12-Lipoxygenase-derived eicosanoids protect against myocardial ischemia/reperfusion injury *via* activation of neuronal TRPV1

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ABSTRACT **Recent evidence implicates the neuronal transient receptor potential vanilloid receptor 1 (TRPV1), expressed on sensory C-fibers, as playing an important endogenous protective role in limiting the damaging effects of myocardial I/R injury. In neurons the 12-lipoxygenase (12-LOX) arachidonic acid (AA) metabolite, 12(S)-HpETE, has been proposed as the endogenous ligand for TRPV1. However, whether 12(S)-HpETE underlies TRPV1 channel activation during I/R is unknown. Treatment of isolated Langendorff rat hearts with a 12-LOX/AA cocktail significantly attenuated I/R injury (~40% inhibition of infarct size), an effect reversed by the 12-LOX inhibitor baicalein or after chemical desensitization of local sensory C-fiber afferents using capsaicin. Both 12(S)-HpETE and AA caused dose-dependent coronary vasodilatation (~EC_{50}s of 6×10^{-19} and 1×10^{-7}, respectively) that was profoundly suppressed by the TRPV1 antagonist capsazepine, in hearts of TRPV1 knockout mice compared with wild-type mice, or by treatment with a CGRP antagonist. In addition, I/R itself stimulates up-regulation of TRPV1 expression in both the cell bodies located within the dorsal root ganglia and locally within the myocardium. Together, our data identify a novel 12-LOX/AA/TRPV1 pathway activated and up-regulated during I/R injury, providing an endogenous damage-limiting mechanism whose targeting may prove useful in treating myocardial infarction.—Sexton, A., McDonald, M., Cayla, C., Thiemermann, C., Ahluwalia, A. 12-Lipoxygenase-derived eicosanoids protect against myocardial ischemia/reperfusion injury *via* activation of neuronal TRPV1. *FASEB J.* 21, 2695–2703 (2007)**

Key Words: coronary vasodilation · channel activation · capsaicin receptor

Relative to its metabolic requirements, heart tissue is one of the most poorly perfused in the body, and ischemia resulting from compromised coronary flow can have serious detrimental effects. Up to 15 min of myocardial ischemia causes damage to cardiomyocytes, which may be reversed on reperfusion (1); beyond this time, however, more serious damage occurs that may ultimately be irreversible. While reperfusion of ischemic tissue is therefore fundamental to limiting the extent of tissue damage, it has become apparent that reperfusion causes damage in addition to that caused by the ischemia insult *per se*, a phenomenon referred to as ischemia reperfusion (I/R) injury.

Clinically, I/R injury may be manifested by reperfusion arrhythmias, myocardial stunning, cardiomyocyte death, and endothelial and microvascular dysfunction, including the "no-reflow" phenomenon (2). Reperfusion may occur spontaneously, but in a clinical setting myocardial I/R injury is also associated with commonly used procedures for treatment of acute coronary occlusion such as thrombolysis, percutaneous coronary angioplasty, and coronary bypass surgery. With acute coronary occlusion currently standing as the principal cause of morbidity and mortality in the Western world, and predicted by the World Health Organization to be the major cause of death in the world in general by 2020 (3), there is considerable interest in elucidating the mechanisms underlying I/R injury in the hope that novel therapeutic targets may be identified that protect against, or at least minimize the extent of, tissue damage.

Originally identified as the "capsaicin receptor," the molecular target for the pungent component of "hot" chili peppers (4), transient receptor potential vanilloid receptor 1 (TRPV1), is now known to be activated by a variety of ligands and its role to extend beyond that played in nociception, for example, in processes as diverse as hair follicle cycling (5). Indeed, in light of evidence that capsaicin can produce a hypotensive effect in spontaneously hypertensive rats, it has been suggested that TRPV1 may represent a novel target for treatment of hypertension (6). Furthermore, an increasing body of evidence has implicated a role for TRPV1 in cardioprotection; a concept recently confirmed in studies using TRPV1 knockout mice (7). In addition, several lines of evidence support the concept

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that sensory C-fiber activation, the primary site of TRPV1 expression, and the consequent release of sensory neuropeptides, particularly substance P (SP) and calcitonin gene-related peptide (CGRP), underlie this cardioprotective action of TRPV1 (7–9).

Among the growing list of activators of TRPV1 are lipoxygenase (LOX) metabolites, including 12(S)-hydroperoxyeicosatetraenoic acid [12(S)-HpETE] and its more stable metabolite, 12-hydroxyeicosatetraenoic acid [12(S)-HETE] (10, 11). The 12-LOX pathway of arachidonic acid (AA) metabolism is known to be stimulated within myocardium *via* hypoxia or ischemia, and there is now a substantial body of evidence demonstrating that eicosanoids derived *via* the 12-LOX pathway are protective against the damaging effects of myocardial I/R injury.

We therefore investigated the hypothesis that activation of the 12-LOX pathway of AA metabolism by myocardial ischemia leads to generation of 12(S)-HpETE, which activates TRPV1 located on sensory C-fibers, which in turn leads to release of vasoactive peptides such as SP and CGRP, which offer protection against I/R injury.

MATERIALS AND METHODS

Animal preparation

Experiments were conducted in accordance with the Animals (Scientific Procedures) Act, UK 1986, with full ethical permission from The Queen Mary, University of London Animal Ethical Review Committee. Male Wistar rats (250–350 g; Charles River, UK), C57BL6 (WT), or TRPV1 knockout mice (TRPV1 KO, 18–22 g, Jackson Laboratories, Bar Harbor, ME, USA) were heparinized (heparin sodium, 1000 IU/kg, i.p.) and anesthetized (pentobarbitone sodium, 120 mg/kg, i.p.). Hearts were then rapidly excised and submerged in ice-cold Krebs solution [mM: NaCl (118.5), KCl (4.7), Mg_2SO_4.7H2O (1.2), KH_2SO_4 (2), sodium bicarbonate (25.0), $CaCl_2$ (1.7), and glucose (12)], where they were left until they had stopped beating. For murine hearts, Krebs buffer also contained 2 mM sodium pyruvate.

Measurement of coronary hemodynamics and cardiac function in rat hearts

Hearts were rapidly excised, then cannulated *via* the aorta and perfused in the Langendorff mode as described previously (12). Briefly, hearts were retrogradely perfused with Krebs solution maintained at 37°C under constant flow (10 ml/min) using a calibrated roller pump (Gilson Minipuls 2). The reservoir of Krebs solution was continuously bubbled with 95% O_2/5% CO_2 and air temperature was maintained by means of a heated (37°C) water jacket. Coronary perfusion pressure (CPP) was measured using an inline pressure transducer (Isotec, Hugo Sachs Elektronik-Harvard Apparatus GmbH, Germany). Left ventricular developed pressure (LVDP=left ventricular systolic pressure−left ventricular end-diastolic pressure) was measured by means of an inline pressure transducer (Isotec, Hugo Sachs Elektronik-Harvard Apparatus GmbH, Germany) attached to a fluid-filled latex balloon inserted into the left ventricle, and inflated to obtain an end diastolic pressure of 8–10 mmHg and an initial LVDP of 80 mmHg minimum. Transducers were connected to ADInstruments PowerLab/4SP equipment (Colorado Springs, CO, USA), and measurements were recorded using ADInstruments PowerLab® Chart 4 for Windows software.

Having established a stable CPP (20–30 min after cannulation), endothelium-dependent and -independent function were tested by administering a bolus dose of the vasodilators bradykinin (BK, 30 pmol) and sodium nitroprusside (SNP, 1 nmol), respectively, *via* the aorta. Hearts were excluded if responses to BK or SNP were <10 mmHg or if postequilibration CPP was <60 mmHg. Drugs were either administered by bolus injection in a volume of 10 μl *via* a side arm in the perfusion line or infused *via* an infusion port at a rate of 0.1 ml/min.

Mechanism of 12(S)-HpETE and arachidonic acid-induced vasodilator activity

Since our previous evidence suggested that a 12-LOX product—namely, 12(S)-HpETE—might be activating sensory C-fibers and, in the coronary vasculature, C-fiber activation is associated with vasodilation, we initially sought to investigate the possibility that 12-LOX metabolites might activate C-fibers by measuring changes in coronary vasodilator function. 12(S)-HpETE (10^{-20}–10^{-15} mol) dose-response curves were constructed. Hearts were then treated with either a desensitizing dose of capsaicin (1 μM for 20 min, 30 min washout) (13) or the selective TRPV1 antagonist capsazepine (3 μM, 20 min) before repeating the dose-response curve to 12(S)-HpETE. Control hearts were treated with vehicle alone. We found considerable variability in these responses, an effect likely due to the instability of 12(S)-HpETE in solution, since it is highly prone to oxidation. To overcome these difficulties, we perfused the Langendorff preparation with12-LOX (2 μg/ml), adapting a protocol devised by Gryglewski and Vane (14); then AA (10^{-10}–10^{-6} mol) dose-response curves were constructed in the absence and presence of the nonselective LOX inhibitor nordihydroguaiaretic acid (NDGA; 10 μM, 30 min) (10), the selective 12-LOX inhibitor baicalein (10 μM, 30 min) (15), a desensitizing dose of capsaicin (as above) capsazepine (3 μM, 20 min) (16), the selective NK1 receptor antagonist SR140333 (1 μM, 15 min) (17), the selective $CGRP_1$ receptor antagonist $\alpha CGRP_{8-37}$ (0.3 μM, 20 min) (18), or a combination of SR140333 and $\alpha CGRP_{8-37}$. All experiments were conducted in the presence of the cyclooxygenase inhibitor indomethacin (5 μM) and exogenous 12-LOX (2 μg/ml) (14).

Effects of 12-LOX and AA on I/R injury

After equilibration, hearts were treated with 12-LOX (2 μg/ml) and AA (30 μM) or vehicle control for 30 min in the absence and presence of baicalein (10 μM, 15 min pretreatment). To determine the role of sensory C-fibers in some experiments, hearts were taken from animals that had been treated with capsaicin *in vivo* (50 mg/kg, 2 mg/ml, s.c.) 3 days earlier (19, 20). This treatment with capsaicin effectively desensitizes C-fibers *in vivo*, resulting in a >95% depletion of neuropeptide content. Hearts were then subjected to global ischemia (25 min), followed by reperfusion (120 min), and infarct size was determined as described previously (21). Experiments were conducted in the presence of indomethacin (5 μM).

Measurement of coronary hemodynamics in TRPV1 KO mice

Hearts of male TRPV1 KO mice were mounted in the Langendorff mode as above and perfused with Krebs solution

at a constant flow rate of 2 ml/min. Basal CPP was 110.4 ± 5.0 (n=7) and 97.6 ± 14.2 (n=5) for WT and TRPV1 KO hearts, respectively. Dose-response curves to AA (10^{-10}–10^{-6} mol) were constructed.

Effects of I/R on TRPV1 expression

In vitro hearts were perfused in the Langendorff mode and subjected to 25 min ischemia/120 min reperfusion. Control hearts were perfused for 145 min. At the end of (re)perfusion, left ventricles were snap frozen in liquid nitrogen and stored at −80°C until use. *In vivo* male Wistar rats were anesthetized (sodium thiopentone, 120 mg/kg i.p.) and myocardial I/R injury was induced by occlusion of the left anterior descending coronary artery for 25 min, followed by 120 min reperfusion. Control animals underwent a sham operation. After reperfusion, hearts were rapidly excised and left ventricles were bisected longitudinally. In addition, dorsal root ganglia (DRG, the location of the cell bodies of cardiac sensory neurons) were removed from each side of the spinal cord, from the cervical enlargement to the fifth or sixth thoracic spinal nerves. All tissue samples were snap frozen in liquid nitrogen and stored at −80°C until use.

Western blot

Samples were homogenized in a standard lysis buffer, supernatants were collected, and protein concentration was determined by Bradford assay. Twenty micrograms of each sample was subjected to SDS-PAGE (7%), followed by electrotransfer onto a nitrocellulose membrane. Protein was detected using rabbit anti-VR1 (TRPV1, 1:2000) N terminus antibody (Neuromics, Edina, MN, USA) or anti-α-actin and goat anti-rabbit HRP-linked antibody (1:2000, Dako, Glostrup, Denmark). Detection of labeled antigen was performed using an enhanced chemiluminescence detection system and autoradiographic film. Densitometric analysis was conducted using Scion Image® software (National Institutes of Health, Bethesda, MD, USA) and TRPV1 protein levels were expressed relative to levels of α-actin.

Quantitative real-time PCR analysis

RNA was extracted from left ventricles using TRIzol® (Invitrogen™ Life Technologies, Gaithersburg, MD, USA) or, for DRG samples, using a Nucleospin® RNA II isolation kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. Total RNA in each ventricle sample was determined using spectrophotometric analysis; for DRG samples, a Ribogreen® RNA Quantitation Kit (Molecular Probes, Carlsbad, CA, USA) was used according to the manufacturer's instructions. cDNA was prepared, then subjected to real-time PCR. Results were analyzed using the ABI PRISM® 7900HT software package SDS 2.1 (Advanced Biotechnologies Ltd., Surrey, UK) and relative changes in gene expression (TRPV1, TRPV2, and TRPV4) were determined using the $2^{-\Delta\Delta C}{}_{T}$ method. mRNA levels were normalized for each sample with respect to the corresponding β-actin levels.

Primers used were:

TRPV1 forward primer (5′-TCATGGGTGAGACCGTCAACAAG-3′) and reverse primer (5′-TCCTTGCCGTCAGGAGTGAA-3′),

TRPV2 forward primer (5′-ACTCGGTGCGGGAGATCATC-3′) and reverse primer (5′-ACCAAGTAGCAGGCGAAGTT-3′),

TRPV4 forward primer (5′-CGCCTGACTGATGAGGAGTT-3′) and reverse primer (5′-TGCCGTCTGCCCTCGGTAGTA-3′),

β-Actin forward primer (5′-GAAATCGTGCGTGACATCAAA-3′) and reverse primer (5′-TGTAGTTTCATGGATGCCACAG-3′).

Reagents and data analysis

BK and $\alpha CGRP_{8-37}$ were obtained from Bachem (St. Helens, UK); AA, 12-LOX, and 12(S)-HpETE were from Cayman Chemicals (Ann Arbor, MI, USA); and SR140333 was a kind gift from Dr. Sandro Giuliani (Menarini Ricerche S.p.A., Florence, Italy). All other drugs were obtained from Sigma (Poole, UK). Indomethacin, L-NAME, SNP, 12-LOX, NDGA, baicalein, and NBT were made up fresh each day. All other drugs were made up as stock solutions and stored at −20°C until needed, except for capsaicin and capsazepine, which were stored at 4°C.

Indomethacin was dissolved in 5% sodium bicarbonate, and baicalein and SR140333 were dissolved in DMSO. Stock solutions of BK, SP, CGRP, and $\alpha CGRP_{8-37}$ were made in distilled water. Capsaicin, capsazepine, 12(S)-HpETE, NDGA, and AA were dissolved in 100% ethanol. All dilutions were then made in saline or Krebs. Final bath concentrations of DMSO and ethanol never exceeded 0.05% and 0.15%, respectively.

Data were expressed as means ± SE, n = number of observations. Differences between groups were examined for statistical significance using 2-way ANOVA or 1-way ANOVA with Bonferroni's multiple comparison test, or unpaired Student's t test, as appropriate. All tests were conducted using GraphPad Prism® Version 4.00 (GraphPad, San Diego, CA, USA).

RESULTS

Mechanism of arachidonic acid/12(S)-HpETE-induced vasodilator activity

AA caused dose-dependent vasodilatation (pEC_{50}= 7.1±1.2, max response=22.4±1.6 mmHg, n=5), which was abolished by treatment with NDGA (P<0.0001, n=5; **Fig. 1*A***) or baicalein (P<0.0001, n=6; Fig. 1*B*). Similarly, 12(S)-HpETE also caused a dose-dependent vasodilation (pEC_{50}=18.2±0.7, max response=10.2±3.2 mmHg, n=6, Fig. 1*B*). The vasodilator responses to AA (**Fig. 2*A***) were blocked by capsazepine (P<0.01, n=6) or sensory C-fiber desensitization (P<0.0001, n=5). Similarly, 12(S)-HpETE-induced vasodilatation (Fig. 2*B*) was abolished by capsazepine (P<0.01, n=6) or sensory C-fiber desensitization (P<0.0001, n=5).

SR140333 significantly attenuated responses to AA, causing a rightward shift (~10-fold) of the dose-response curve (P<0.0001, n=6; **Fig. 3*A***). $\alpha CGRP_{8-37}$ also profoundly attenuated responses to AA (P<0.0001, n=4; Fig. 3*B*), suppressing the maximum response by ~80%. Combined treatment with SR140333 and $\alpha CGRP_{8-37}$ did not result in greater suppression of responses to AA than did infusion of either antagonist alone (data not shown).

AA also caused dose-dependent vasodilatation in perfused WT mouse hearts (pEC_{50}= 8.5±1.4, max response=22.1±8.5 mmHg, n=5) that was significantly (P<0.05) shifted to the right by ~30-fold in hearts of TRPV1 KO mice, although the maximum response was

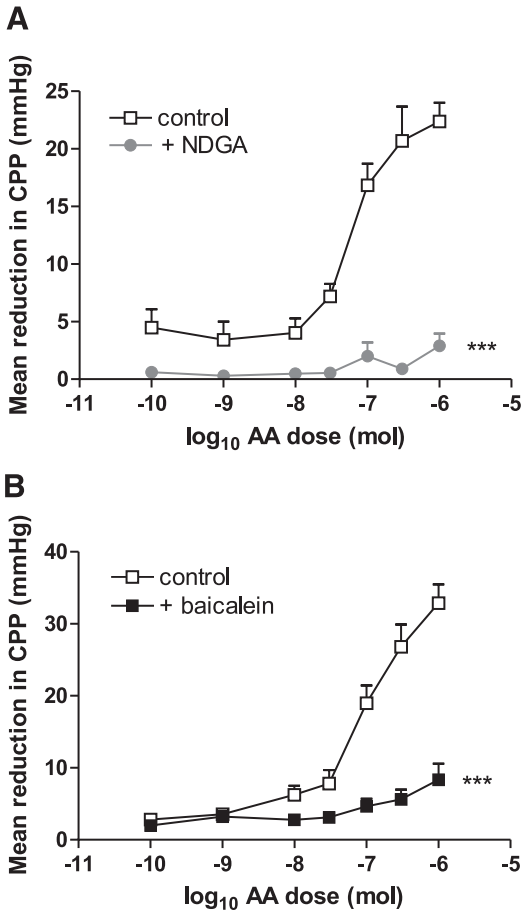

Figure 1. Effect of *A)* nonselective LOX inhibition, using NDGA (10 μM), and *B)* selective 12-LOX inhibition, using baicalein (10 μM), on arachidonic acid (AA) -induced coronary vasodilatation in the isolated rat heart. Vasodilator responses are expressed as a reduction in CPP (mmHg). Each point represents the mean ± SE (n=4–6). Statistical analysis performed using 2-way ANOVA. ***$P < 0.0001$ compared with control.

unaffected (pEC_{50}=7.0±0.4, max response=20.9±6.3 mmHg, n=7).

Effects of 12-LOX and AA on I/R injury

I/R insult produced an infarct size of ~60% in control untreated hearts (**Fig. 4*A***) that was significantly attenuated by 12-LOX/AA treatment prior to ischemia (P<0.01, n=7). This protective effect of 12-LOX/AA was lost in the presence of baicalein or prior C-fiber desensitization. Neither baicalein nor capsaicin treatment alone had any effect on infarct size *per se* (Fig. 4*A*). 12-LOX/AA treatment improved coronary perfusion postischemia as evidenced by a significantly improved postischemic LVDP (P<0.0001, n=7; Fig. 3*B*). As with infarct size, whereas baicalein or C-fiber desensitization had no effect on LVDP *per se*, separately each treatment did block the improvements associated with 12-LOX/AA treatment in the initial stages of reperfusion; however, no significant inhibitory effects were observed at the later stages of reperfusion (Fig. 3*B*). Reperfusion of hearts was associated with a typical increase in CPP, reflecting coronary vasoconstriction, and perfusion with 12-LOX/AA significantly attenuated this increase (Fig. 3*C*). However, unlike the other indices of I/R injury, baicalein treatment or C-fiber desensitization *per se* profoundly and significantly suppressed the postischemic rise in CPP in the absence of 12-LOX/AA: at 150 min reperfusion CPP = 265 ± 22% (n=6), +baicalein CPP = 170 ± 34 (n=6, P<0.05), +capsaicin CPP = 146.7 ± 9.5 (n=4, P<0.05). Addition of 12-LOX/AA in these conditions had no effect *vs.* the appropriate control (data not shown).

Effects of I/R on TRPV1 expression

After an I/R insult *in vitro*, TRPV1 protein expression was significantly lower than that in control sham hearts (P<0.05, n=4, **Fig. 5*A***). In contrast, after an I/R insult *in vivo*, expression of TRPV1 protein was significantly

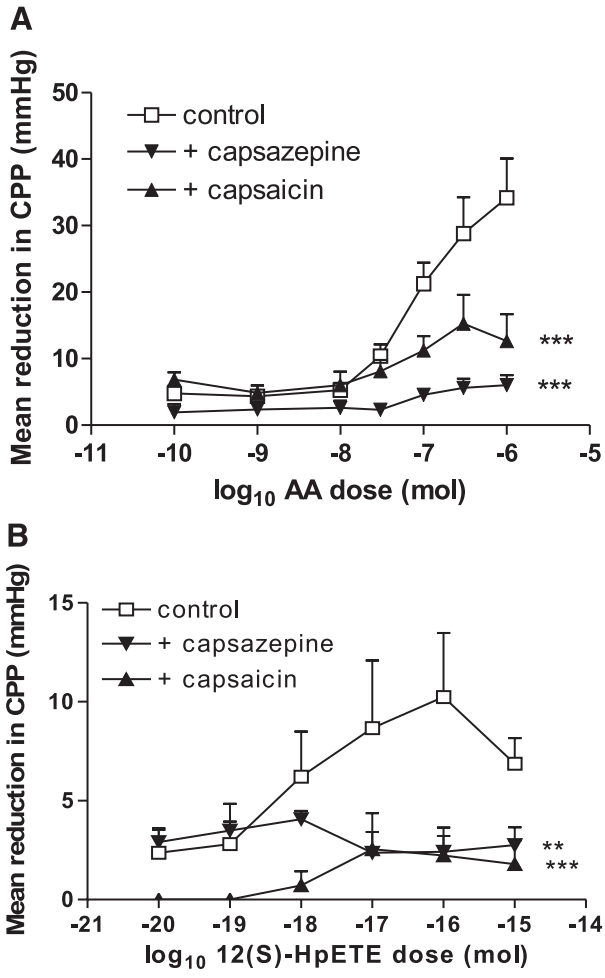

Figure 2. Effect of C-fiber desensitization (capsaicin 1 μM) and TRPV1 blockade (capsazepine 3 μM) on *A)* arachidonic acid (AA) -induced and *B)* 12(S)-HpETE-induced coronary vasodilatation in isolated rat heart. Vasodilator responses are expressed as a reduction in CPP (mmHg). Each point represents the mean ± SE (n=5–7). Statistical analysis performed using 2-way ANOVA. ***$P < 0.0001$ and **$P < 0.01$ compared with control.

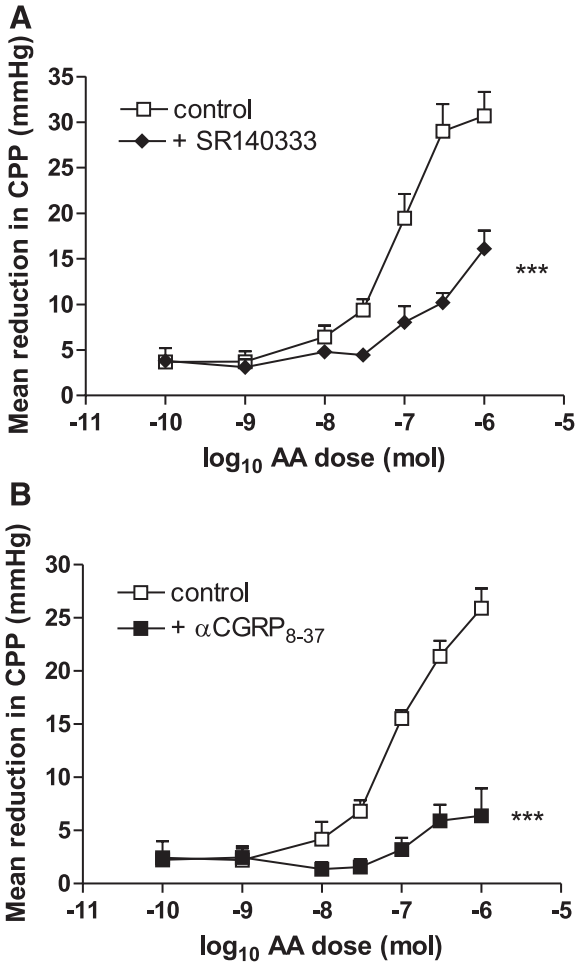

Figure 3. The effect of *A)* NK1 (SR140333 1 μM) and *B)* CGRP1 receptor ($CGRP_{8-37}$ 0.3 μM) blockade on arachidonic acid (AA) -induced coronary vasodilatation in isolated rat heart. Vasodilator responses are expressed as a reduction in CPP (mmHg). Each point represents the mean ± SE (n=4–6). Statistical analysis performed using 2-way ANOVA. ***$P < 0.0001$ compared with control.

elevated compared with control hearts (P<0.05, n=6; Fig. 5*B*). In addition, TRPV1 protein expression was elevated above sham controls in the DRG following I/R (P<0.05, n=6; Fig. 5*C*). Real-time quantitative PCR revealed TRPV1 mRNA expression in all samples. While TRPV1 mRNA expression in hearts subject to an *in vitro* I/R insult was no different from that in sham control (n=5; Fig. 5*D*), differences were evident in samples taken from *in vivo* studies, where TRPV1 mRNA levels following I/R were significantly lower than in sham control hearts (P<0.05, n=7; Fig. 5*E*). However, no difference in TRPV1 mRNA levels was found in DRG removed from these animals (P>0.05, n=6; Fig. 4*F*). There were no significant changes in TRPV2 or TRPV4 expression following ischemia *in vitro* or *in vivo* in ventricle or DRG (data not shown).

DISCUSSION

The 12-LOX pathway of arachidonic acid (AA) metabolism is known to be stimulated within myocardium *via* hypoxia or ischemia, and a substantial body of evidence demonstrates that eicosanoids derived *via* this pathway are protective against the damaging effects of myocardial I/R injury (22–24). Our data suggest that 12-LOX-mediated cardioprotection is mediated in part by the activity of sensory neuropeptides as a consequence of 12(S)-HpETE-induced neuronal TRPV1 activation. In addition our data show that I/R injury *in vivo* results in an increase in TRPV1 synthesis in DRG and transport of TRPV1 to cardiac nerve terminals. It is likely, therefore, that during I/R injury up-regulation/activation of the 12-LOX/AA/TRPV1 pathway represents an endogenous damage-limiting mechanism.

Our previous studies have demonstrated that the 12-LOX metabolite 12(S)-HpETE causes transient dose-dependent coronary vasodilatation (25). We veri-

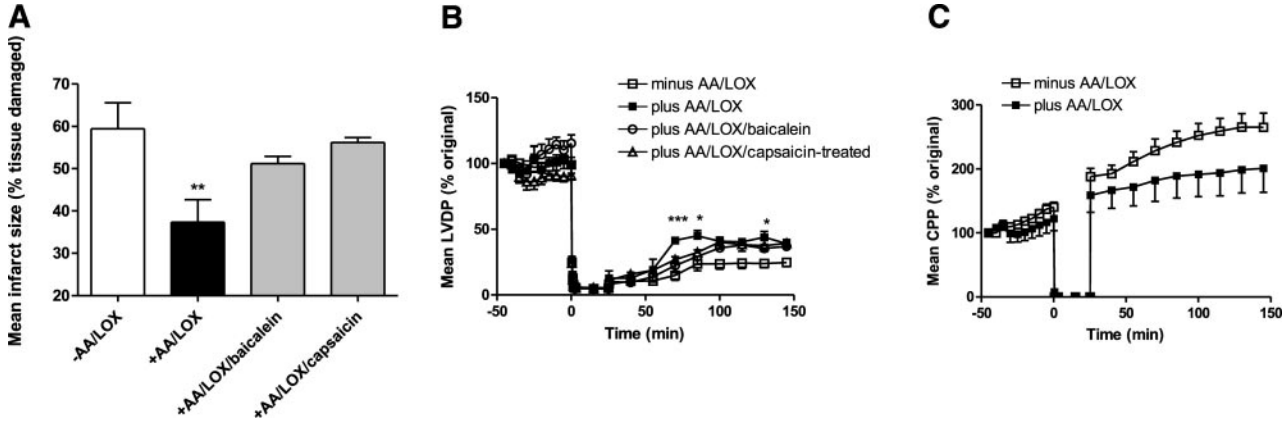

Figure 4. *A)* Infarct size, expressed as percentage wet weight in isolated rat heart subjected to 25 min global ischemia followed by 120 min reperfusion in the presence and absence of 12-LOX (2 μg/ml) and arachidonic acid (AA, 30 μM), and the effect of 12-LOX inhibition and C-fiber desensitization on 12-LOX/AA-mediated effects. Statistical analysis using 1-way ANOVA with Bonferroni's multiple comparison test (n=4–8). *B)* LVDP in isolated rat heart in the presence and absence of 12-LOX (2 μg/ml) and AA (30 μM), and after 12-LOX inhibition and C-fiber desensitization. *C)* Effect of 12-LOX (2 μg/ml) and AA (30 μM) on CPP. Data are means ± SE. Statistical analysis using 2-way ANOVA (n=4–8). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.0001$ compared with control (−AA/LOX).

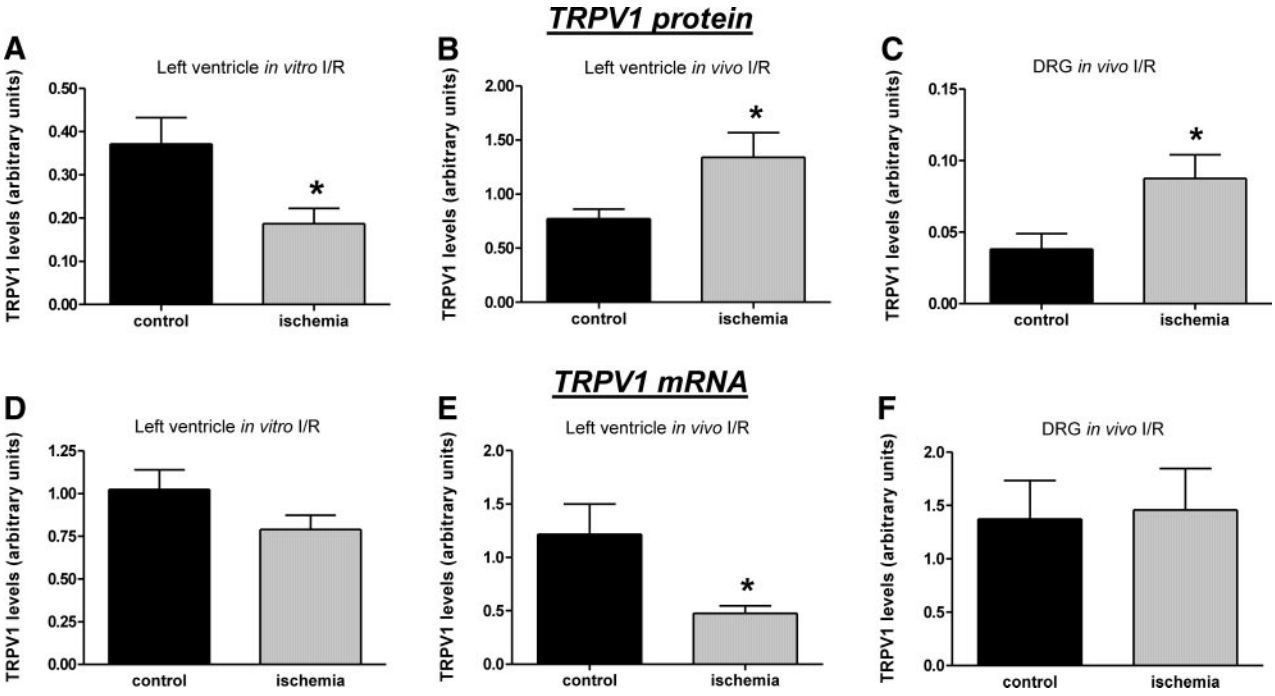

Figure 5. TRPV1 expression after I/R injury *in vitro* or *in vivo*. TRPV1 protein expression in *A)* left ventricle *in vitro*, *B)* left ventricle *in vivo*, and *C)* DRG *in vivo*. TRPV1 mRNA levels in *D)* left ventricle *in vitro*, *E)* left ventricle *in vivo*, and *F)* DRG *in vivo*. Data are means ± SE (ratio of levels of TRPV1 protein or mRNA relative to the control, α- or β-actin, respectively, expressed in arbitrary units). Statistical analysis performed using unpaired Student's *t* test (n=4–7). *$P < 0.05$ compared with controls.

fied this finding in the present study but, as before, we found considerable variability in these responses. This is likely to be due to the instability of 12(S)-HpETE in solution since it is highly prone to oxidation. To overcome these difficulties, we perfused the Langendorff preparation with AA and 12-LOX, adapting a protocol devised by Gryglewski and Vane (14) to allow for local generation of the 12-LOX metabolites within the coronary preparation and, in this way, avoid premature oxidation. This method of application proved vastly superior to direct administration of the 12-LOX metabolites, with considerably improved stability and reproducibility. In isolated perfused rat hearts, AA elicited a dose-dependent vasodilatation. This effect was blocked by treatment with the nonselective LOX inhibitor, NDGA, and profoundly attenuated by the selective 12-LOX inhibitor baicalein. These findings indicate that AA-induced coronary vasodilatation was due to 12-LOX metabolism of AA leading to 12(S)-HpETE, then to 12(S)-HETE generation. Products of the 12-LOX pathway of AA metabolism mediate the vasoactive effects of AA in several different vessel types, including the basilar artery (26, 27) and mesenteric arteries (28), and also in the coronary microcirculation (29). It is unlikely that any vasoactive effects of 12-LOX/AA treatment are due to prostaglandin generation since the nonisoform selective COX inhibitor indomethacin was present in all experiments. Infusion of 12-LOX alone did cause a small transient decrease in perfusion pressure, but this effect was not sustained despite continued perfusion of the 12-LOX. It is possible that perfused 12-LOX in the absence of exogenous AA may use an endothelial lipid as a substrate, but it is unlikely that 12-LOX crosses the endothelial membrane. The transient nature of this effect further supports the contention that this brief action is likely an artifact of infusion. Likewise, application of AA in the absence of 12-LOX also caused vasodilatation; however, these responses were not reproducible and were prone to significant tachyphylaxis in the present system (data not shown), which precluded the use of AA in the absence of 12-LOX. Whether this effect is due to conversion by endothelial enzymes is uncertain, but it cannot be excluded that a proportion, likely small, of the response to AA is due to conversion by an endogenous enzyme other than the perfused 12-LOX.

We previously demonstrated that 12(S)-HpETE-induced vasodilatation involves activation of cardiac sensory C-fiber afferents. Indeed, *in vitro* desensitization of sensory C-fibers, using capsaicin, abolished the response to both AA and direct administration of 12(S)-HpETE. *In vitro* desensitization of C-fibers, using capsaicin, is a standard method for isolating C-fibers from tissue *in vitro* and is caused in part by depletion of neuropeptide content. Washing the preparation for 30 min after capsaicin application ensures removal of any neuropeptides released during the desensitization process while still maintaining a desensitized state of nerve endings (30). Further support for an involvement of sensory C-fibers is provided by the findings that selective receptor antagonists for the sensory neuropeptides, CGRP and SP, also suppressed AA-induced vasodilatation. The $CGRP_1$ receptor antagonist $\alpha CGRP_{8-37}$, and to a lesser extent the NK1 receptor antagonist

SR140333, attenuated responses to AA, suggesting that both CGRP and SP are involved in AA- and 12(S)-HpETE-induced vasodilator responses. It is well established that tachykinin nerve processes are present within the heart (principally located within intrinsic cardiac ganglia and the adventitia of coronary arteries) (31), and immunohistochemical studies in rat, guinea pig, porcine, and human tissues have confirmed that the predominant neuropeptides contained within these nerve terminals are SP and CGRP, although neurokinin A (NKA) has also been identified (for review, see refs. 6, 32). CGRP acts as a powerful vasodilator in the cardiovascular system (33), predominantly *via* activation of $CGRP_1$ receptors. SP also vasodilates the coronary vasculature, albeit to a lesser extent than CGRP, *via* activation of the tachykinin NK1 (32, 34). Our findings suggest that CGRP plays a major role in AA-induced vasodilatation, with a lesser role for SP; a combination of the antagonists produced no greater inhibitory effect than to block CGRP receptors alone, suggesting that SP likely activates the same signaling pathway as CGRP to result in smooth muscle relaxation.

Despite previous observations demonstrating the coronary vasodilator actions of 12-LOX products, identification of the receptor mediating this effect has not been forthcoming. In 2000 Hwang *et al.* (10) demonstrated that AA metabolites, the strongest being 12(S)-HpETE, were potent activators of the nonselective cation channel TRPV1 on sensory C-fiber afferents (4). In the current study, selective blockade of TRPV1 using capsazepine abolished the coronary vasodilator effects of both AA and 12(S)-HpETE. In addition, the dose-response curve to AA in hearts from TRPV1 knockout mice was shifted rightward ~30-fold compared with hearts of WT mice, demonstrating that TRPV1 activation mediates a major component of 12-LOX-mediated activity in the heart.

Having identified the primary mechanism underlying the vasoactive effects of AA, we went on to investigate whether this pathway might be involved in 12-LOX-dependent cardioprotection against I/R injury. Good evidence supports the thesis that both 12-LOX metabolites and sensory neuropeptides (8, 35, 36) act as endogenous factors, the local production of which is elevated during ischemia, that limit the damage caused by I/R injury. LOX metabolites have also been implicated in ischemic preconditioning (whereby repeated sublethal periods of ischemia offer protection against a subsequent prolonged ischemic episode), in particular, 12(S)-HETE (15, 24) and 12(S)-HpETE (23). Indeed, it is interesting to note the recent demonstration that TRPV1 and 12-LOX mRNA are coexpressed in rat DRG neurons (11). In addition, a growing body of evidence points to a protective role for TRPV1 against myocardial I/R injury, and it is thought that such protection is mediated by sensory neuropeptide release and activity. Indeed, a wealth of evidence has demonstrated that CGRP protects against experimentally induced I/R injury in a number of organs, including the heart (8, 36), an effect absent in $CGRP_1$ receptor knockout mice or after selective receptor antagonism (37). Moreover, I/R injury is associated with substantial release of CGRP both in experimental models and in patients with an acute myocardial infarction (38). In addition, a recent study conducted in the hearts of various acid-sensing channel knockout mice demonstrated that TRPV1 activation is the primary mediator of acid-evoked release of CGRP (9). Since acidosis is an important characteristic of myocardial I/R injury, these findings raise the hypothesis that TRPV1-mediated CGRP release in the heart may represent an endogenous damage-limiting mechanism. However, whether a link exists between the 12-LOX pathway and the TRPV1/C-fiber/neuropeptide pathways in I/R injury has not previously been explored.

In control hearts, global ischemia followed by reperfusion produced a characteristic increase in CPP, reduction in LVDP, and an infarct size of ~60%. Treatment with 12-LOX/AA prior to ischemia reduced infarct size substantially and improved postischemic LVDP, although it had no effect on CPP. The protective effects of 12-LOX and AA were inhibited by treatment with baicalein or C-fiber desensitization. These findings establish a definitive link between sensory neuropeptide- and 12-LOX-mediated cardioprotection, and identify a novel pathway whose activation limits I/R-induced injury. The exact mechanisms of this protective effect are uncertain, although activation of PKC has been implicated in the beneficial effects of both 12-LOX metabolites and CGRP (23, 39). However, it is surprising that no improvements in coronary flow were evidenced in response to AA, although this is similar to studies in TRPV1 knockout animals, where I/R infarct size was considerably enhanced compared with wild-types but no differences in coronary flow were evident (7). This group suggested that a total lack of effect on flow was unlikely and that perhaps flow distribution had been altered to improve cardiac function rather than a net increase in flow; it is possible that such a mechanism underlies the lack of effect in the current study.

A particular characteristic of TRPV1 is its plasticity whereby in inflammatory situations the expression of this receptor is up-regulated (40). Several studies demonstrate that TRPV1 protein expression is elevated in response to peripheral inflammation (41–43), although some studies report no change in TRPV1 mRNA (44). In the present study, TRPV1 protein expression in hearts subjected to an I/R insult *in vitro* was decreased by ~50% compared with sham control hearts. This suggests that the I/R insult itself results in an apparent "consumption" of TRPV1 due possibly to decreased protein stability or increased protein processing, both phenomena associated with ischemic injury. This decrease would theoretically result in suppression of the activity of the protective 12-LOX/TRPV1 pathway. However, while it was long believed that neuronal proteins are synthesized exclusively in the neuronal cell bodies (*i.e.*, within the DRG of C-fibers) and then transported to peripheral and central nerve terminals by anterograde transport, it has become evident that mRNA

is also present in the distal domains of the neuron and that protein synthesis can take place en route within the axon (45) as well as within the nerve terminals themselves (46). Indeed, in response to injection of carrageenan into the hindpaw of rats, TRPV1 mRNA is transported from the DRG along the axon to both the central and peripheral terminals of primary afferents, where it is then translated, the resulting increase in TRPV1 receptors apparently contributing to an increase in sensitivity to capsaicin (47). Such regulation is believed to provide for rapid increases in local protein synthesis after appropriate stimulation. Clearly, in the *in vitro* model of I/R injury, the neurons innervating the heart were no longer associated with their cell bodies, and therefore transport of mRNA to the peripheral terminals and subsequent translation to increase TRPV1 expression were not possible. Indeed, measurement of cardiac TRPV1 mRNA levels demonstrated no change in tissues subjected to I/R injury compared with control. However, based on previous findings, we hypothesized that repeating the I/R injury studies *in vivo* would expose an increase in TRPV1 protein. Indeed, TRPV1 protein expression was elevated in ischemic heart samples associated with a reduction in local TRPV1 mRNA levels. These changes were accompanied by an increase in expression of TRPV1 protein in DRG while mRNA levels remained unchanged. These changes in TRPV1 were not due to an artifact, or indeed a generalized effect of ischemic injury, since no change in expression of TRPV2 or TRPV4 mRNA was evident in tissues from either the *in vitro* or *in vivo* experiments. These results suggest that the I/R insult perhaps triggers transport from the DRG to the periphery of TRPV1 mRNA, and possibly also protein, as a protective/compensatory mechanism.

In conclusion, this study has demonstrated that in the coronary circulation, C-fibers and TRPV1 are involved in the vasodilator response to AA and 12(S)-HpETE, supporting the notion that AA-induced vasodilatation is mediated by the 12-LOX product 12(S)-HpETE and that C-fiber/TRPV1-mediated responses to AA/12(S)-HpETE involve the vasoactive neuropeptides CGRP and SP. We have also shown that 12-LOX and AA are protective against myocardial I/R injury and that such protection is in part a consequence of C-fiber activation. The data from this study also suggest that I/R injury results in a decrease in TRPV1 expression locally within the heart, but that an increase of TRPV1 synthesis in DRG and transport to cardiac nerve terminals occurs *in vivo* to maintain expression levels. Finally, it is likely that, during I/R injury, up-regulation or activation of the 12-LOX/AA/TRPV1 pathway represents an endogenous damage-limiting mechanism. FJ

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