Protease-activated receptor 2 protects against myocardial ischemia-reperfusion injury through the lipoxygenase pathway and TRPV1 channels

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Abstract. This study tests the hypothesis that the lipoxygenase (LOX) pathway mediates protease-activated receptor (PAR) 2-induced activation of the transient receptor potential vanilloid receptor 1 (TRPV1) to protect the heart from ischemia/reperfusion (I/R) injury. SLIGRL, a PAR2 activating peptide, was administered prior to reperfusion following left anterior descending coronary artery ligation in wild type (WT) and TRPV1 knockout (TRPV1$^{-/-}$) mice. In a Langendorffly perfused heart I/R model, hemodynamic parameters, including left ventricular end-diastolic pressure, left ventricular developed pressure, coronary blood flow and left ventricular peak +dP/dt were evaluated after I/R. SLIGRL reduced the cardiac infarct size in WT and TRPV1$^{-/-}$ mice with a greater effect in the former strain ($P<0.05$). SLIGRL increased plasma levels of calcitonin gene-related peptide (CGRP) and substance P in WT (both $P<0.05$) but not in TRPV1$^{-/-}$ mice. Pretreatment with CGRP8-37 (a CGRP receptor antagonist) or RP67580 (a neurokinin-1 receptor antagonist) alone had no effect on SLIGRL-induced cardiac protection in either strain. However, combined administration of CGRP8-37 and RP67580 abolished SLIGRL-induced cardiac protection in WT but not in TRPV1$^{-/-}$ mice. Nordihydroguaiaretic acid (a general LOX inhibitor) and baicalein (a 12-LOX inhibitor), but not indomethacin (a cyclooxygenase inhibitor) and hexanamide (a selective cytochrome P450 epoxygenase inhibitor), abolished the protective effects of SLIGRL in WT (all $P<0.05$) but not in TRPV1$^{-/-}$ hearts. These data suggested that PAR2, possibly via 12-LOX, activates TRPV1 and leads to CGRP and substance P release to prevent I/R injury in the heart, indicating that the 12-LOX-TRPV1 pathway conveys cardiac protection to alleviate myocardial infarction.

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Introduction

The protease-activated receptor 2 (PAR) belongs to the family of G protein-coupled receptors expressed in the cell membrane, which is activated by proteolytic cleavage of the N-terminal domain to unmask a tethered ligand (1). SLIGRL, a PAR2 agonist peptide consisting of six amino acids, is able to selectively activate PAR2 in the absence of serine proteases. PAR2 activation has been shown to play a role in inflammation (2), effects that are attributed in part to a neurogenic mechanism possibly involving activation of the transient receptor potential vanilloid 1 (TRPV1) channels (3). TRPV1 is a non-selective cation channel mainly expressed in primary sensory neurons and sensory C- and Aδ-fibers (4) that play a role in inflammatory processes through release of neuropeptides including calcitonin gene-related peptide (CGRP) and substance P (SP) from the sensory nerve endings (3). TRPV1 may be activated by noxious heat and various inflammatory mediators such as protons, lipoxygenase products, and other endogenous arachidonic acid derivatives (5). PAR2 has been shown to co-express with TRPV1 in neurons containing SP and CGRP in dorsal root ganglia (DRG) (3). Activation of PAR2 sensitizes TRPV1 expressed in DRG neurons *in vitro* and *in vivo* (6) and in particular, PAR2 agonists but not other PARs selectively stimulate release of CGRP and SP from primary spinal afferent neurons (6).

Activation of PAR2 with SLIGRL protects against myocardial ischemia and reperfusion (I/R) injury (7). Our previous study demonstrated that SLIGRL-induced protective effects in *ex vivo* myocardial I/R injury model was impaired in TRPV1$^{-/-}$ hearts (8). The present study, using an *in vivo* myocardial I/R injury model, investigated whether PAR2-induced cardioprotection is mediated by activation of TRPV1 and release of CGRP and SP.

It has been shown that PAR2 protects against myocardial I/R injury via lipoxygenase (LOX)-derived eicosanoids released from endothelium to regulate the coronary circulation (7,9). PAR-2 activation causes vasodilation, which can be attenuated by inhibition of nitric oxide (NO) or prostaglandins

(PGs) synthesis (10). Vasodilation responses to acetylcholine in the perfused heart were impaired after I/R injury whereas vasodilatory response to PAR2 was preserved (7). Given that PGs and LOX products have been indicated to be able to activate or sensitize TRPV1 channel (11), PAR2-mediated activation of the LOX pathways may contribute to cardioprotection after I/R injury via activation of TRPV1. Despite a substantial body of evidence demonstrates that arachidonic acid (AA) derivatives play an important role in myocardial I/R injury (12), it is unknown whether LOX or cyclooxygenase (COX) pathways contribute to PAR2-induced activation or sensitization of TRPV1 channel.

In this study, we tested the hypothesis that the LOX pathway mediates PAR2-induced activation of TRPV1 to protect against myocardial I/R injury. We investigated the involvement of TRPV1 channel and its induced CGRP and SP in PAR2-induced cardioprotection in *in vivo* myocardial I/R injury model using TRPV1 null ($TRPV1^{-/-}$) mouse model and pharmacological antagonists, respectively. The involvement of LOX and COX in PAR2-induced activation of TRPV1 was dissected by using various pharmacological inhibitors in *ex vivo* perfused heart model.

Materials and methods

In vivo myocardial I/R model. All experimental procedures involving animals were approved by the Michigan State University Animal Care and Use Committee (East Lansing, USA) and conform to The National Institutes of Health guidelines (Bethesda, MD, USA). The 12-week-old male $TRPV1^{-/-}$ strain B6.129S4-$TRPV1^{tm1Jul}$ and matching control wild type (WT) strain C57BL/6J mice (Jackson Laboratory) were used. Acute myocardial I/R models were established as described (13). Briefly, mice were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight). The left anterior descending (LAD) artery was ligated with an 8-0 silk suture for 45 min, and the myocardial ischemia was confirmed by pale color in the occluded distal. Then, the ligature was released for 3 h reperfusion, and the reperfusion was confirmed by return of a red color in the pale region.

Experimental protocols in vivo. Mice were randomly assigned to one of following treatment, all peptides injection combined with amastatin (1.25 mg/kg), an aminopeptidase inhibitor given simultaneously to avoid peptide degradation; all treatments were given through intraperitoneal injection (i.p.) 10 min before reperfusion. Control groups: LSIGRL (2.5 mg/kg, i.p.) a SLIGRL negative control peptide (Peptides international Inc.) was given; PAR2 activation group: SLIGRL (2.5 mg/kg, i.p., EC_{50}=5 μmol/l) was given 10 min before reperfusion; RP67580 pretreatment group: RP67580 (2 mg/kg, i.p.), a NK1 receptor antagonist was given 5 min before SLIGRL; CGRP8-37 pretreatment group: CGRP8-37, a CGRP receptor antagonist (1 mg/kg, i.p., IC_{50}=4.9 nmol/l) was given 5 min before SLIGRL and then CGRP8-37 was continually intravenous injection at 2 nmol/kg/min. The dosage of was determined by our pilot study and based on previous report (14).

Evaluation of myocardial infarct size. Infarct size was measured according to the method as described (13). Briefly, after 3 h of reperfusion, the LAD was occluded with a suture at the same site of the initial ligation. To demarcate the ischemic area at risk, Evans blue dye (1%) was perfused into the aorta. Then hearts were excised and sliced into five cross sections below the ligature. The heart sections were then incubated with a 1% triphenyltetrazolium chloride (TTC) for 15 min at 37˚C. Once the color was established, the slices were fixed in 10% formalin for 24 h and weighed. Both sides of each slice were quantified by Image J. The infarcted area to risk area ratio (% infarct size) was calculated and multiplied by the weight of the slice.

Measurement of SP and CGRP (15). The blood samples were collected at 10 min after SLIGRL treatment. Commercially available CGRP and SP radioimmunoassay kits (Peninsula Laboratories Inc.) were used to determine the concentrations of CGRP and SP. The plasma samples were purified by the supplier recommended methods.

Langendorff heart preparation and measurements of cardiac function (13,15). Mice (12-week-old) were heparinized (500 U/kg i.p.) and anesthetized with pentobarbital sodium (50 mg/kg i.p.). Isolated hearts from $TRPV1^{-/-}$ and WT mice were perfused at 37˚C and 80 mmHg with Krebs-Henseleit buffer (118 mmol/l NaCl, 4.7 mmol/l KCl, 1.2 mmol/l $MgSO_4$, 1.2 mmol/l KH_2PO_4, 2.5 mmol/l $CaCl_2$, 25 mmol/l $NaHCO_3$, 0.5 mmol/l Na-EDTA, and 11 mmol/l glucose, saturated with 95% O_2-5% CO_2, pH 7.4) with Langendorff apparatus. A water-filled balloon was inserted into the left ventricle and adjusted to a left ventricular end-diastolic pressure (LVEDP) of 5-8 mmHg. The distal end of the catheter was connected to a Digi-Med Heart Performance Analyzer via a pressure transducer. Coronary flow (CF) was continuously measured using an ultrasonic flow probe. Hearts were paced at 400 bpm except during sustained global ischemia, and pacing was reinitiated 3 min after reperfusion. Left ventricular developed pressure (LVDP), left ventricular peak positive dP/dt (+dP/dt) during isovolumic contraction were used as indices of left ventricular (LV) systolic function; LVEDP were used as indices of LV diastolic function.

Experimental protocols ex vivo. All hearts were allowed to stabilize for 25 min, then perfused at 1% of the coronary flow rate with LSIGRL control (10^{-7} M), vehicle control (DMSO), or SLIGRL (10^{-7} M). For the groups of SLIGRL plus inhibitors, LOX inhibitor nordihydroguaiaretic acid (NDGA, $5x10^{-6}$ M), 12-LOX inhibitor baicalein ($1x10^{-5}$ M), COX inhibitor indomethacin ($1x10^{-5}$ M) (16), CYP450 inhibitor miconazole ($1x10^{-6}$ M), and selective CYP450 epoxygenase inhibitor *N*-methylsulphonyl-6-(2-proparglyloxy-phenyl) hexanamide (PPOH, $2x10^{-5}$ M) (17) were added into the perfusate 5 min before adding SLIGRL and continued for additional 5 min after SLIGRL perfusion. Hearts were subsequently subjected to 35 min of no-flow normothermic global ischemia followed by 40 min of reperfusion.

Statistical analysis. All values are expressed as the mean ± standard error of the mean. Comparisons among groups measured at the end of the I/R experiments in the bar charts of each figure and in SP, CGRP release and infarct size

experiments were performed by one-way analysis of variance followed by the Tukey-Kramer multiple comparison test. To compare two groups in infarct size decrease (%), a t-test was used. The results were considered statistically significant at P<0.05.

Results

Protective effects of SLIGRL on myocardial I/R injury. In vivo myocardial I/R experiments showed that SLIGRL significantly reduced infarct size in both WT and TRPV1$^{-/-}$ mice (both P<0.05, Fig. 1A), when compared to LSIGRL-treated control groups. More importantly, the reduction percentage of infarct size when compared to LSIGRL-treated control group was greater in WT than that in TRPV1$^{-/-}$ mice (P<0.05; Fig. 1B).

The release of SP and CGRP. Compared to LSIGRL-treated groups, SLIGRL significantly increased the plasma levels of both CGRP and SP in WT mice (both P<0.05) but not in TRPV1$^{-/-}$ mice (Fig. 2).

Blockade of the CGRP and SP receptors on SLIGRL-induced cardioprotection. Pretreatment with CGRP8-37 or RP67580 alone did not have a significant effect on SLIGRL-induced reduction of infarct size in both WT and TRPV1$^{-/-}$ mice (Fig. 3). Interestingly, the protective effects of SLIGRL were abolished by combined treatment with CGRP8-37 and RP67580 in WT mice but not in TRVR1$^{-/-}$ mice (Fig. 3).

Effects of COX inhibition on SLIGRL-induced cardioprotection. The improvement of hemodynamic parameters after treated with SLIGRL were reported in our previous publication (18). The hemodynamic parameters were impaired in TRPV1$^{-/-}$ mice when compared to WT mice in *ex vivo* myocardial I/R model (all P<0.05, Table I). Inhibition of COX with indomethacin did not affect the hemodynamics of both WT and TRPV1$^{-/-}$ hearts treated with SLIGRL (Table I).

Effects of LOX inhibition on SLIGRL-induced cardioprotection. The cardioprotective effects of SLIGRL were attenuated by inhibition of either non-specific LOX inhibitor NDGA or 12-LOX inhibitor baicalein in WT hearts but not in TRVR1$^{-/-}$ hearts (Table I).

Effects of CYP450 inhibition on SLIGRL-induced cardioprotection. The cardioprotective effects of SLIGRL were significantly attenuated by inhibition of CYP450 with miconazole in WT hearts expressed as increased LVED and decreased CF, LVDP, and +dP/dt, as well as in TRPV1$^{-/-}$ hearts expressed as increased LVED and decreased +dP/dt (all P<0.05, Table I). However, selective inhibition of CYP450 epoxygenase with MS-PPOH did not affect SLIGRL-induced protective effects in WT and TRPV1$^{-/-}$ hearts (Table I).

Discussion

A possible link between PAR2 and TRPV1 in sensory neurons has been suggested in the literatures (19). Accumulating evidence suggests that PAR2 activation exerts a cardioprotective effect during myocardial I/R and produces potent

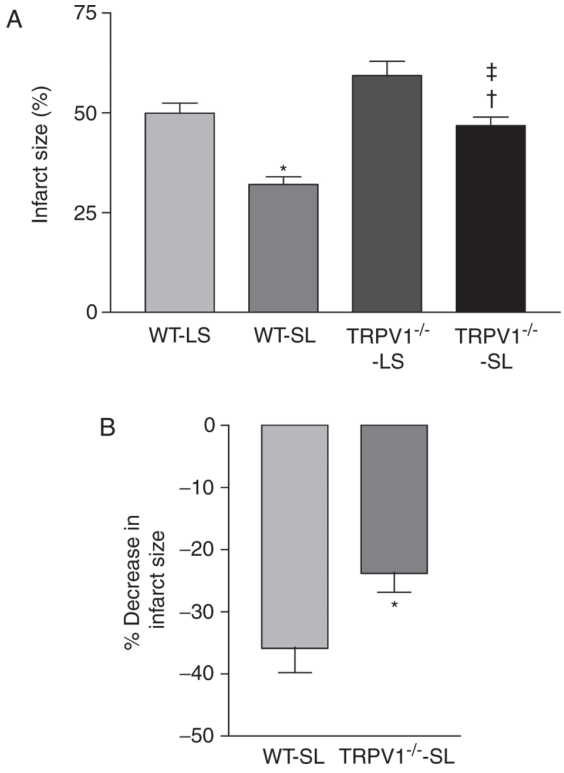

Figure 1. SL decreases infarct size in an *in vivo* myocardial ischemia/reperfusion model. 12-week-old male mice were subjected to 45 min ischemia and 180 min reperfusion and were treated with SL or LS 10 min before reperfusion. Myocardial infarcts were assessed by Evans blue and TTC staining. (A) Infarct size is expressed as a percentage of the area at risk. (B) Decreases in infarct size (%) after treatment with SL were calculated using LS as the control. Values are presented as the mean ± standard error of the mean. n=5-6. *P<0.05 vs. WT-LS; †P<0.05 vs. TRPV1$^{-/-}$-LS; ‡P<0.05 vs. WT-SL. WT, wild-type; TRPV1, transient receptor potential vanilloid receptor 1; SL, SLIGRL; LS, LSIGRL.

dose-dependent coronary vasodilatation in NO-dependent or -independent manner (7,9). It is postulated that PAR2 induces vasodilatation via releasing lipoxygenase-derived eicosanoid and activating sensory C-fibers along the coronary arteries (7). Activation of PAR2 led to sensitization or activation of TRPV1 and increased CGRP and SP release (3), and these neuropeptides may induce coronary vasodilatation, (20) which are expected to protect heart from I/R injury. Pretreatment with capsaicin, leading to CGRP and SP release, significantly improved the recovery of hemodynamics after I/R injury (21). Our previous study has also shown that treatment with exogenous CGRP and SP before ischemia protected heart from I/R injury in both WT and TRPV1$^{-/-}$ hearts (22). In addition, PAR2 agonists-induced vasodilatation was profoundly attenuated by the TRPV1 selective antagonist capsazepine (7). However, capsazepine inhibits not only TRPV1 but also mitochondrial function to induce cellular apoptosis and necrosis via non-receptor mediated mechanisms (23). To further examine whether TRPV1 is involved in PAR2-mediated cardioprotection during I/R injury *in vivo*, TRPV1$^{-/-}$ and WT mice were used, and we demonstrated that WT mice showed greater improvement of heart function and larger reduction of infarct size after treated with SLIGRL when compared with TRPV1$^{-/-}$ mice, suggesting that TRPV1 plays a critical role in PAR2-induced cardioprotection.

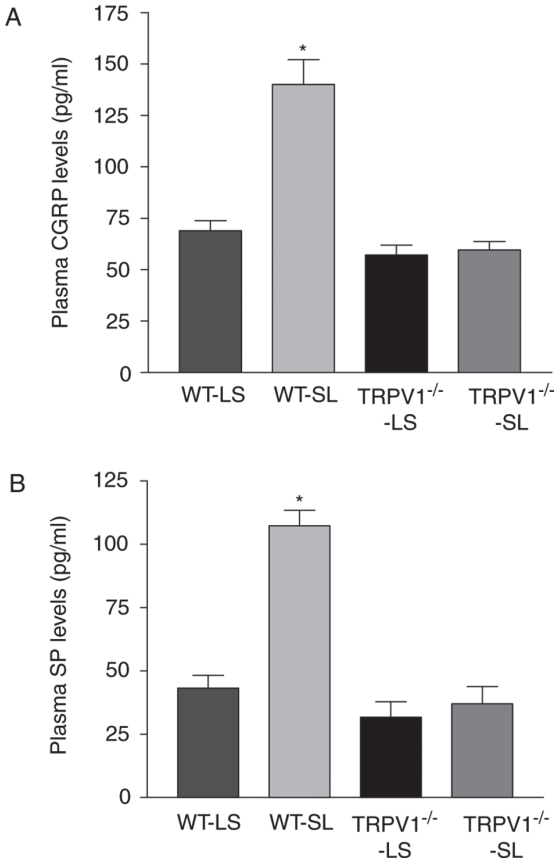

Figure 2. Plasma levels of CGRP and SP after treatment with SL. Plasma levels of (A) CGRP and (B) SP were measured in WT and TRPV1$^{-/-}$ mice subjected to myocardial ischemia/reperfusion injury and treated with SL or LS. Values are presented as the mean ± standard error of the mean. n=5-6. *P<0.05 vs. WT-LS. WT, wild-type; TRPV1, transient receptor potential vanilloid receptor 1; CGRP, calcitonin gene-related peptide; SP, substance P; SL, SLIGRL; LS, LSIGRL.

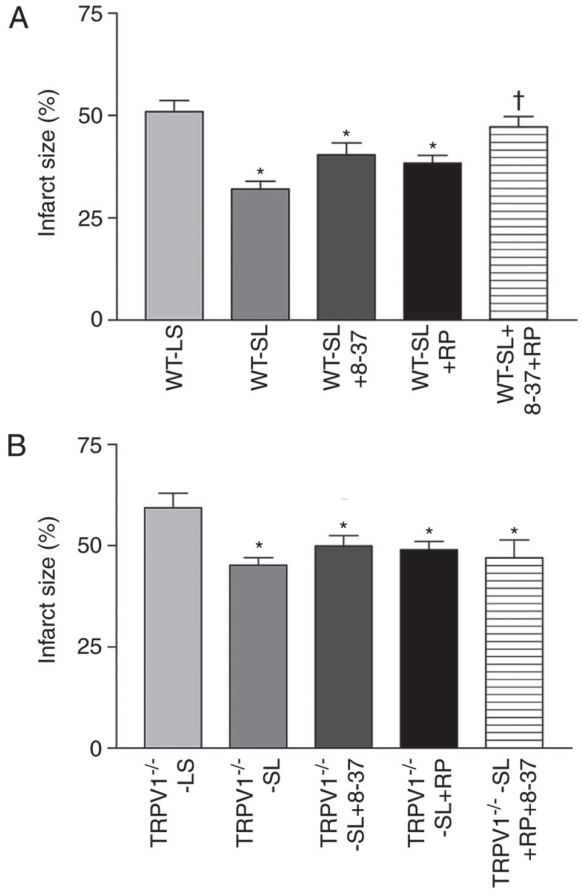

Figure 3. Effects of the SP and CGRP receptor antagonists on SL-induced cardioprotection. WT and TRPV1$^{-/-}$ mice were subjected to the same SL treatment and myocardial ischemia/reperfusion protocol as described in Fig. 1. (A) WT mice were treated with SL or LS 10 min before reperfusion in the presence or absence of antagonists 8-37 and/or RP. *P<0.05 vs. WT-LS; †P<0.05 vs. WT-SL. (B) TRPV1$^{-/-}$ mice were treated with SL or LS 10 min before reperfusion in the presence or absence of antagonists 8-37 and/or RP. Values are presented as the mean ± standard error of the mean. n=5-6. *P<0.05 vs. TRPV1$^{-/-}$-LS. WT, wild-type; TRPV1, transient receptor potential vanilloid receptor 1; SP, substance P; CGRP, calcitonin gene-related peptide; SL, SLIGRL; LS, LSIGRL; 8-37, CGRP8-37; RP, RP67580.

Reperfusion of ischemic tissue results in increased production of oxidants and radicals that can initiate inflammatory response at reflow. It was reported that ROS participate in mediating TRPV1-dependent and neuropeptide-dependent vasodilatation (20). Moreover, SP and CGRP are not only related to vasodilatation but also to inflammatory response. There is increasing evidence that both TRPV1 and PAR2 play an important role in inflammation acting as either a pro-inflammatory or anti-inflammatory mediator, which depends on the activated cells and the type of inflammation (2,24-26). Those pro-inflammatory and anti-inflammatory effects involve neurogenic mechanisms, because it is prevented by ablation of sensory nerves or using TRPV1$^{-/-}$ mice (25,26). CGRP is one of the most potent vasodilators identified to date, and has been shown particularly sensitive to coronary vasculature (27). In addition to vasodilation, CGRP exerts positive chronotropic and inotropic effects (28). CGRP also regulates inflammatory processes, including inhibiting NF-κB activation and lowering ROS, interleukin-2, and monocyte chemoattractant protein-1, suggesting that CGRP-induced cardioprotection might also be mediated by its anti-inflammatory effects (29,30). SP can act on inflammatory cell *in vivo* and has pro-inflammatory effects (31). Studies showed that pretreatment with a tachykinin NK_1 receptor antagonist markedly inhibited I/R injury (32,33). However, in intestinal I/R model, tissue damage was significantly reduced by SLIGRL and this effect was abolished by pretreatment with RP67580, indicating that NK_1 receptor mediates PAR2-induced protective effects. In the present study, SLIGRL induced higher plasma levels of SP and CGRP in WT than TRPV1$^{-/-}$ mice. The protective effect of SLIGRL on myocardial I/R injury was not able to be abolished by pretreatment with CGRP8-37 or RP67580 alone. However, pretreatment with CGRP8-37 and RP67580 together can significantly increase infarct size in SLIGRL-treated WT mice but not in TRPV1$^{-/-}$ mice. The results indicate that SP and CGRP may have a cooperative effect on cardioprotection, and PAR2 activator-induced myocardial protection is, at least partially, mediated by SP and CGRP. PAR2 activator sensitizes not only TRPV1 but also TRPV4, the latter is also co-expressed with PAR2 in DRG (34). Thus, the TRPV4 channel may also contribute to the cardioprotective effect of PAR2 activation. This is probably a mechanism through which the infarct size was slightly reduced by PAR2 activation in TRPV1$^{-/-}$ mice. Interestingly, the protective effects of PAR2 on TRPV1$^{-/-}$ mice

Table I. Effects of cyclooxygenase, lipoxygenase and epoxygenase inhibitors on SL-induced cardiac protection.

Groups	CF, %	+dP/dt, mmHg/s	LVEDP, mmHg	LVDP, mmHg
WT-SL	81.8±2.2	3736.8±137.6	10.4±1.5	67.0±2.4
TRPV1$^{-/-}$ -SL	57.3±5.0[a]	2740.6±185.4[a]	19.2±1.9[a]	52.8±3.4[a]
WT-SL+Indo	71.4±4.9	3479.2±145.4	13.0±1.7	59.5±3.9
TRPV1$^{-/-}$-SL+Indo	66.8±3.9	2732.4±271.9	18.8±2.1	51.7±2.9
WT-SL+NDGA	58.9±5.2[a]	2961.7±202.0[a]	19.0±1.7[a]	54.4±3.1[a]
TRPV1$^{-/-}$-SL+NDGA	53.9±4.4[a]	2347.8±184.8[a]	24.5±3.3[a]	47.1±2.3[a]
WT-SL+Bai	55.0±9.6[a]	2680.6±180.5[a]	20.4±2.7[a]	51.0±1.8[a]
TRPV1$^{-/-}$-SL+Bai	52.5±2.9[a]	2431.0±214.3[a]	21.0±1.7[a]	46.1±3.5[a]
WT-SL+Mico	57.1±3.3[a]	2468.8±159.8[a]	21.6±0.7[a]	44.9±4.0[a]
TRPV1$^{-/-}$-SL+Mico	44.2±8.7[a]	2113.0±255.9[a,b]	26.2±2.3[a,b]	40.6±4.4[a]
WT-SL+PPOH	70.5±5.1	3063.8±234.7	18.1±2.1	61.4±4.3
TRPV1$^{-/-}$-SL+PPOH	56.5±7.1[a]	2696.2±205.9[a]	22.4±2.6[a]	52.3±3.4[a]

[a]P<0.05 vs. WT-SL; [b]P<0.05 vs. TRPV1$^{-/-}$-SL. n=5-6/group. SL, SLIGRL (a PAR2 activating peptide); Indo, indomethacin (a COX inhibitor); NDGA, nordihydroguaiaretic acid (a lipoxygenase inhibitor); Bai, baicalein (a 12-LOX inhibitor); Mico, miconazole (a CYP450 inhibitor); PPOH, N-methylsulphonyl-6-(2-proparglyloxy-phenyl) hexanamide (a CYP450 epoxygenase inhibitor); CF, coronary flow; +dP/dt, left ventricular peak positive dP/dt; LVEDP, left ventricular end-diastolic pressure; LVDP, left ventricular developed pressure. N=5-6 per group.

was not attenuated by combination treatment with CGRP8-37 and RP67580, suggesting a different mechanism between WT and TRPV1$^{-/-}$ mice. One of the possible reasons could be that TRPV4 might compensate the function of TRPV1 in the knockout mice.

Evidence demonstrates that various metabolic products of AA such as LOX products and PGs can activate or sensitize TRPV1 (11,35). Studies showed that PAR2 activators stimulated the release of PGs. PGs can enhance release of SP and CGRP (35). Moreover, PGI_2 protects the heart from myocardial I/R injury and decreases oxidative stress (36). However, in present study, COX inhibition with indomethacin did not affect SLIGRL-induced protection in both WT and TRPV1$^{-/-}$ hearts. It is consistent with other studies showing that perfusion with indomethacin before ischemia did not significantly alter LVDP recovery (37). These results indicate that metabolic products of COX pathway might not be related to PAR2-induced myocardial protection.

Products of LOX have been implicated in mediating inflammatory responses and can function as potent vasodilator, particularly in the setting of oxidative stress. LOX products, such as hydroperoxyeicosatetraenoic acids, hydroxyeicosatetraenoic acids, and leukotriene B_4 directly activate TRPV1 in sensory neurons (11). Moreover, 12-LOX-derived eicosanoids protect against myocardial I/R injury via activation of neuronal TRPV1 (38). The present study showed that the protective effects of SLIGRL were suppressed in the presence of NDGA and baicalein in WT mice but there were no significant differences in TRVR1$^{-/-}$ hearts. These results are in line with previous study demonstrated that baicalein suppressed SLIGRL-induced coronary vasodilatation (7), indicating that the 12-LOX/TRPV1 pathway is involved in PAR2 activation-induced myocardial protection.

AA can also be metabolized by the CYP450 pathway. A group of products of CYP450 metabolites of the epoxidation, including epoxyeicosatrienoic acid (EETs), have protective effects in I/R injury in the heart and vasculature, and EETs possess anti-inflammatory properties (39). EETs induce an endothelium-independent relaxation of coronary arteries and cause vasodilation when NO synthesis is impaired

40). In this study, the protective effects of SLIGRL were suppressed in the presence of miconazole not only in WT but also in TRVR1$^{-/-}$ hearts. The effects of miconazole in hearts may be due to inhibition of either CYP450 or adenylyl cyclase.

This study provides direct evidence that the TRPV1 receptor plays a role in mediating PAR2 activator SLIGRL-induced cardiac protection via at least in part, increased endogenous CGRP and SP release. The 12-LOX/TRPV1 pathway is involved in PAR2 activator-induced myocardial protection.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

BZ performed experiments, contributed to the acquisition, analysis and interpretation of the data, and drafted the manuscript. SM contributed to the interpretation of the data and revision of the manuscript. DHW was responsible for the

conception and design of the experiments, interpretation of the data, and revision of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experimental procedures involving animals were approved by the Michigan State University Animal Care and Use Committee (East Lansing, USA) and conform to The National Institutes of Health Guidelines (Bethesda, MD, USA).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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