or morphine (MOR) was given 5 min before ischemia. The two TRPA1 inhibitors (TCS and AP) were given 10 min before morphine treatment. (*C*) Infarct size per area at risk percentage. Data are presented as mean ± SD (n = 6); **P* < 0.001 *versus* DMSO; #*P* < 0.001 *versus* MOR. For comparison purposes, the DMSO group data presented in figure 2 are also presented here in this figure. (*D*) Representative images of left ventricle area at risk for each group.

the cardiac myocyte itself. Therefore, we further determined how TRPA1 modulates cellular death of cardiac myocytes when independent of the nervous system. We isolated primary cardiac myocytes from adult rat hearts and the following day subjected the cardiac myocytes to either sham or hypoxia–reoxygenation. Primary adult cardiac myocytes are viable in cell culture for 48 h, and as also shown by others,[20] a percentage of cell death will occur for a sham-treated group. We administered the TRPA1 activators and inhibitors without the presence of hypoxia–reoxygenation (Supplemental Digital Content 5A, which describes the experimental protocol, http://links.lww.com/ALN/B321). Without hypoxia–reoxygenation, these agents showed no differences in cell death by percentage of LDH release (sham, 14 ± 6; DMSO, 14 ± 3; ASP, 13 ± 4; optovin, 13 ± 2; AP, 15 ± 7; TCS, 14 ± 7; percentage of LDH release of total amount in cells; n = 6 per group; Supplemental Digital Content 5B, which describes LDH release data, http://links.lww.com/ALN/B321). Further, no differences were noted in trypan blue exclusion when compared to untreated or DMSO-treated cardiac myocytes (sham, 11 ± 2; DMSO, 11 ± 2; ASP, 11 ± 4; optovin, 10 ± 2; AP, 10 ± 2; TCS, 11 ± 2; percentage of trypan blue–positive cells; n = 6 per group; Supplemental Digital Content 5, C and D, which provides images and quantification of trypan blue exclusion assay, http://links.lww.com/ALN/B321). We also tested the TRPA1 inhibitors when given before reoxygenation (Supplemental Digital Content 6A, which describes the experimental protocol, http://links.lww.com/ALN/B321). Similar to the *in vivo* model findings, the TRPA1 inhibitors did not affect LDH release (sham, 13 ± 2; DMSO, 49 ± 11*; AP, 49 ± 6*; TCS, 49 ± 10*; n = 6 per group; *P < 0.05 *vs.* sham; Supplemental Digital Content 6B, which describes LDH release data, http://links.lww.com/ALN/B321). The TRPA1 inhibitors also did not change the number of trypan blue–positive cells when compared to DMSO-treated adult cardiac myocytes (sham, 9 ± 3; DMSO, 38 ± 3*; AP, 33 ± 3*; TCS, 33 ± 4*; n = 6 per group; *P < 0.05 *vs.* sham; Supplemental Digital Content 6, C and D, which provides images and quantification of trypan blue exclusion assay, http://links.lww.com/ALN/B321).

Further, we tested whether the TRPA1 activators affected cardiac myocyte viability when the TRPA1 activators were

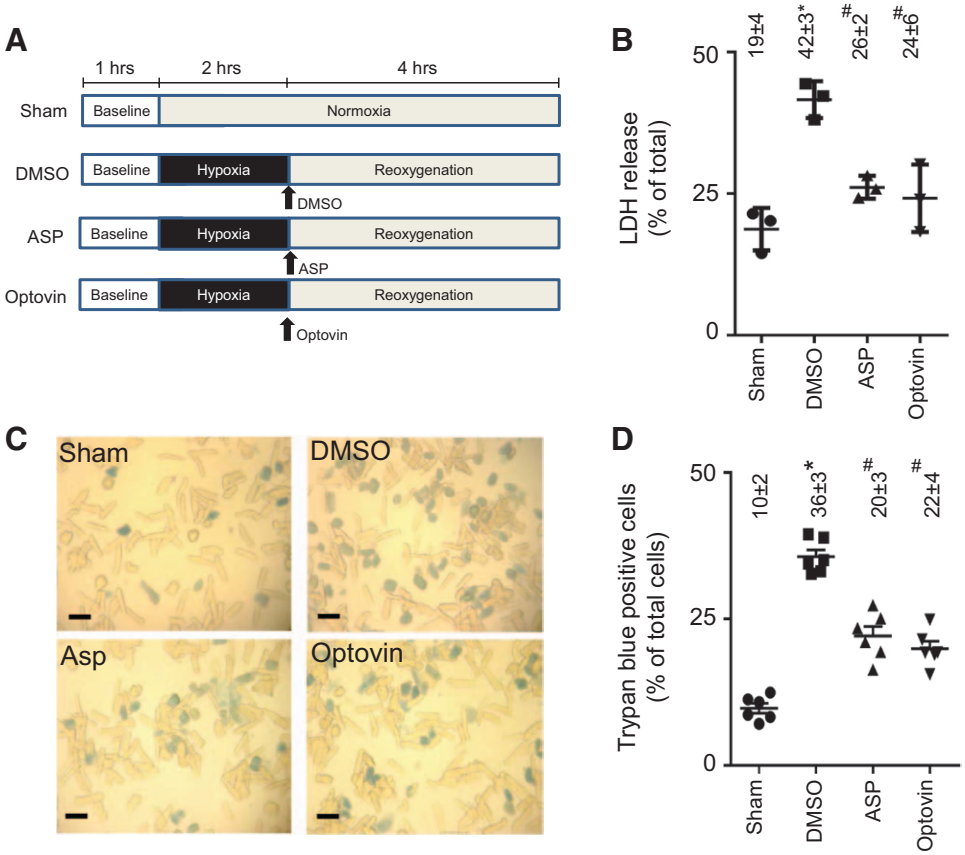

Fig. 4. Activation of transient receptor potential ankyrin 1 (TRPA1) at reoxygenation in isolated adult cardiac myocytes reduces cell death. (*A*) Experimental protocol for cardiac myocyte hypoxia–reoxygenation studies. The two TRPA1 activators (ASP 7663 [ASP] and optovin) were given immediately after hypoxia. (*B*) Percentage of lactate dehydrogenase (LDH) release for each experimental group (n = 3 per group). (*C*) Representative images of trypan blue–positive and trypan blue–negative cardiac myocytes for each group (*black bar* represents 50 μm). (*D*) Percentage of dead cells for each experimental group (n = 6 per group). Data points represent individual biologic results for each experiment in addition to values presented as mean ± SD; *P < 0.05 *versus* sham; #P < 0.05 *versus* dimethyl sulfoxide (DMSO).

given before reoxygenation (fig. 4A). We determined that when either TRPA1 activator was given, the amount of LDH released was less when compared to vehicle-treated adult rat cardiac myocytes (ASP, 26 ± 2%# and optovin, 24 ± 6%# *vs.* DMSO, 42 ± 3%* and sham, 19 ± 4%; percentage of LDH release; n = 3 per group; mean ± SE; *P < 0.05 *vs.* all other groups; #P < 0.05 *vs.* sham or DMSO; fig. 4B). Further, the percentage of cardiac myocytes that died was significantly reduced by almost 40% when assessed by using a trypan blue exclusion assay (ASP, 20 ± 3%# and optovin, 22 ± 4%# *vs.* DMSO, 36 ± 3*% and sham, 10 ± 2%, percentage of trypan blue–positive cells; n = 6 per group; mean ± SE; *P < 0.05 *vs.* all other groups; #P < 0.05 *vs.* sham or DMSO; fig. 4, C and D). Together, these data suggest that TRPA1 activators are effective agents to reduce injury from hypoxia–reoxygenation when TRPA1 activators are directly applied to the cardiac myocyte before reoxygenation.

About 50% of the myocardial damage can be attributed to reperfusion, and accordingly, drugs to limit reperfusion injury are importantly needed.[21,22] Therefore, we further determined whether administration of a TRPA1 activator just before reperfusion also reduces cellular death in an *in vivo* rodent heart attack injury model (experimental protocol; fig. 5A). Interestingly, TRPA1 activators when given before reperfusion reduced myocardial injury (ASP, 42 ± 8,* optovin, 43 ± 7,* and DMSO 60 ± 5, n = 6 per group; *P < 0.001 *vs.* DMSO; fig. 5, B and C). The reduction in heart damage seen was also equal to that when the agents were given before ischemia (fig. 2C), implying that the beneficial effect of TRPA1 activation occurs mainly during reperfusion to limit reperfusion injury.

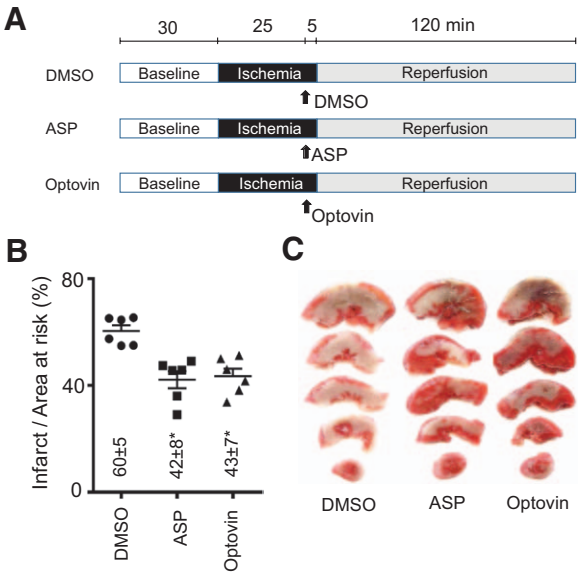

Fig. 5. Transient receptor potential ankyrin 1 (TRPA1) activation reduces infarct size at reperfusion. (*A*) Experimental protocol for myocardial ischemia–reperfusion studies. TRPA1 activator (ASP 7663 [ASP] and optovin) was given 5 min before reperfusion. (*B*) Infarct size per area at risk percentage. Data points represent individual biologic results for each experiment in addition to values presented as mean ± SD (n = 6). *P < 0.05 *versus* dimethyl sulfoxide (DMSO). (*C*) Representative images of left ventricle area at risk for each group.

Discussion

Here, we report that TRPA1 is present within the cardiac myocytes and regulates cardiac reperfusion injury. This finding is important since it suggests that the TRPA1 receptor, largely considered only in the cells of the nervous system,[23] also contributes an important physiologic role in the heart that regulates cellular damage from ischemia–reperfusion injury (fig. 6).

TRPA1 can also be modulated by different pain relievers including NSAIDs, acetaminophen, and COX-2.[7–9] The presence and function of TRPA1 within the cardiac myocyte must be considered since drugs specifically targeting TRPA1 for pain relief may be detrimental to the heart when at risk of ischemia–reperfusion. If TRPA1 is inhibited, this should be considered when selecting pain relievers for patients during the perioperative period and when treating acute or chronic pain conditions. This will be particularly important in the future when more specific TRPA1 small molecules are designed as analgesics.

Prostaglandins including A1, A2, and J2 can activate neuronal TRPA1.[9] One can surmise that some NSAIDs, COX-2 inhibitors, and acetaminophen modulate TRPA1 by altering the production of arachidonic acid metabolites that occur during reperfusion injury. This is supported since either prostaglandin A1 or J2, when given exogenously, reduces myocardial infarct size in experimental models.[24,25] Although further studies are needed, NSAIDs or acetaminophen, by limiting the production of prostaglandins interacting with TRPA1, may potentially block this mechanism that reduces cellular injury.

Opioid-dependent activation of TRPA1 is reported in neuronal cells.[19] Receptors associated with pain relief, such as the opioid receptor family, are also present in the cardiac myocytes.[26,27] When activated by opioids, opioid receptors

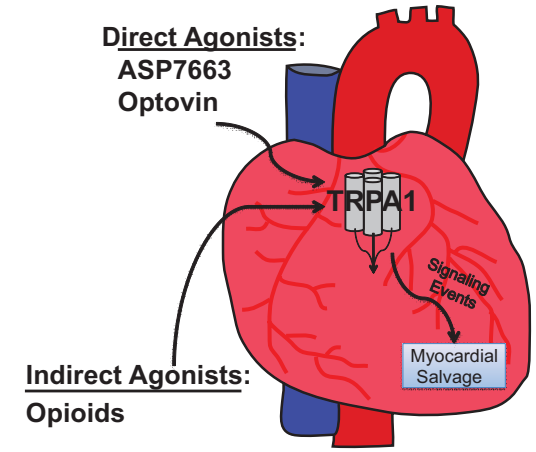

Fig. 6. Hypothetical pathway summary. Activation of transient receptor potential ankyrin 1 (TRPA1) before or during ischemia within the cardiac myocyte reduces ischemia–-reperfusion injury.

contribute an initiation of a signaling cascade that reduces damage from ischemia–reperfusion injury.[28,29] Our study suggests that opioids, frequently given by anesthesiologists for analgesia, reduce myocardial injury *via* a TRPA1-dependent mechanism. This finding suggests that adjuvants to opioids, which may block TRPA1, may limit the beneficial effects of opioids in regard to reducing reperfusion injury. We previously showed that aspirin, unlike ibuprofen, can block the ability of opioids to reduce myocardial reperfusion injury.[30] Although more studies are needed, potentially other myocardial salvaging techniques (such as using volatile anesthetics[31] or implementing remote conditioning[31]) may also have a reduced efficacy for limiting myocardial ischemia–reperfusion injury when TRPA1 is inhibited.

Reactive aldehydes, produced at the highest levels during the initial minutes of reperfusion, are established to modify critical cysteines present on TRPA1, which in turn regulate function.[12] These cysteine modifications, which occur through Michael adduct addition, can change the cellular gating of calcium by TRPA1.[32] For our study, the binding site for optovin is identified to be within a region of critical cysteines present on TRPA1 that is also essential for reactive aldehyde-induced TRPA1 activation.[12,31] Although further studies will be needed, the TRPA1 activators we used may reduce injury during a heart attack by occupying critical cysteines of TRPA1, limiting the amount of irreversible reactions that occur by reactive aldehydes during the initial minutes of reperfusion.

In contrast to the reversible TRPA1 activators used, cinnamaldehyde, the fragrant component of cinnamon oil, is a reactive aldehyde. Administering this reactive aldehyde before ischemia–reperfusion injury resulted in an increase in blood pressure, which was previously shown by a previous study from others in rodents. This particular study described that the cardiovascular hemodynamic responses when cinnamaldehyde was given were lost in TRPA1 knockout mice, suggesting the specificity for cinnamaldehyde within the cardiovascular system to interact with TRPA1.[33] We further extend the findings of this study by describing how cinnamaldehyde does not reduce myocardial injury unlike the reversible TRPA1 agonists given. It is also interesting to note that the blood pressure elevation seen for cinnamaldehyde did not occur for the reversible TRPA1 activators optovin and ASP 7663. Although more work is needed, we believe that this difference in effect seen by cinnamaldehyde (with an end result of increased blood pressure and lack of infarct size reduction compared to the other TRPA1 agonists optovin and ASP 7663) is secondary to the aldehyde that is part of the chemical structure of cinnamaldehyde. The aldehyde may form an irreversible adduct by Michael addition as opposed to the other activators, optovin and ASP 7663, which may instead cause a reversible modification of TRPA1.

Our study should be interpreted within the context of several potential limitations. With the discovery of TRPA1 within the cardiac myocyte, more in-depth molecular analysis is needed. This includes determining the critical cysteines within TRPA1 that are important in regulating cellular injury. Further, we administered optovin intravenously without optogenetic activation of the drug before delivery as described in a previous study.[31] Since ultraviolet light in this previous study was used to activate optovin, we can only assume that the effect we see for our studies, which is similar to another TRPA1 agonist, ASP 7763, is possibly secondary to the reactive oxygen species produced during reperfusion, converting optovin into an active form. This is an interesting idea that will require further study and could suggest a means to develop therapeutic modulators of cellular injury that are selectively cleaved into an active form only during ischemia–reperfusion injury.

Our findings can also lead to developing more cardiac safe analgesics that will not block endogenous pathways involving TRPA1, which are important for cardiac protection. Further, once the pathway is understood, design of agents may be possible to provide a beneficial effect of pain relief, with a secondary benefit of reducing reperfusion injury. This could improve upon using opioids to reduce myocardial ischemia–reperfusion injury, which have deleterious side effects that can lead to addiction, overdose, and death.[34]

In summary, this report describes the presence of TRPA1 in the heart and how TRPA1 agonists can reduce damage from cardiac reperfusion injury. This may need to be considered when developing drugs to target TRPA1 for analgesia. Further, these findings identify that adjuvants given with opioids that target TRPA1 may block the ability for opioids to reduce myocardial injury. This is important when considering treatment strategies for patients with acute or chronic pain who may have a potential risk of suffering a heart attack.

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Competing Interests

The authors declare no competing interests.

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