Aldehyde Dehydrogenase Activation Prevents Reperfusion Arrhythmias by Inhibiting Local Renin Release From Cardiac Mast Cells

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Background—Renin released by ischemia/reperfusion from cardiac mast cells activates a local renin-angiotensin system (RAS). This exacerbates norepinephrine release and reperfusion arrhythmias (ventricular tachycardia and fibrillation), making RAS a new therapeutic target in myocardial ischemia.

Methods and Results—We investigated whether ischemic preconditioning (IPC) prevents cardiac RAS activation in guinea pig hearts ex vivo. When ischemia/reperfusion (20 minutes of ischemia/30 minutes of reperfusion) was preceded by IPC (two 5-minute ischemia/reperfusion cycles), renin and norepinephrine release and ventricular tachycardia and fibrillation duration were markedly decreased, a cardioprotective anti-RAS effect. Activation and blockade of adenosine A2b/A3 receptors and activation and inhibition of protein kinase C (PKCe) mimicked and prevented, respectively, the anti-RAS effects of IPC. Moreover, activation of A2b/A3 receptors or activation of PKCe prevented degranulation and renin release elicited by peroxide in cultured mast cells (HMC-1). Activation and inhibition of mitochondrial aldehyde dehydrogenase type-2 (ALDH2) also mimicked and prevented, respectively, the cardioprotective anti-RAS effects of IPC. Furthermore, ALDH2 activation inhibited degranulation and renin release by reactive aldehydes in HMC-1. Notably, PKCe and ALDH2 were both activated by A2b/A3 receptor stimulation in HMC-1, and PKCe inhibition prevented ALDH2 activation.

Conclusions—The results uncover a signaling cascade initiated by A2b/A3 receptors, which triggers PKCe-mediated ALDH2 activation in cardiac mast cells, contributing to IPC-induced cardioprotection by preventing mast cell renin release and the dysfunctional consequences of local RAS activation. Thus, unlike classic IPC in which cardiac myocytes are the main target, cardiac mast cells are the critical site at which the cardioprotective anti-RAS effects of IPC develop. (Circulation. 2010;122:771-781.)

Key Words: arrhythmia ■ ischemia ■ norepinephrine ■ renin ■ reperfusion

How critical mast cells are in cardiac pathophysiology is not well understood. Numerous mast cells are present in the mammalian heart (=50,000 mast cells per 1 g human heart tissue) in close proximity to vessels and nerves, and their density markedly increases in heart failure, ischemic cardiomyopathy, and experimental infarct models.1 Mast cells synthesize, store, and release a variety of mediators. We recently reported that cardiac mast cells are also an important source of the aspartyl protease renin.2 When released by ischemia/reperfusion (I/R), this renin initiates the activation of a local renin-angiotensin system (RAS); the locally formed angiotensin II (Ang II) exacerbates norepinephrine release from cardiac sympathetic nerves and elicits reperfusion arrhythmias.3 When mast cells are depleted or pharmacologically stabilized, renin and norepinephrine release and reperfusion arrhythmias are markedly reduced.3 Thus, the release of mast cell–derived renin represents a new target in the prevention and treatment of ischemic cardiac dysfunction.

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protection afforded by classic IPC (eg, infarct size reduction). The PKCε isoenform has been shown to play a key cardioprotective role against I/R and to exhibit antisecretory activity in mast cells. Accordingly, we set out to determine whether IPC might also prevent the activation of a local cardiac RAS initiated by the release of mast cell renin and, if so, whether this novel IPC paradigm involves the activation of PKCe in mast cells. Given that phosphorylation of mitochondrial aldehyde dehydrogenase type-2 (ALDH2) by PKCe correlates with cardioprotection, we hypothesized that IPC could promote PKCe-induced activation of ALDH2, which would then remove the toxic aldehydes known to degranulate mast cells such as acetaldehyde and 4-hydroxynonenal (4-HNE), which are formed by lipid peroxidation. Our study outlines a novel protective anti-RAS effect of IPC; we find that the sequential activation of adenosine A2b and A3 receptors, PKCe, and ALDH2 in cardiac mast cells diminishes the release of renin elicited by I/R and thus curtails Ang II– and norepinephrine-induced arrhythmias.

Methods

See the online-only Data Supplement for the complete Methods section.

Ex Vivo Hearts

In total, 132 male Hartley guinea pigs (weight, 300 to 350 g; Charles River Laboratories, Kingston, NY) were anesthetized with CO2 and euthanized by stunning with approval from the Institutional Animal Care and Use Committee. Isolated hearts were perfused at constant pressure with oxygenated Ringer at 37°C in a Langendorff apparatus (Radnoti Glass Technology, Monrovia, Calif).

Ischemia/Reperfusion

After equilibration, all hearts were subjected to 20 minutes of global ischemia followed by 30 minutes of reperfusion. For IPC, two 5-minute cycles of ischemia were each followed by 5 minutes of reperfusion. For pharmacological prevention of IPC, antagonists were perfused for 20 minutes (glyceryl trinitrate [GTN] for 30 minutes) before and during IPC and then washed out for 15 minutes before I/R. For pharmacological preconditioning, given agents were perfused for two 5-minute cycles before I/R except for 8V1-1 (PKCε inhibitor), which was administered during the entire 30-minute reperfusion following the 20-minute ischemia. For prevention of pharmacological preconditioning, antagonists were perfused for 20 minutes (GTN for 30 minutes) before and during pharmacological preconditioning and then washed out for 15 minutes before I/R. Coronary flow was measured every 2 minutes; samples were assayed for renin, norepinephrine, β-hexosaminidase, and creatine phosphokinase (CPK). Surface ECG was obtained from the left ventricle and right atrium, recorded in digital format, and analyzed with Power Laboratory/8SP (AdInstrument, Colorado Springs, Colo).

Cell Culture

The human mastocytoma cell line (HMC-1) was a gift of from Dr I. Biaggioni (Vanderbilt University, Nashville, Tenn). Cells were maintained in suspension culture as previously described.  

β-Hexosaminidase and Renin Assay

β-Hexosaminidase and renin coronary overflow was measured as previously described. HMC-1 cells were suspended in Ringer buffer, and equal volumes were divided into aliquots in Eppendorf tubes and incubated at 37°C with a given agent (ie, Alda-1, ϕeRACK, or LUF5835 plus IBMECa) for 10 minutes (preceded or not by a 30-minute incubation with GTN). Acetaldehyde, H2O2, or 4-HNE was subsequently added for 20 minutes. All results were normalized and expressed as percent above control.

Norepinephrine Assay

Coronary effluent was assayed for norepinephrine by high-performance liquid chromatography with electrochemical detection as previously described.  

CPK Assay

Coronary effluent was assayed for CPK release with a CPK assay kit (Genzyme Diagnostics, Charlottetown, Prince Edward Island, Canada).

Polymerase Chain Reaction and Immunostaining

For reverse-transcription polymerase chain reaction (RT-PCR), total RNA was extracted from HMC-1 cells with TRIZol reagent (Invitrogen, Carlsbad, Calif), 1 μg total RNA from each sample was reverse transcribed, and complementary DNA was amplified by RT-PCR with a Taqman primer (Valencia, Calif) 1-step RT-PCR kit. PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining. For immunostaining, HMC-1 cells were fixed and permeabilized on glass slides and stained with the goat anti–A2b receptor antibody (Santa Cruz Biotechnology, Santa Cruz, Calif) conjugated to Alexa Fluor 488 donkey anti-goat IgG and with rabbit anti–A3 receptor antibody (Santa Cruz) conjugated to Alexa Fluor 488 donkey anti-rabbit IgG. Nuclei were stained with DAPI. For immunofluorescence, cells were examined with an inverted fluorescence microscope (Nikon Eclipse TE 2000-U, Morrell Instruments, Melville, NY) interfaced to an electron multiplying charge-coupled device (Hamamatsu Photonics, Bridgewater, NJ) and processed with Metamorph software (version 6.2, Universal Imaging Corp, Downingtown, Pa).

Translocation of PKCe

Cytosolic and membrane fractions of HMC-1 cells were separated, and Western blot analysis was performed with a PKCe-specific antibody (Santa Cruz).

ALDH2 Enzymatic Activity Assay

ALDH2 activity in HMC-1 cells was determined spectrophotometrically by monitoring the reductive reaction of NAD+ to NADH at 340 nm as previously described.  

Drugs and Chemicals

Acetaldehyde, H2O2, IBMECa, MRS1754, MRS1523, DPCPX, chelerythrine, 5-hydroxydecanoate, and cyanamide were purchased from Sigma-Aldrich (St Louis, Mo); 4-HNE in ethanol solution was synthesized in Dr Mochly-Rosen’s laboratory (Stanford University School of Medicine, Palo Alto, Calif). Phorbol 12-myristate 13-acetate was purchased from LC Laboratories (Woburn, Mass). GTN was a gift from Dr M.W. Beukers (University of Leiden, Leiden, the Netherlands); EXP3174 was a gift from Merck Sharp & Dohme Ltd (Whitehouse Station, NJ); ϕeRACK, 8V1-1, and Alda-1 were synthesized in Dr Mochly-Rosen’s laboratory (Stanford University School of Medicine, Palo Alto, Calif). Phorbol 12-myristate 13-acetate was purchased from LC Laboratories (Woburn, Mass). GTN was purchased from Hospira Inc (Lake Forest, Ill). Human plasma angiotensinogen was purchased from Calbiochem (San Diego, Calif).

Statistics

Data are presented as mean±SEM. Nonparametric tests were used throughout the study. For 2-group comparisons, the Mann–Whitney test was used (Figures 1 and 2). For comparisons among >2 groups, the Kruskal-Wallis test followed by the posthoc Dunn test was used (Figures 1 through 3, 4D, 4F, and 5 through 7). GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, Calif) was used. Values of P<0.05 were considered statistically significant.
Kruskal-Wallis with the Dunn multiple comparison test (for all others). (IBMECA, 50 nmol/L) for two 5-minute cycles plus a 5-minute washout before I/R (n = 6).

B. Hearts were pretreated with the selective PKC δ isoform activator ωV1-2 (1 μmol/L) and a combination of A2b (LUF 5835, 50 nmol/L) and A3 receptor agonists (IBMECA, 50 nmol/L) for two 5-minute cycles plus a 5-minute washout instead of IPC before I/R (n = 6).

Instead of IPC, other hearts were perfused with a combination of selective A2b (LUF 5835) and A3 (IBMECA) receptor agonists for two 5-minute cycles plus a 5-minute washout instead of IPC before I/R (n = 6). Other hearts were perfused with the selective PKC δ activator ωRACK, 500 nmol/L for two 5-minute cycles plus a 5-minute washout before I/R (n = 5). Other hearts were perfused with the ALDH2 desensitizer GTN (2 μmol/L, perfused for 30 minutes), followed by ωRACK for two 5-minute cycles plus a 5-minute washout instead of IPC before I/R (n = 5). Bars represent mean±SEM of overflows during the first 4 minutes of reperfusion or duration of VT/VF. **P<0.01, ***P<0.001 from I/R; †P<0.05, ††P<0.01 from IPC by Mann–Whitney test (for I/R versus IPC) or Kruskal-Wallis test with the Dunn multiple comparison test (for all others).

had previously shown that the enhanced norepinephrine overflow and arrhythmias result from the activation of a local RAS by renin released from cardiac mast cells.3

When I/R was preceded by IPC (ie, two 5-minute cycles of ischemia, each followed by 5 minutes of reperfusion), mast cell degranulation was only approximately half that occurring with I/R alone, as indicated by a marked decrease in β-hexosaminidase overflow (ie, the overflow of β-hexosaminidase increased by 202±31% and 109±14% with I/R and I/R preceded by IPC, respectively; n = 5 and 5; P<0.05). IPC also greatly reduced the overflow of renin and norepinephrine and the duration of VT/VF (ie, an overall 70% to 85% decrease; Figure 1), clearly indicating a cardioprotective anti-RAS effect of IPC.

Results

IPC Prevents the Activation of a Cardiac RAS and Alleviates Arrhythmic Dysfunction

Spontaneously beating Langendorff-perfused guinea pig hearts were subjected to 20 minutes of global ischemia followed by 30 minutes of reperfusion. This resulted in mast cell degranulation, demonstrated by a 202±31% (n = 5; mean±SEM) increase in β-hexosaminidase overflow into the coronary effluent. I/R also caused large increases in renin and norepinephrine overflow (ie, ∼2.5-fold and ∼75-fold, respectively) and severe ventricular arrhythmias (tachycardia and fibrillation [VT/VF]) that lasted ∼12 minutes (Figure 1). We

Figure 1. IPC reduces renin and norepinephrine (NE) release and shortens arrhythmias caused by I/R in guinea pig hearts ex vivo. This cardioprotective anti-RAS effect is mimicked or prevented by activation or blockade of adenosine A2b and A3 receptors in combination but is unaffected by adenosine A1 receptor blockade (A), mimicked by PKCδ activation, and prevented by PKCδ inhibition (B). A. Coronary overflow of renin and norepinephrine and duration of reperfusion arrhythmias (VT/VF) in I/R (n = 6) and I/R preceded by IPC (n = 8) or I/R preceded by IPC in the presence of DPCPX (adenosine A1 receptor antagonist, 300 nmol/L) or MRS1754 and MRS1523 (adenosine A2b and A3 receptor antagonists; 50 and 100 nmol/L) (n = 6 each). Instead of IPC, other hearts were perfused with a combination of A2b (LUF 5835, 50 nmol/L) and A3 receptor agonists (IBMECA, 50 nmol/L) for two 5-minute cycles plus a 5-minute washout before I/R (n = 6). Additional hearts were perfused with the selective PKCδ inhibitor ωV1-2 (1 μmol/L) and then subjected to I/R preceded by IPC (n = 5). Additional hearts were perfused with the selective PKCδ activator ωRACK (500 nmol/L) instead of IPC (two 5-minute cycles plus a 5-minute reperfusion; n = 5) before I/R. Bars represent mean±SEM of overflows during the first 4 minutes of reperfusion or duration of VT/VF. **P<0.01, ***P<0.001 from I/R; †P<0.05, ††P<0.01 from IPC by Mann–Whitney test (for I/R versus IPC) or Kruskal-Wallis test with the Dunn multiple comparison test (for all others).

Figure 2. Selective inhibition of the PKCδ isoform prevents the cardioprotective IPC-like anti-RAS effects generated by the combined activation of adenosine A2b and A3 receptors (A). Selective desensitization of ALDH2 with GTN prevents the cardioprotective effects of PKCδ activation (B). A. Coronary overflow of renin and norepinephrine (NE) and duration of VT/VF in guinea pig hearts subjected to I/R (n = 6). Instead of IPC, other hearts were perfused with a combination of selective A2b (LUF 5835, 50 nmol/L) and A3 receptor agonists (IBMECA, 50 nmol/L) for two 5-minute cycles plus a 5-minute washout before I/R (n = 6). Other hearts were perfused with the selective PKCδ inhibitor ωV1-2 (1 μmol/L) followed by a combination of A2b (LUF 5835) and A3 (IBMECA) receptor agonists for two 5-minute cycles plus a 5-minute washout instead of IPC before I/R (n = 6). B. Instead of IPC, hearts were perfused with the selective PKCδ activator ωRACK, 500 nmol/L for two 5-minute cycles plus a 5-minute washout before I/R (n = 5). Other hearts were perfused with the ALDH2 desensitizer GTN (2 μmol/L, perfused for 30 minutes), followed by ωRACK for two 5-minute cycles plus a 5-minute washout instead of IPC before I/R (n = 5). Bars represent mean±SEM of overflows during the first 4 minutes of reperfusion or duration of VT/VF. **P<0.01, ***P<0.001 from I/R; †P<0.05, ††P<0.01 from the A2b and A3 receptor agonist combination (A); †P<0.05 from ωRACK (B) by Mann–Whitney test (for I/R versus IPC) or Kruskal-Wallis test with the Dunn multiple comparison test (for all others).
First, we assessed whether inhibition or activation of adenosine receptors prevents or mimics, respectively, the IPC-mediated attenuation of renin release in hearts subjected to I/R. We found that the combined blockade of adenosine A<sub>2b</sub> and A<sub>3</sub> receptors with the MRS1754 (50 nmol/L)<sup>20</sup> and MRS1523 (100 nmol/L)<sup>21</sup> compounds prevented the IPC-induced attenuation of renin and norepinephrine release and the alleviation of reperfusion arrhythmias (Figure 1A). Conversely, the combined activation of A<sub>2b</sub> and A<sub>3</sub> receptors with the LUF5835 (50 nmol/L)<sup>22</sup> and IBMЕCA (50 nmol/L)<sup>23</sup> compounds mimicked the cardioprotective anti-RAS effects of IPC (Figure 1A). In contrast, activation of A<sub>2b</sub> or A<sub>3</sub> receptors alone failed to mimic the effects of IPC (renin overflow, 26.94±2.80, 19.77±3.9, and 24.37±2.58 pg·h<sup>-1</sup>·g<sup>-1</sup> Ang I formed during IPC and 6.81±1.77 pmol/g in the presence and absence of EXP3174 (Figure 1A). In fact, the IPC-induced attenuation of renin and norepinephrine release and the abbreviation of reperfusion arrhythmias were the same in the presence and absence of 5-hydroxydecanoate (Figure 1A). Hence, the cardioprotective anti-RAS effects of IPC do not appear to depend on the opening of mKATP channels.

According to early reports in rabbits and rats, Ang II mimicked the cardioprotective effects of classic IPC. Thus, we determined whether Ang II, which is locally produced in the heart subjected to I/R, contributes to the anti-RAS effects of IPC. For this, we induced IPC in hearts perfused with the AT<sub>1</sub> receptor antagonist EXP3174. EXP3174 (300 nmol/L; IC<sub>50</sub>=18 nmol/L)<sup>24</sup> failed to affect the cardioprotective anti-RAS effects of IPC. In fact, the IPC-induced attenuation of renin and norepinephrine release and the abbreviation of reperfusion arrhythmias were the same in the presence and absence of 5-hydroxydecanoate (Figure IA in the online-only Data Supplement). Therefore, AT<sub>1</sub> receptors are probably not involved in the mediation of the cardioprotective anti-RAS effects of IPC.

Translocation of PKC<sub>e</sub> Mediates the Cardioprotective Anti-RAS Effects of Adenosine A<sub>2b</sub> and A<sub>3</sub> Receptor Activation

Because PKC activation/translocation is likely to be involved in the cardioprotective effects of classic IPC<sup>8,29</sup> we next investigated the role of PKC in the cardioprotective anti-RAS
effects of IPC. Treatment of hearts with the general, non–isoform-selective PKC activator phorbol 12-myristate 13-acetate (PMA; 0.05 mmol/L, two 5-minute cycles before I/R) mimicked the protective effects of IPC on renin and norepinephrine release and reperfusion arrhythmia duration (Figure IB in the online-only Data Supplement). Moreover, inhibition of PKC with the specific but non–isoform-selective chelerythrine (2.8 µmol/L) prevented the effects of IPC on the same parameters (Figure IB in the online-only Data Supplement). Thus, general PKC activation appears to mediate the cardioprotective anti-RAS effects of IPC.

Of the PKC family of serine/threonine kinases, the PKCe isoform has been shown to play a key cardioprotective role against I/R. Thus, we tested whether yeRACK, a selective activator peptide of PKCe, mimics the anti-RAS effects of IPC. yeRACK (500 nmol/L), perfused for two 5-minute cycles followed by a 5-minute washout before I/R, mimicked the protective anti-RAS effects of IPC. Indeed, the overflow of renin and norepinephrine and the duration of VT/VF were reduced by ~55% to 90% compared with I/R hearts (Figure 1B). Moreover, selective inhibition of PKCe with εV1-2 (1 µmol/L) prevented the effects of IPC on the same parameters (Figure 1B). Thus, PKCe activation appears to be required and sufficient for the genesis of the cardioprotective anti-RAS effects of IPC.

Stimulation of adenosine A<sub>2b</sub> and A<sub>1</sub> receptors mimicked the anti-RAS effects of IPC (see Figure 1A); selective activation of the PKCe isoform also had anti-RAS effects similar to IPC (see Figure 1B). Given that adenosine is known to activate PKC, thus initiating the traditional preconditioning cascade, we determined whether the anti-RAS IPC-like effects of A<sub>2b</sub> and A<sub>1</sub> receptors rely on the consequent activation of PKCe. To verify this notion, we assessed whether PKCe blockade would prevent the IPC-like effects of A<sub>2b</sub> and A<sub>1</sub> receptor agonists. We found that selective inhibition of the PKCe isozyyme with εV1-2 (1 µmol/L) prevented the anti-RAS IPC-like effects resulting from the combined activation of A<sub>2b</sub> and A<sub>1</sub> receptors (Figure 2A). Thus, A<sub>2b</sub> and A<sub>1</sub> receptor–mediated activation of PKCe appears to be the first significant step in the anti-RAS preconditioning pathway.

Activation of ALDH2 Is Pivotal for the Cardioprotective Anti-RAS Effects of PKCe

Because the cardioprotective infarct-sparing effects of PKCe activation have been found to depend on phosphorylation of mitochondrial ALDH2, we next assessed whether the anti-RAS effects of IPC are also determined by ALDH2 activation. For this, we assessed whether inhibition/inactivation of ALDH2 would abolish the anti-RAS effects of IPC and whether activation of ALDH2 would mimic them. We found that GTN, perfused for 30 minutes at a concentration known to inactivate ALDH2 (2 µmol/L), prevented the cardioprotective anti-RAS effects of yeRACK (ie, GTN abolished the yeRACK-induced inhibition of renin and norepinephrine release and the alleviation of VT/VF; Figure 2B). We also found that the general ALDH inhibitors cyanamide (5 mmol/L) and GTN prevented the anti-RAS effects of IPC (Figure 3A and 3B). Conversely, selective activation of ALDH2 with Alda-1 (20 µmol/L, two 5-minute cycles) reproduced all of the anti-RAS effects of IPC, an action that was also prevented by cyanamide (Figure 3A) and by pretreatment with GTN (Figure 3B). Collectively, these findings suggest that ALDH2 activation by PKCe is a crucial mechanistic step in the development of the anti-RAS effects of IPC.

Mast Cells Are the Site of the Cardioprotective Anti-RAS Action of IPC

Given the pivotal role that mast cells play in the activation of RAS in the heart, cardiac mast cells are likely to be the site at which the anti-RAS effects of IPC develop. Because combined activation of adenosine A<sub>2b</sub> and A<sub>1</sub> receptors mimics the cardioprotective anti-RAS effects of IPC, whereas the combined blockade of the same receptors prevents the anti-RAS effects (see Figure 1A), we first ascertained the presence of A<sub>2b</sub> and A<sub>1</sub> receptors on mast cells. For this, we used human mast cells in culture (HMC-1 cells). Total RNA (1 µg) was extracted from HMC-1 cells, reverse transcribed, and amplified by PCR using sense and antisense primers specific for human A<sub>2b</sub> and A<sub>1</sub> receptor genes. Figure 4A depicts an ethidium bromide–stained gel showing that the HMC-1 PCR products for these adenosine receptor subtypes are consistent with those reported by others. HMC-1 cells were also immunopositive for the 2 adenosine receptor subtypes (Figure 4B).

We next made certain that mast cell PKCe can be activated. Using Western analysis in cytosolic and membrane fractions of HMC-1 cells, we found that the phorbol ester PMA (positive control) markedly increased the translocation of PKCe from cytosol to membrane (ie, a hallmark of PKCe activation; Figure 4C through 4F). Incubation of HMC-1 cells with the PKCe activator yeRACK (500 nmol/L for 30 minutes) before a 7-minute incubation with a below-threshold concentration of PMA (3 mmol/L) also significantly translocated PKCe from cytosol to membrane (Figure 4C and 4D). Moreover, incubating HMC-1 cells with the adenosine A<sub>2b</sub> and A<sub>1</sub> receptor agonists in combination (LUF5835 and IBMECA, 50 nmol/L each for 1 hour) also translocated PKCe (Figure 4E and 4F).

Our findings in isolated guinea pig hearts and cultured mast cells suggested that IPC may result from the activation of adenosine A<sub>2b</sub> and A<sub>1</sub> receptors expressed by cardiac mast cells and consequent PKCe-dependent activation of mitochondrial ALDH2. Thus, we next investigated the role of ALDH2 in mast cell degranulation and renin release elicited by prototypic toxic compounds formed in I/R. For this, we measured mast cell degranulation in response to acetaldelyde, 4-HNE, another toxic aldehyde that accumulates during cardiac ischemia, and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), which triggers toxic aldehydes formation by membrane lipid peroxidation. Incubation of HMC-1 cells with acetaldelyde (300 to 700 µmol/L), H<sub>2</sub>O<sub>2</sub> (0.1 to 1 mmol/L), or 4-HNE (3 to 30 µmol/L) elicited a concentration-dependent increase in the release of β-hexosaminidase (~15% to 42%, an indication of mast cell degranulation) and renin (~15% to 80%; Figure 5). Notably, preincubation of HMC-1 cells with the ALDH2 activator Alda-1 (20 µmol/L) prevented the
degranulating effects of each acetaldehyde, H2O2, and 4-HNE (Figure 5A through 5F). Moreover, pretreatment of HMC-1 cells with the ALDH2 desensitizer GTN (2 μmol/L for 30 minutes) prevented the antidegranulating effects of Alda-1 (Figure 5). These findings suggested that ALDH2 activation in mast cells prevents their degranulation by toxic aldehydes produced in the I/R heart and that this represents a crucial mechanistic step in the anti-RAS effects of IPC.

We next measured ALDH2 activity in HMC-1 cells in response to the specific ALDH2 activator Alda-1,37 the non–isoform-specific PKC activator PMA, the PKC-selective agonist ψeRACK, and the adenosine A2b and A3 receptor agonists in combination. We found that the ALDH2 enzymatic activity (ie, NADH production) was enhanced by Alda-1 (100 μmol/L), PMA (300 nmol/L), ψeRACK (0.5 and 1 μmol/L), and LUF5835 (50 nmol/L) and IBMECA (50 nmol/L) combined (Figure 6). Notably, selective PKCe inhibition with eV1-2 (1 μmol/L) prevented the increase in ALDH2 activity elicited by Alda-1 and A3 receptor agonists in combination (Figure 6).

Given that activation of A2b and A3 receptors in HMC-1 cells increased ALDH2 activity and that this was inhibited by the PKCe antagonist (see Figure 6), we next examined whether activation of A2b and A3 receptors and PKCe would each protect mast cells from degranulation and renin release. We found that the large concentration-dependent increase in β-hexosaminidase and renin release elicited by incubation with H2O2 (0.1 to 1 mmol/L) was markedly inhibited by LUF5835 (50 nmol/L) and IBMECA (50 nmol/L) combined (Figure 7A and 7C) and by ψeRACK (500 nmol/L; Figure 7B and 7D). These effects were prevented by prior ALDH2 desensitization with GTN pretreatment (2 μmol/L for 30 minutes; Figure 7A through 7D). Collectively, these findings indicate that activation of adenosine A2b and A3 receptors on the mast cell membrane leads to an increase in PKCe activity and thus ALDH2 activation, which prevents the degranulating effects of toxic aldehydes such as those produced in I/R.

Finally, we sought to establish whether the cardioprotective anti-RAS effects of IPC, which most likely result from an action at the mast cell level, are independent of the IPC-induced reduction of myocyte damage. Because both PKCe activation38 and PKCe inhibition12 protect cardiac myocytes from I/R-induced damage, we compared PKCe activation with PKCe inhibition in terms of CPK release, as well as...
renin and norepinephrine release and VT/VF duration. We found that I/R (ie, 20 minutes of global ischemia followed by 30 minutes of reperfusion) caused a characteristic increase in CPK overflow into the coronary effluent of isolated guinea pig hearts that peaked between the 4th and 10th minutes of reperfusion (Figure IIA in the online-only Data Supplement). IPC, ψeRACK pretreatment (500 nmol/L, two 5-minute cycles), and reperfusion with the PKCδ antagonist V1-1 (500 nmol/L) each and all reduced total CPK overflow (0 to 20 minutes of reperfusion) by ~50% (Figure IIB in the online-only Data Supplement). However, whereas ψeRACK pretreatment markedly reduced the activation of RAS, thus displaying cardioprotective anti-RAS effects, reperfusion with δV1-1 did not affect renin and norepinephrine release or alleviate VT/VF, thus failing to protect the heart from the consequences of mast cell degranulation, including induction of VT/VF (Figure IIC in the online-only Data Supplement). These findings indicate that the cardioprotective anti-RAS effects of IPC are mediated by a separate pathway that is independent of cardiac myocyte salvage but directly dependent on the modulation of renin release from cardiac mast cells.

**Discussion**

The notion of a local tissue-specific RAS, in addition to the classic circulating RAS, has now gained general recognition.38,39 Renin, the rate-limiting step in RAS activation, has been found in cardiac myocytes,38,40 and renin mRNA has been identified in heart fibroblasts and endothelial and smooth muscle cells of coronary vessels.38 Our laboratories recently demonstrated that mast cells can synthesize and secrete renin.2,3 I/R causes the release of renin from cardiac mast cells, activating a local RAS, which results in severe arrhythmic dysfunction.3

We have now uncovered a novel cardioprotective anti-RAS paradigm of IPC and delineated its transductional pathway. IPC prevents I/R-induced renin release from cardiac mast cells via an adenosine-mediated activation of PKCδ in these cells, followed by activation of mitochondrial ALDH2, which effectively prevents mast cell degranulation. Given the pivotal role played by mast cell renin in local RAS activation,1–3 we propose that the cardioprotective anti-RAS effect of IPC, typified by a marked decrease in the overflow of β-hexosaminidase, renin, and norepinephrine, and the curtailing of VT/VF are based on an inhibitory action at the mast cell level. This novel IPC paradigm is unlike classic IPC. In classic IPC, myocytes are the main target of cardioprotection, and IPC is characterized by infarct-size reduction and improved recovery of contractility.4–6

Although the adenosine A1 receptor has often been associated with IPC-induced protection of cardiac myocytes,5,8 we found here that the anti-RAS effects of IPC do not involve A1 receptors because A1 receptor blockade failed to modify the anti-RAS effects of IPC. This agrees with the reported lack of A1 receptors in mast cells41 and with our proposal that mast cells are the critical site at which the cardioprotective anti-RAS effects of IPC develop.
Figure 6. Activation of adenosine A2b and A3 receptors in combination or PKC ε increases ALDH2 activity in HMC-1 cells. Selective inhibition of the PKC ε isoform prevents the increase in ALDH2 activity by the combined activation of adenosine A2b and A3 receptors. Incubation of HMC-1 cells with the selective ALDH2 activator Alda-1 (100 μmol/L for 10 minutes), the PKC ε activator PMA (300 nmol/L for 10 minutes), the PKC ε-selective activator ωRACK (0.5 and 1 μmol/L for 30 minutes), or the adenosine A2b and A3 receptor agonists LUF5835 and IB-MECA in combination (both at 50 nmol/L for 1 hour) increases ALDH2 activity (measured by the rate of NADH production at 340 nm). Pretreatment of HMC-1 cells with the PKC ε antagonist ωV1-2 (1 μmol/L for 20 minutes) prevents the effects of A2b and A3 receptor activation. Bars are mean percent increases from control (#SEM; n = 4 to 8). Basal NADH production was 3.63 ± 0.25 μmol·min⁻¹·mg⁻¹ protein. *P < 0.05, **P < 0.01, ***P < 0.001 from control; #P < 0.05 from the combination of A2b and A3 receptor agonists by Kruskal-Wallis test followed by the posthoc Dunn test.

Mast cells are known to express both adenosine A2b and A3 receptors42,43 (see also Figure 4). We found that the combined activation of A2b and A3 receptors displayed IPC-like effects: It attenuated the release of renin and norepinephrine and alleviated reperfusion arrhythmias associated with I/R. These cardioprotective anti-RAS effects were similar to those afforded by IPC, which in fact was abolished by blockade of adenosine A2b and A3 receptors in combination. Although A2b and A3 receptors are known as low-affinity receptors (Kᵢ ≥5 and 1 μmol/L for A2b and A3, respectively),44 both were likely activated by endogenous adenosine during IPC. Indeed, interstitial adenosine was shown to reach a 7-μmol/L level after 6 minutes of IPC in the isolated heart.45

Significantly, the combined activation of A2b and A3 receptors in HMC-1 cells in culture also prevented peroxide-induced degranulation and renin release, supporting our conclusion that these mast cell receptors play a major role in the anti-RAS effects of IPC. Activation of either A2b or A3 receptor alone failed to mimic the cardioprotective effects of IPC, demonstrating the necessity that both receptors be activated for the initiation of IPC. Other actions of adenosine such as coronary dilatation have also been shown to require a concomitant activation of both A2b and A3 receptors.46

That the activation of A2b and A3 receptors in HMC-1 cells prevents degranulation and renin release concurs with the protective antisecretory effect of A2b receptors, demonstrated by enhanced mast cell activation when A2b receptors are deleted in mice.47 Yet, other investigators have shown that activation of A2b and A3 receptors promotes the release of mediators and cytokines from human lung fragments, rat RBL-2H3 cells, HMC-1 cells, and macrophages from A2b receptor–deleted mice.42,43,44 The discrepancy between these findings and ours most likely depends on differences in cells and animal species and on the different stimuli used to degranulate mast cells.

Opening of Kᵢ,ATP channels in the inner membrane of mitochondria in cardiac myocytes has been found to contribute to the protective effects of classic IPC.6–8 Yet, the mKᵢ,ATP channel antagonist 5-hydroxydecanoate27 failed to modify the cardioprotective anti-RAS effects of IPC, indicating that these mitochondrial channels are not involved in the mast cell–dependent anti-RAS effects of IPC.

Having established that activation of A2b and A3 receptors contributes to the anti-RAS effects of IPC, we asked whether...
A2b and A3 receptors might signal via PKCε, given that this isoform has both cardioprotective and antiproteolytic properties in mast cell–like RBL-H3 cells. Indeed, we found that selective blockade of PKCε with ψeRACK mimicked them. Notably, the IPC-like antiproteolytic effects of A2b and A3 receptor agonists in combination were prevented by selective PKCε inhibition. Collectively, at this point, our findings suggested that the initial step in the anti-RAS signaling sequence of IPC could be an adenosine-induced stimulation of A2b and A3 receptors resulting in PKCε translocation in cardiac mast cells. In fact, A2b and A3 receptor activation in HMC-1 cells caused the translocation of PKCε from cytosol to membrane, the hallmark of PKCε activation.

Acetaldehyde and 4-HNE are formed during I/R, in part by lipid peroxidation caused by reactive oxygen species such as hydrogen peroxide. These toxic aldehydes, known to elicit mast cell degranulation, can be removed by mitochondrial ALDH2, a phosphorylation target of PKCε. Indeed, we found that Alda-1, a selective ALDH2-activating drug, not only mimicked the cardioprotective anti-RAS effects of IPC in the guinea pig heart ex vivo but also prevented the degranulating effects of acetaldehyde, H2O2, and 4-HNE in HMC-1 cells in culture. These effects were abolished by selective inactivation of ALDH2 with GTN. Similarly, pretreatment of HMC-1 cells with GTN prevented the antidegranulating effects caused by activation of A2b and A3 receptors or PKCε. Hence, these findings add further support to the proposal that activation of ALDH2 in cardiac mast cells is the final crucial step of the protective anti-RAS pathway.

Because A2b and A3 receptors, PKCε, and ALDH2 are also present in cardiac myocytes, and ALDH2 prevents renin release and RAS activation. As the search for cardioprotective drugs continues unabated, our findings elucidate novel basic mechanisms of pharmacological cardioprotection (ie, the detoxification of reactive aldehydes and ROS in I/R by increasing the catalytic activity of mitochondrial ALDH2, thus alleviating the dysfunctional consequences of RAS activation in the heart). This new finding suggests that in addition to protecting cardiac myocytes from I/R-induced injury, drugs that prevent cardiac mast cell degranulation may prevent the activation of a local RAS, thus providing an additional benefit to patients with myocardial infarction and perhaps in other cardiac oxidative stress conditions such as those occurring in heart failure.

Conclusions

We propose that adenosine released from various cells during I/R and IPC activates A2b and A3 receptors on the surface of cardiac mast cells and that this is followed by activation/translocation of PKCε, which then increases the catalytic activity of mitochondrial ALDH2 (see Figure 8). By eliminating reactive aldehydes and their mast cell–degranulating effects, ALDH2 prevents renin release and RAS activation. This reduces Ang II formation, inhibits excessive norepinephrine release, and prevents the generation of reperfusion arrhythmias. Notably, the relevance of our findings extends beyond the disclosure of a new IPC mechanism. Indeed, although the discovery of IPC has generated a wealth of studies, its clinical translation has yet to come to fruition.

The heart may contribute to renin production and RAS activation in I/R. In fact, the incidence of reperfusion arrhythmias was markedly reduced, but not completely abolished, in hearts isolated from mice lacking mast cells or in guinea pig hearts perfused with mast cell–stabilizing agents. This suggests that other cells in the heart could release renin in response to I/R.

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Disclosure

Dr Mochly-Rosen is the founder of KAI Pharmaceuticals Inc, a company that plans to bring PKC regulators to the clinic. However, none of the work in her laboratory is in collaboration with or supported by the company. The other authors report no disclosures.

References


**CLINICAL PERSPECTIVE**

Ischemia/reperfusion is a known cause of cardiac dysfunction that is often accompanied by infarction, contractile failure, and severe arrhythmias. The production of reactive oxygen species and reactive aldehydes is considered a prominent cause because aldehyde detoxification by mitochondrial aldehyde dehydrogenase type-2 displays cardioprotective effects. We recently reported that ischemia/reperfusion elicits the release of renin from cardiac mast cells, which in turn activates a local renin-angiotensin system, causing severe arrhythmic dysfunction. Reactive oxygen species and reactive aldehydes are likely responsible for mast cell degranulation and renin release; hence, means to prevent such effects could be clinically beneficial. We describe here a novel pathway that is independent of cardiomyocyte salvage and relies instead on aldehyde dehydrogenase type-2 activation in mast cell mitochondria. Aldehyde dehydrogenase type-2 detoxifies reactive aldehydes produced in ischemia/reperfusion, thus preventing mast cell degranulation and renin release. This avoids the activation of a local renin-angiotensin system and reperfusion arrhythmias. This new finding suggests that in addition to protecting cardiac myocytes from ischemia/reperfusion-induced injury, drugs that prevent cardiac mast cell degranulation may prevent the activation of a local renin-angiotensin system, thus providing an additional benefit to patients with myocardial infarction and perhaps in other cardiac oxidative stress conditions such as those occurring in heart failure.
Aldehyde Dehydrogenase Activation Prevents Reperfusion Arrhythmias by Inhibiting Local Renin Release From Cardiac Mast Cells
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Supplemental Materials

MATERIALS AND METHODS

Animals. 132 male Hartley guinea pigs weighing 300-350 grams (Charles River Laboratories, Kingston, NY) were used for the study. All experiments were approved by the Institutional Animal Care and Use Committee of Weill Cornell Medical College.

Perfusion of guinea-pig hearts ex vivo. Guinea pigs were anesthetized with CO₂ and euthanized by stunning. Hearts were rapidly isolated and perfused at constant pressure (55 cm H₂O) with oxygenated Ringer's solution (NaCl 154 mmol/L, KCl 5.61 mmol/L, CaCl₂ 2.16 mmol/L, NaHCO₃ 5.95 mmol/L and dextrose 5.55 mmol/L) at 37°C in a Langendorff apparatus (Radnoti Glass Technology Inc., Monrovia, CA).

Ischemia/reperfusion (I/R): following an equilibration period all hearts were subjected to 20-min global ischemia induced by complete cessation of coronary perfusion, followed by 30-min reperfusion (I/R). Ischemic preconditioning (IPC): hearts were subjected to 2 x 5-min cycles of ischemia, each followed by 5-min reperfusion. Pharmacological prevention of IPC: hearts were perfused with a given antagonist for 20 min (30 min for GTN) before and during IPC, and then washed out for 15 min before I/R. Pharmacological preconditioning: hearts were perfused for 2 x 5-min cycles with a given agent, except for the selective PKCδ inhibitor δV1-1 which was administered during the entire 30-min reperfusion following the 20-min ischemia. Prevention of pharmacological preconditioning: hearts were perfused with a given antagonist for 20 min (30 min for GTN) before
and during pharmacological preconditioning. All agents were then washed out for 15 min before I/R.

Coronary flow was measured by timed collections of the effluent every 2-min; all samples were assayed for renin and norepinephrine, some for β-hexosaminidase and creatine phosphokinase (CPK). Surface ECG was obtained from leads attached to the left ventricle and the right atrium, recorded in digital format through each experiment, and analyzed using Power Lab/8SP (AdInstrument, Colorado Springs, CO). Onset and duration of reperfusion arrhythmias were recorded and evaluated according to the Lambeth conventions.1

Cell culture. The human mastocytoma cell line (HMC-1) was kindly provided to us by Dr. I. Biaggioni (Vanderbilt University, Nashville, TN) and J. H. Butterfield (Mayo Clinic, Rochester, MN). Cells were maintained in suspension culture at high density in Iscove’s modified Dulbecco’s medium supplemented with 10% FBS and 1.2 mmol/L monothioglycerol and kept at 37°C, 5% CO₂.

β-Hexosaminidase and renin assay. Coronary effluent from guinea-pig hearts ex vivo was assayed for β-hexosaminidase using the method of Schwartz et al.2 Samples of coronary effluent (20 µL) were placed in the well of a 96-well plate and total β-hexosaminidase content was determined. For the measurement of renin overflow, coronary effluent was immediately concentrated 8-fold by centrifugal filtration (Millipore, Billerica, MA). Concentrated samples were incubated overnight with human angiotensinogen and then renin activity (angiotensin I formed) was determined by GammaCoat Plasma Renin Activity ¹²⁵I
Radioimmunoassay (DiaSorin, Stillwater, MN). The detection limit was approximately 0.01 pmol.

For HMC-1 cells, 4 to 6 pooled confluent flasks were pelleted and cells washed twice with Ringer buffer (pH 7.4). Pelleted HMC-1 were then resuspended in Ringer buffer and equal volumes of cell suspension were aliquoted in Eppendorf tubes and incubated at 37°C with gentle agitation. Cell suspensions were then incubated with a given agent (i.e., Alda-1, ψεRACK or LUF5835 + IBMECA) for 10 min (preceded or not by a 30-min incubation with GTN). Acetaldehyde, H₂O₂ or 4-HNE was subsequently added to the incubation mixture for 20 minutes. At the end of protocol, samples were transferred to ice and then centrifuged at 1,000 g for 10 min. Supernatants were collected and kept in ice until β-hexosaminidase and renin release was evaluated. Cell pellets were lysed with 0.5% triton X-100 and centrifuged at 13,000 g. Supernatants were then used for protein and total β-hexosaminidase content determination. All results were normalized and expressed as percent above control.

**NE assay.** Coronary effluent was assayed for norepinephrine by HPLC with electrochemical detection as previously described.³ The detection limit was approximately 0.2 pmol.

**CPK assay.** Coronary effluent was assayed for creatine phosphokinase release using a creatine kinase assay kit (Genzyme Diagnostics, Charlottetown, PE, Canada). Substrate and buffer reagent were added to the collected coronary effluent and then incubated at 37°C for 2 min. The change in absorbance at 340
nm at one-min intervals was recorded in the spectrometer until the change in absorbance was constant.

**PCR and immunostaining.** **RT-PCR:** total RNA was extracted from HMC-1 cells using TRIzol reagent (Invitrogen, Carlsbad, CA), 1 µg of total RNA from each sample was reverse-transcribed and cDNA amplified by RT-PCR using a QIAGEN One-step RT-PCR kit. The sense primers specific for adenosine A<sub>2b</sub>- and A<sub>3</sub>-receptors were: 5'-TAAGATCTTCCCTGTGGCCT-3', and 5'-AGATGCCCAACACAGCAG-3', respectively. Antisense primers specific for adenosine A<sub>2b</sub>- and A<sub>3</sub>-receptors were: 5'-GCTTGGCAGAGAAGATAC-3', and 5'-ATCTGCCGTAAGCTTGACC-3', respectively. The amplification profile used was: 50°C for 30 min, 95°C for 15 min, then 94°C for 30 sec, 55°C for 30 sec, 72°C for 1.5 min (40 cycles), and finally 72°C for 10 min. PCR products generated were, ~330 and ~354 bp for adenosine A<sub>2b</sub>- and A<sub>3</sub>-receptor, respectively. PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining.

**Immunostaining:** HMC-1 cells were fixed and permeabilized on glass slides and stained with the goat anti-A<sub>2b</sub>-receptor Ab (Santa Cruz Biotechnology, Santa Cruz, CA)(1:200) conjugated to Alexa Fluor 488 donkey anti-goat IgG (1:500) and with rabbit anti-A<sub>3</sub>-receptor Ab (Santa Cruz) (1:200) conjugated to Alexa Fluor 488 donkey anti-rabbit IgG (1:500). Nuclei were stained with DAPI. For viewing immunofluorescence, cells were examined with an inverted fluorescence microscope (Nikon Eclipse TE 2000-U) interfaced to an electron
multiplying charge coupled device (Hamamatsu) and processed with Metamorph software (version 6.2; Universal Imaging Corp.).

**Translocation of PKCε.** Following incubation of HMC-1 cells with PMA, the PKCε-selective agonist ψεRACK, or the adenosine A2b- and A3-receptor agonists LUF5835 and IB-MECA in combination, cells were homogenized in buffer (200 µL) (20 mmol/L Tris- HCl, 2 mmol/L EDTA, 10 mmol/L EGTA, 0.25 M sucrose, β-mercaptoethanol and 1X protease inhibitors cocktail). Cell homogenates were then passed through syringes with needle (30 Gauge) 10 times, and cell lysates were centrifuged at 100,000 g for 30 min to collect cytosolic fractions (supernatant). The pellets were resuspended in homogenization buffer (50 µL) with 1% Triton X-100, and then centrifuged at 100,000 g for 30 min to collect membrane fractions (supernatant). Translocation of PKCε was assessed by using a PKCε-specific antibody (Santa Cruz; 1:1000 dilution) in Western blot analysis. Methods for Western blot analysis were as previously described. The ratio of PKCε in membrane to that in cytosol was expressed as PKCε translocation.

**ALDH2 enzymatic activity assay.** Enzymatic activity of ALDH2 in HMC-1 cells was determined spectrophotometrically by monitoring the reductive reaction of NAD⁺ to NADH at 340 nm as previously described. The assays were carried out in 50 mmol/L sodium pyrophosphate buffer, pH = 9.0, at 25°C. 300 µg of cell lysates and 2.5 mmol/L NAD were added to the buffer. To start the reaction, 10 mmol/L acetaldehyde was added and the accumulation of NADH was recorded for 3 min with measurements being taken every 15 s. ALDH2 reaction rates were
calculated as μmol NADH/min/mg proteins, and compared to cells that were treated with Na Ringer (control) and expressed as % increase from control.

**Drugs and chemicals.** Acetaldehyde (17.8 M) and H$_2$O$_2$ (30% w/w, 9.8 M) were purchased from Sigma-Aldrich (St. Louis, MO); 4-hydroxy Nonenal (4-HNE) in ethanol solution was purchased from Cayman Chemical and dissolved in DMSO to 300 mmol/L stock concentration after solvent evaporation. DMSO concentration during experiments was always below 0.1% and did not affect the cell response. LUF5825 was a gift from Dr. M.W. Beukers (University of Leiden, Leiden, Netherlands); EXP3174 was a gift from Merck Sharp & Dohme Ltd (Whitehouse Station, NJ); ψεRACK, δV1-1 and Alda-1 were synthesized in the Mochly-Rosen lab (Stanford University School of Medicine, Palo Alto, CA). IBMECA, MRS1754, MRS1523, DPCPX, chelerythrine, 5-hydroxydecanoate and cyanamide were purchased from Sigma-Aldrich. Phorbol 12-myristate 13-acetate was purchased from LC Laboratories (Woburn, MA). Glyceryl trinitrate was purchased from Hospira Inc. (Lake Forest, IL). Human plasma angiotensinogen was purchased from Calbiochem (San Diego, CA). Cyanamide and chelerythrine were dissolved in water; EXP3174, DPCPX and 5-hydroxydecanoate were dissolved in ethanol; IBMECA, MRS1754, MRS1523, phorbol 12-myristate 13-acetate, and Alda-1 were dissolved in DMSO.

**Figure Legends**

**Figure 1.** IPC reduces renin and NE release, and shortens arrhythmias caused by I/R in guinea-pig hearts ex vivo. This cardioprotective anti-RAS
effect is mimicked or prevented by activation or blockade of adenosine A$_{2b}$- and A$_{3}$-receptors in combination, but unaffected by adenosine A$_{1}$-receptor blockade (Panel A), and mimicked by PKC$\varepsilon$ activation and prevented by PKC$\varepsilon$ inhibition (Panel B). Panel A: Coronary overflow of renin and NE, and duration of reperfusion arrhythmias (VT/VF) in isolated hearts subjected to 20-min global ischemia and 30-min reperfusion ($n=6$). In other hearts, I/R was preceded by IPC (2 x 5-min ischemia cycles each followed by 5-min reperfusion; $n=8$). Other hearts were subjected to I/R preceded by IPC in the presence of DPCPX (300 nmol/L, adenosine A$_{1}$-receptor antagonist) or MRS1754 and MRS1523 (50 and 100 nmol/L, adenosine A$_{2b}$- and A$_{3}$-receptor antagonists, respectively) ($n=6$ each). Instead of IPC, other hearts were perfused with a combination of A$_{2b}$- (LUF 5835; 50 nmol/L) and A$_{3}$-receptor agonists (IBMECA; 50 nmol/L) for 2 x 5-min cycles each followed by 5-min washout before I/R ($n=6$).

Panel B: Hearts underwent I/R preceded by IPC either in the absence ($n=8$) or presence ($n=5$) of the selective PKC$\varepsilon$ inhibitor V1-2 (1 µmol/L). Additional hearts were perfused with the selective PKC$\varepsilon$ activator $\psi$V1-2 instead of IPC (500 nmol/L, 2 x 5-min cycles each followed by 5-min reperfusion; $n=5$) before I/R. Basal, pre-ischemic overflows of active renin (i.e., ANG I formed) and NE were $6.81 \pm 2.12$ pg/hr/g and $4.02 \pm 0.77$ pmol/g, respectively. Bars represent means ± SEM of overflows during the first 4 min of reperfusion or duration of VT/VF. ** And ***, $P<0.01$ and $P<0.001$, respectively, from I/R; † and ††, $P<0.05$ and $P<0.01$ from IPC, respectively, by Mann-Whitney test (for I/R vs LUF5835 + IBMECA or $\psi$V1-2) or Kruskal-Wallis with Dunn’s multiple comparison test (for all others).
Figure 2. Selective inhibition of the PKCε isoform prevents the cardioprotective IPC-like anti-RAS effects generated by the combined activation of adenosine A2b- and A3-receptors (Panel A). Selective desensitization of ALDH2 with glyceryl trinitrate (GTN) prevents the cardioprotective IPC-like anti-RAS effects generated by PKCε activation (Panel B). Panel A: Coronary overflow of renin and NE, and duration of VT/VF in ex vivo guinea-pig hearts subjected to I/R (n=6). Instead of IPC, other hearts were perfused with a combination of selective A2b- (LUF 5835; 50 nmol/L) and A3-receptor agonists (IBMECA; 50 nmol/L) for 2 x 5-min cycles each followed by 5-min washout before I/R (n=5). Other hearts were perfused with the selective PKCε inhibitor εV1-2 (1 µmol/L), followed by a combination of A2b- (LUF 5835; 50 nmol/L) and A3-receptor agonists (IBMECA; 50 nmol/L) for 2 x 5-min cycles each followed by 5-min washout instead of IPC before I/R (n=6). Panel B: Instead of IPC, hearts were perfused with the selective PKCε activator (ψεRACK; 500 nmol/L) for 2 x 5-min cycles each followed by 5-min washout before I/R (n=5). Other hearts were perfused with the ALDH2 desensitizer glyceryl trinitrate (GTN; 2µmol/L, perfused for 30 min), followed by ψεRACK (500 nmol/L) for 2 x 5-min cycles each followed by 5-min washout instead of IPC before I/R (n=5). Bars represent means ± SEM of overflows during the first 4 min of reperfusion or duration of VT/VF. ** And ***, P<0.01 and P<0.001 from I/R, respectively; † and ††, P<0.05 and P<0.01, respectively from the A2bR and A3R agonist combination.
(Panel A), and †, P<0.05 from ψεRACK (Panel B), by Mann-Whitney test (for I/R vs IPC) or Kruskal-Wallis test with Dunn’s multiple comparison test (for all others).

**Figure 3.** Selective activation of ALDH2 with Alda-1 mimics the cardioprotective anti-RAS effects of IPC. General ALDH inhibition with cyanamide or selective desensitization of ALDH2 with glyceryl trinitrate (GTN), each prevents the cardioprotective anti-RAS effects of IPC and Alda-1. Panel A: Coronary overflow of renin and NE, and duration of VT/VF in guinea-pigs hearts subjected to I/R (n=6) or I/R preceded by IPC either in the absence (n=8) or presence (n=5) of the ALDH inhibitor cyanamide (CYA; 5 mmol/L). Other hearts were perfused with the selective ALDH-2 activator Alda-1 instead of IPC (20 µmol/L, 2 x 5-min cycles each followed by 5-min washout; n=5) before I/R. Other hearts were perfused with CYA (5 mmol/L), followed by Alda-1 (20 µmol/L, 2 x 5 min cycles each followed by 5-min reperfusion) instead of IPC before I/R (n=5). Panel B: Hearts were pretreated with the ALDH2 desensitizer glyceryl trinitrate (nitroglycerin, GTN; 2 µmol/L, perfused for 30 min) and then subjected to I/R preceded by IPC (n=5). Other hearts were perfused with the selective ALDH-2 activator Alda-1 instead of IPC (20 µmol/L, 2 x 5-min cycles each followed by 5-min reperfusion; n=5) before I/R. Other hearts were perfused with GTN (2 µmol/L) for 30 min, followed by Alda-1 (20 µmol/L, 2 x 5 min cycles each followed by 5-min reperfusion) instead of IPC before I/R (n=5). Bars represent means ± SEM of overflows collected during the first 4 min of reperfusion or duration of VT/VF. *, ** And ***, P<0.05, P<0.01 and P<0.001 from I/R,
respectively; *, ** and ###, P<0.05, P<0.01 and <0.001 from IPC, respectively; † and ††, P<0.05 and P<0.01 from Alda-1, respectively, by Kruskal-Wallis test with Dunn’s multiple comparison test.

Figure 4. Expression of adenosine receptors by HMC-1 cells. ψεRACK or adenosine A2b- and A3-receptor agonists in combination induces selective activation of PKCε in HMC-1 cells. A, Ethidium bromide gel of transcripts of the adenosine A2b- and A3-receptor sub-types in HMC-1 cells. B, Immuno-staining of HMC-1 cells expressing the adenosine A2b- and A3-receptor sub-types. Nuclei (blue) are stained with DAPI. Scale bar = 10 μm. C and E, Translocation of PKCε (i.e., activity of PKCε) was determined by Western blot analysis in cytosolic and membrane fractions of HMC-1 cells. C, Control cells were incubated with Na Ringer for 30 min demonstrating the basal activity of PKCε. Incubation of HMC-1 cells with the PKC activator phorbol ester myristate (PMA) 300 nmol/L for 7 min (positive control) or the PKCε activator ψεRACK 500 nmol/L for 30 min, prior to an additional 7-min incubation with a below-threshold concentration of PMA (3 nmol/L), elicits an increase in PKCε translocation from cytosol to membrane (i.e., PKCε activation). Notably, treatment with 3 nmol/L PMA for 7 min alone does not cause PKCε translocation. E, Control cells were incubated with Na Ringer for 1 hr demonstrating the basal activity of PKCε. Incubation of HMC-1 cells with 300 nmol/L PMA for 7 min (positive control) or the adenosine A2b- and A3-receptor agonists LUF5835 and IB-MECA in combination (both at 50 nmol/L; 1 hour) elicits an increase in PKCε translocation from cytosol to membrane (i.e., PKCε
activation). D and F, quantification of PKC\(_{\varepsilon}\) translocation from cytosolic to membrane fractions, expressed as % of control. Bars are means ± SEM (D, \(n=4-6\); F, \(n=7\)) normalized relative to \(\beta\)-actin. * And **, \(P<0.05\) and \(P<0.01\), from control respectively, by Kruskal-Wallis test followed by post-hoc Dunn’s test.

Figure 5. Mast cell degranulation and renin release elicited by acetaldehyde (A and D), \(H_2O_2\) (B and E) and 4-HNE (C and F): blockade by Alda-1 and its reversal by pretreatment with GTN. Incubation of HMC-1 cells with acetaldehyde (300-700 \(\mu\)mol/L, 20 min), \(H_2O_2\) (0.1-1 mmol/L, 20 min) or 4-HNE (3-30 \(\mu\)mol/L, 20 min) elicits a concentration-dependent release of both \(\beta\)-HEX and renin. Pre-incubation of HMC-1 cells with the ALDH2 activator Alda-1 (20 \(\mu\)mol/L, 10 min) attenuates the acetaldehyde-, \(H_2O_2\)- and 4-HNE-induced release of \(\beta\)-HEX and renin. Pre-treatment of HMC-1 cells with the ALDH2-desensitizer glyceryl trinitrate (GTN, 2 \(\mu\)mol/L, 30 min) prevents the effects of Alda-1. Points are means (± SEM; \(n=3-17\)). *, ** and ***, \(P<0.05\), \(P<0.01\) and \(P<0.001\), from control, respectively; #, ## and ###, \(P<0.05\) and \(P<0.01\), from Alda-1 + GTN respectively, by Kruskal-Wallis with Dunn’s multiple comparison test.

Figure 6. Activation of adenosine A\(_{2b}\)- and A\(_3\)-receptors in combination or PKC\(_{\varepsilon}\) increases ALDH2 activity in HMC-1 cells. Selective inhibition of the PKC\(_{\varepsilon}\) isoform prevents the increase in ALDH2 activity by the combined activation of adenosine A\(_{2b}\)- and A\(_3\)-receptors. Incubation of HMC-1 cells with the selective ALDH2 activator Alda-1 (100 \(\mu\)mol/L; 10 min), the PKC activator
phorbol ester myristate (PMA) (300 nmol/L; 10 min), the PKCε-selective agonist γεRACK (0.5 and 1 µmol/L; 30 min), or the adenosine A2b- and A3-receptor agonists LUF5835 and IB-MECA in combination (both at 50 nmol/L; 1 hour) increases ALDH2 activity (measured by the rate of NADH production at 340 nm).

Pre-treatment of HMC-1 cells with the PKCε antagonist εV1-2 (1 µmol/L; 20 min) prevents the effects of adenosine A2b- and A3-receptor activation. Bars are mean percent increases from control (±SEM; n=4-8). Basal NADH production was 3.63 ± 0.25 µmol/min/mg protein. *, ** and ***, P<0.05, P<0.01 and P<0.001 from control, respectively, by Kruskal-Wallis test followed by post-hoc Dunn’s test. #, P<0.05 from the A2b- and A3-receptor agonists combination by Kruskal-Wallis test followed by post-hoc Dunn’s test.

Figure 7. Activation of adenosine A2b- and A3-receptors (A and C), or PKCε (B and D), inhibits mast cell degranulation and renin release elicited by H2O2: prevention by GTN pretreatment. Incubation of HMC-1 cells with H2O2 for 20 min elicits a concentration-dependent release of both β-HEX and renin. Pre-incubation of HMC-1 cells with the adenosine A2b- and A3-receptor agonists LUF5835 and IB-MECA (both at 50 nmol/L, 10 min) in combination, or with the PKCε activator (γεRACK, 500 nmol/L, 10 min) attenuates the H2O2-induced release of β-HEX and renin. Pre-treatment of HMC-1 cells with the ALDH2 desensitizer glyceryl trinitrate (GTN, 2 µmol/L, 30 min) prevents the effects resulting from the activation of A2b- and A3-receptors and PKCε. Points are means (± SEM; n=3-19). *, ** and ***, P<0.05, P<0.01 and P<0.001, from control,
respectively; #, ## and ###, P<0.05, P<0.01 and P<0.001 from LUF5835 + IB-MECA + GTN or ψεRACK + GTN, respectively, by Kruskal-Wallis with Dunn’s multiple comparison test.

Figure 8. Proposed mechanisms of the cardioprotective anti-RAS effects of ischemic preconditioning.

Legend to Supplemental Figure I. The cardioprotective anti-RAS effects of IPC are unaffected by mitochondrial K$_{ATP}$ channel blockade or angiotensin AT$_1$-receptor blockade (Panel A), and mimicked by general PKC activation and prevented by general PKC inhibition (Panel B). Panel A: Coronary overflow of renin and NE, and duration of VT/VF in ex vivo guinea-pig hearts subjected to I/R (as in Fig.1; n=6). Other hearts underwent I/R preceded by IPC in the presence of 5-hydroxydecanoate, a mitochondrial K$_{ATP}$ channel blocker (5-HD; 100 µmol/L; n=6), or EXP3174, an angiotensin AT$_1$-receptor antagonist (300 nmol/L; n=5). Panel B: Hearts were pretreated with the general PKC inhibitor chelerythrine (2.8 µmol/L; Chel) and then subjected to I/R preceded by IPC (n=6). Additional hearts were perfused with the general PKC activator phorbol ester myristate (0.05 nmol/L; PMA) instead of IPC (2 x 5-min cycles + 5-min reperfusion; n=6) before I/R. Bars are means ± SEM of overflows during the first 4 min of reperfusion or duration of VT/VF. ** And ***, P<0.01 and <0.001 from I/R, respectively; †† and †††, P<0.01 and P<0.001 from IPC, respectively, by Mann-
Whitney test (for I/R vs PMA) or Kruskal-Wallis with Dunn’s multiple comparison test (for all others).

**Legend to Supplemental Figure II.** Selective inhibition of PKCδ during reperfusion following ischemia prevents CPK release from isolated guinea-pig hearts (Panel A and B), but fails to affect the cardioprotective anti-RAS effect of IPC (Panel C). Time course of CPK overflow (Panel A) and total CPK release during 20 min of reperfusion (Panel B) in *ex vivo* guinea-pig hearts subjected to I/R preceded or not by IPC or PKCε activation (with ψεRACK, 500 nmol/L) (n=5-7). Other hearts underwent I/R and received the selective PKCδ inhibitor δV1-1 (500 nmol/L, n=4) during the 30-min reperfusion. Panel C: Coronary overflow of renin, NE, and duration of VT/VF, in *ex vivo* guinea-pig hearts subjected to I/R, preceded or not by IPC (n=8 and 6, respectively) or the selective PKCε activator ψεRACK (500 nmol/L) for 2 x 5-min cycles each followed by 5-min washout (n=5). Other hearts underwent I/R and received the selective PKCδ inhibitor δV1-1 (500 nmol/L) during the 30-min reperfusion (n=4). In Panel A and B, bars represent means ± SEM of overflows collected during the first 20 min of reperfusion. In Panel C, bars represent means ± SEM of overflows collected during the first 4 min of reperfusion or duration of VT/VF. *, ** And ***, P<0.05, P<0.01 and P<0.001 from I/R, respectively, by Mann-Whitney test.

**Reference List**


Supplemental Figure I

**VT/VF duration (seconds)**

- **A**: NS
- **B**: NS

*** NE overflow (pmol/g)

- **A**: NS
- **B**: NS

**** Renin overflow (ANG I formed; pg/hr/g)

- **A**: NS
- **B**: NS

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Experiment conditions:
- 5-HD
- EXP3174
- IPC
- Chel
- PMA

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Ischemia/Reperfusion