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Purine release: A protective signaling mechanism of the mitochondrial permeability transition pore in ischemia

Відомо, що реперфузійні порушення функції серця є наслідком процесів, які зумовлені відкриванням мітохондріальної пори (МП). Утворення МП є ключовим моментом у розвитку апоптозу та супроводжується вивільненням з мітохондрій цілої низки сигнальних молекул. Автори припустили можливість появи та сигнальної ролі пуринів при ішемічному прекондиціюванні серця. Перевірці цієї гіпотези присвячена наша робота. Показано, що при відкриванні під дією Ca^{2+} МП з ізольованих мітохондрій вивільнювались аденозин, інозин і 3'-рибозил монофосфат сечової кислоти (3'-РМФСК), а з ізольованих сердець після ішемії-реперфузії - інозин та похідні 3'-РМФСК. В обох випадках – на мітохондріях та ізольованому серці – поява зазначених сполук блокувалася інгібіторами пори циклоспорином А і сангліферином А. Так само вивільнення цих сполук пригнічувалося при ішемічному прекондиціюванні серця. Обговорюється можливе сигнальне значення пуринів при відкриванні МП при ішемічному прекондиціюванні серця та їх роль в кардіопротекції.

INTRODUCTION

A decisive event in the pathology of cardiac ischemia-reperfusion (IR) injury is opening of the permeability transition pore (PTP), resulting in cytochrome *c* release and cell death [7, 11, 19, 22]. Several factors that regulate the PTP are implicated in either IR injury or cardioprotection, including Ca^{2+} [33], reactive oxygen species (ROS) [6], nitric oxide [6], ATP/ADP ratio [13], and pH balance [29]. Consistent with this, PTP inhibitors such as cyclosporin A (CsA) and sanglifehrin A (SfA) afford protection against IR injury [10,18].

Apoptotic signaling by the mitochondrial proteins released upon PTP opening (e.g. cytochrome *c*, AIF) is well-characterized [11, 34], but pore opening also releases mitochondrial solutes (<1500 Da) and little attention has been paid to the downstream signaling roles of such molecules. Given the important role of the PTP in IR injury, it is interesting to note that several studies have characterized solutes released from the heart during IR injury [2, 12,16,20,25,38], in particular purines [2,20,25]. The sub-cellular origin of these purines is unclear, but the observation that CsA decreases tissue AMP levels following cardiac IR [18] led us to hypothesize that purines release from mitochondria during PTP opening may by required for IPC signaling.

Opening of the PTP is generally considered a pathologic phenomenon, but recently it was shown that transient PTP opening is essential for the cardioprotective benefits of ischemic preconditioning (IPC) [23]. Events downstream of the PTP that underlie this phenomenon are poorly understood, but since purines have a well-defined role in IPC signaling [20], we hypothesized that purines may be the missing link between PT pore opening and cardioprotection in IPC.

METHODS

Male Sprague-Dawley rats (150g) were handled in accordance with the *"Guide for the care and use of laboratory animals"* (NIH Publication #85-23, 1996).

Heart perfusions: Langendorff perfusion with Krebs-Henseleit (KH) buffer was performed as previously described [20], in constant flow mode (12 ml/min/gram). Following ~20 min. equilibration, hearts were subject to 25 min global normothermic ischemia then 30 min. of reperfusion. Normoxic controls were perfused for a matching time period. In some experiments CsA (0.2 μM) or SfA (1 μM) were infused for 10 min prior to ischemia. For IPC studies hearts were subject to 3 cycles of 5 min ischemia plus 5 min reperfusion, prior to the 25 min ischemia. For IPC plus CsA studies, CsA was infused 10 min prior to, and during each 5 min reperfusion cycle of the IPC protocol. The coronary sinus was cannulated for effluent collection, and effluent samples were frozen immediately in liquid N_2 for subsequent spectrophotometric analysis (DU-800 Spectrophotometer, Beckman, Carlsbad CA) or LC-MS (see below).

Isolated mitochondrial studies: Heart mitochondria were isolated as previously described [39], and protein determined by the Lowry method [30]. Mitochondria were also purified by Percoll™ gradient centrifugation [15], and their purity gauged by western blotting for the plasma membrane protein GLUT-4. Mitochondrial proteins (50μg/lane) were resolved by SDS-PAGE, and after transfer to nitrocellulose, membranes were blocked with 5% non-fat dry milk and probed with a polyclonal anti-GLUT-4 antibody (diluted 1:1000), followed by a peroxidase-linked goat anti-rabbit secondary antibody (diluted 1:5000) and ECL detection.

Mitochondrial PTP opening was measured as the swelling-induced decrease in light scattering [6], spectrophotometrically at 520nm, with mitochondria suspended at 0.5 mg protein/ml in buffer comprising KCl (120 mM), KH_2PO_4 (3 mM), Tris (50 mM), succinate (5 mM), rotenone (5 мМ), pH 7.35, at 37C. After 4 min equilibration, $CaCl_2$ (200 μM) was added to initiate PTP opening. At subsequent time points CsA (5 мМ) plus EGTA (1 mM) were added to stop swelling, then samples were centrifuged at 14,000 x g for 5 min, 4°C. Supernatants were frozen immediately in liquid N_2 and analyzed as described for coronary effluents.

LC-MS analysis: Preliminary analysis (not shown) revealed that UV-absorbing solutes in cardiac effluents and mitochondrial supernatants were purines. A method was adopted to separate and identify purine standards by their unique signatures of retention time on both UV and mass chromatograms, and their ion fragmentation patterns (m/z) [8,28]. An 1100 series LC-MS system (Agilent, Palo Alto CA) was employed, with 20 μl injection onto a Novapak™ C_{18} column (5 μm mesh, 3.9 mm x 30 cm, Waters, Milford MA). Flow rate was 0.3 ml/min, and the mobile phase was 0.1% formic acid, 96% H_2O, 4% MeOH. Column effluent absorbance was monitored at 254 nm before passing into the MS, with electrospray ionization in positive-ion mode, and monitoring 100-550 m/z.

Chemicals and statistics: Cyclosporin A (CsA) was from Calbiochem (La Jolla CA). Sanglifehrin A (SfA) was a gift from Novartis (Basel, Switzerland). Anti GLUT-4 antibody was from Santa Cruz Biotech (Santa Cruz CA). Secondary antibodies and ECL reagents were from GE Biosciences (Piscataway NJ). All other chemicals were from Sigma (St. Louis MO). Data were analyzed by ANOVA and are presented as means ± S.E.M.

RESULTS

IR injury: Figure 1 shows changes in cardiac function caused by IR injury. Both left ventricular developed pressure and rate pressure product were significantly decreased following IR. Consistent with a role for PTP opening in this pathologic event [7,11,19,22], the adverse effects of IR injury were partially abrogated by the PTP inhibitors CsA and SfA. In addition, a similar magnitude of protection was afforded by IPC.

Solute release in cardiac IR injury: Figure 2A shows absorbance spectra of coronary effluents collected during post-ischemic reperfusion. Absorbance was maximal at 250nm (l_{MAX}). Prior administration of IPC or the PTP inhibitors CsA or SfA significantly decreased the effluent A_{250} (Figure 2B). The highest A_{250} was observed during the first 5 sec of reperfusion, followed by a sharp decrease at 10 sec to a level that was sustained for a further 3 min (Figure 2C), then a slow decrease for the remaining 30 min of reperfusion (Figure 2A). Most notably, release of solutes with l_{MAX} 250nm was seen during the reperfusion cycles in the development of IPC, and this release was also inhibited by CsA (Figure 2D).

Solute release by mitochondrial PTP opening: Since the release of UV-absorbing solutes during IR and IPC was inhibited by PTP inhibitors, these solutes may originate inside mitochondria. To investigate this, isolated heart mitochondria were subjected to PTP opening. Figure 3A shows typical CsA-sensitive, Ca^{2+}-induced PTP opening traces. Spectrophotometric analysis of mitochondrial supernatants revealed that Ca^{2+}-treated mitochondria released solutes with l_{MAX} 260nm, in a CsA-sensitive manner (Figure 3B). Supporting the mitochondrial origin of these solutes, Percoll™ purified heart mitochondria exhibiting no plasma membrane contamination also released solutes with l_{MAX} 260nm upon PTP opening, in a CsA-sensitive manner (Figure 3C).

Identification of mitochondrial solutes: Figure 4 shows the LC-MS method used to identify purines, with the UV and mass chromatograms of each purine standard in panels A and B respectively, and the mass spectrum of the major peak for each species in panels E-J. Using this method, each purine could be identified from its unique fingerprint of two retention times plus m/z pattern. Figure 5 shows the results of the LC-MS analysis on supernatants from Percoll™-purified mitochondria. The UV chromatograms from control and Ca^{2+}-treated mitochondria are in panel A, with the major peaks quantified and identified in panel B, and mass spectra for selected peaks in panels C-E. In addition to adenosine and inosine, a positive ion species at m/z 381 was released, which was putatively identified as 3-N-ribosyluric acid 5'-monophosphate (3-RUAMP) by analyzing its fragmentation pattern. In support of this identification, metabolites of 3-RUAMP were identified in coronary effluents (see below). Overall, these data indicate that mitochondria undergoing PTP opening release adenosine, inosine, and 3-RUAMP in approximately 1:1:1 stoichiometry.

Identification of cardiac effluent solutes: Figure 6A shows UV chromatograms of effluents from control and IR hearts, with the major peaks quantified and identified in panel B, and mass spectra for selected peaks in panels C-E. In addition to the predominance of inosine and

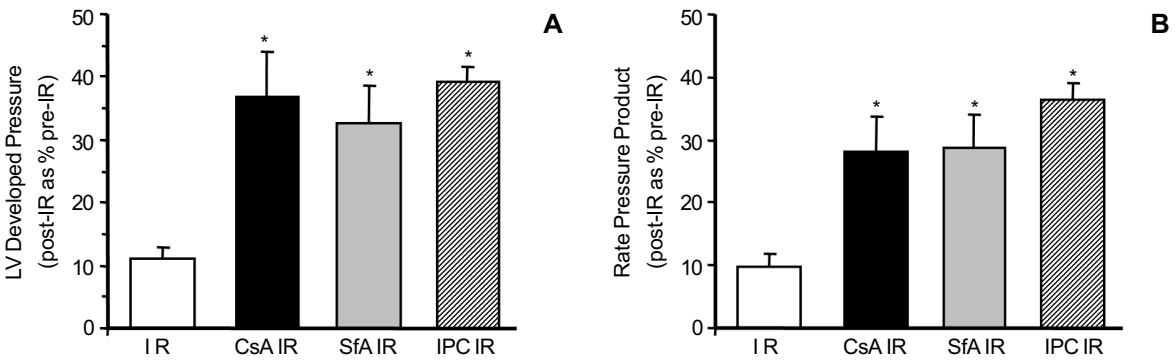


Figure 1. IR-induces cardiac dysfunction. Hearts were subjected to IR as detailed in the methods. A: Recovery of LV developed pressure, with post IR values expressed as a percentage of pre IR values. Experimental groups are: IR (white), CsA plus IR (black), SfA plus IR (gray), and IPC plus IR (hashed). B: Recovery of rate pressure product. Groups are as in panel A. All data are means ± SEM from at least 5 independent experiments. *P<0.05 between IR alone and IR + treatment groups

its metabolite hypoxanthine, the effluents also contained xanthine, ribose and pyrimidine-2,4-dione. The latter 3 species are metabolites of 3-RUAMP, which was released from mitochondria upon PTP opening (Figure 5). Overall these data indicate that the major UV-absorbing solutes released from the reperfused heart are inosine and 3-RUAMP metabolites, in approximately 9:1 stoichiometry.

DISCUSSION

The main finding of this study is that mitochondrial PTP opening is an important upstream event for cardiac purine release in both IR injury and IPC. Although PTP opening has generally been considered pathological in IR injury [7,11,19,22], recent evidence suggests that PTP opening may mediate protection in

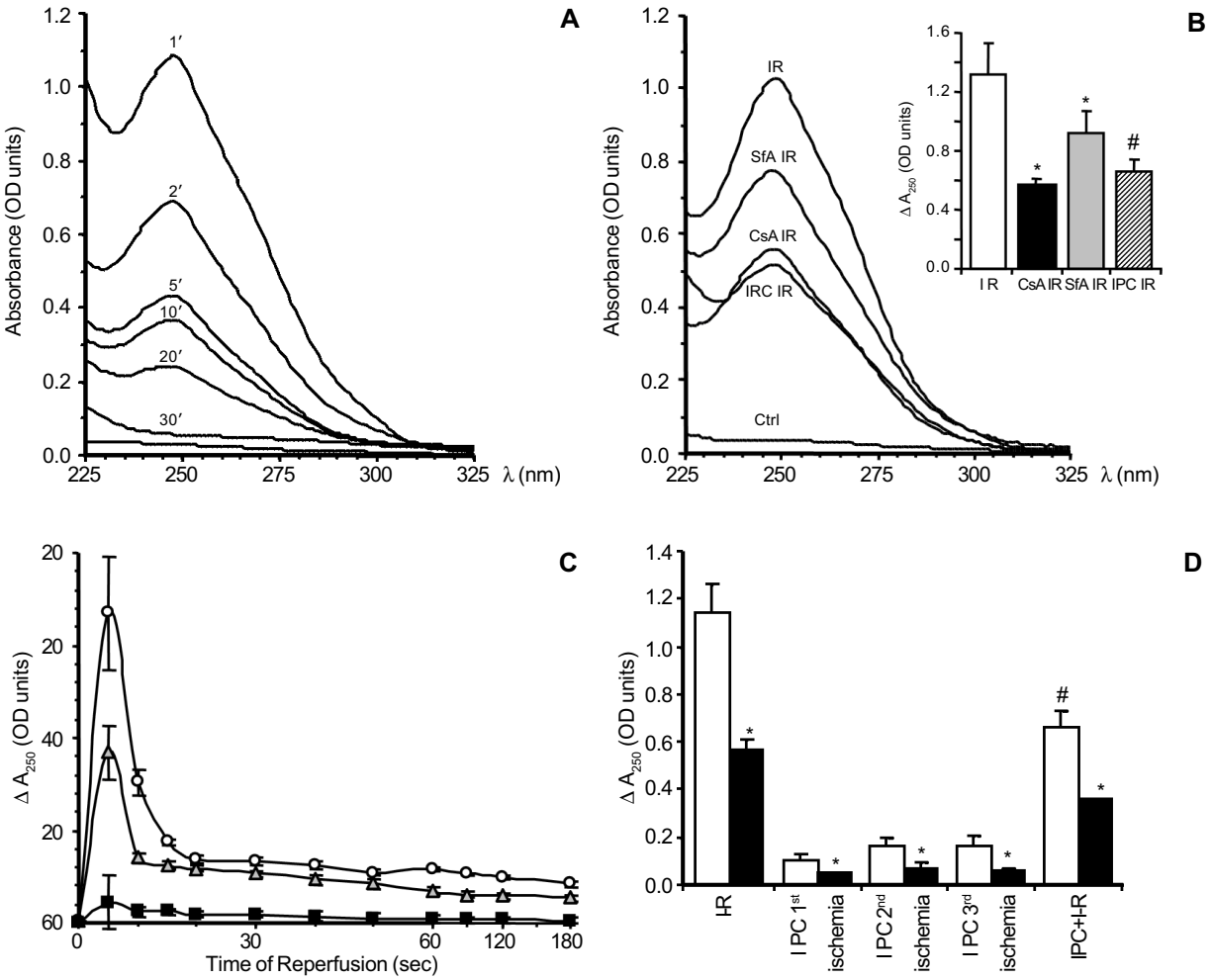


Figure 2. IR and IPC induce cardiac solute release. Hearts were perfused, and coronary effluents collected and analyzed, as detailed in the methods. A: Representative absorbance spectra of coronary effluents collected during reperfusion after prolonged ischemia. Numbers on traces indicate elapsed min. of reperfusion (unlabelled lowest trace is pre-ischemic effluent). B: Effect of CsA, SfA and IPC on spectra of effluents collected in the first min. of reperfusion. Inset: quantitation of spectral data expressed as DA_{250}, (i.e. minus A_{250} of control effluent). C: Kinetics of DA_{250} vs. time in three experimental groups: IR (open circles), SfA + IR (shaded triangles), and CsA + IR (black squares). D: Cardiac solute release in IPC. DA_{250} of coronary effluents from reperfusion after IR alone, or from the 3 short reperfusions during IPC cycles, or from reperfusion after IPC then IR. Open bars: control, Filled bars: plus CsA. Data are representative or means ± SEM of at least 4 independent experiments. *P<0.05 relative to the normal IR or IPC condition (no CsA/SfA treatment), #P<0.05 between IR alone and IPC + IR groups

IPC [23]. Purine signaling is known to be cytoprotective [1,14,21,25,27,32,35,40], and our data suggest that mitochondrial purine release links the PTP and cardioprotection.

Mitochondrial purine release: The release of adenosine and inosine from isolated Percoll™ purified mitochondria (Figures 3 & 5) suggests that either these nucleosides were released as nucleotides (e.g. AMP) and converted to nucleosides by contaminating plasma membrane 5' nucleotidase (5-NT), or that mitochondria contain a free nucleoside pool. Favoring the latter, the Percoll™-purified mitochondria were free of contamination by plasma membranes. In addition, both the mitochondrial catabolism of adenosine [24, 42,43], and a mitochondrial nucleoside transporter [9,26] have been reported, indicating the importance of adenosine/inosine transport for organelle function. Notably, the mitochondrial nucleoside transporter is structurally related to the peripheral benzodiazepine receptor, a putative PTP component [11]. Thus, PTP-linked nucleoside release may be via this transporter. The m/z $^{+}$381 species released from mitochondria upon PTP opening was putatively identified as 3-RUAMP (Figure 5), which has previously only been reported as synthesized by erythrocytes [36]. Consistent with a role for PTP opening upstream of purine release in cardiac IR and IPC, metabolites of 3-RUAMP were released from the heart under these conditions (Figure 6).

Cardiac purine release: It is reported that cardiac NAD^+ released upon reperfusion originates from NAD^+ released by mitochondrial PTP opening [12]. The current data suggest that purines released during cardiac IR and IPC

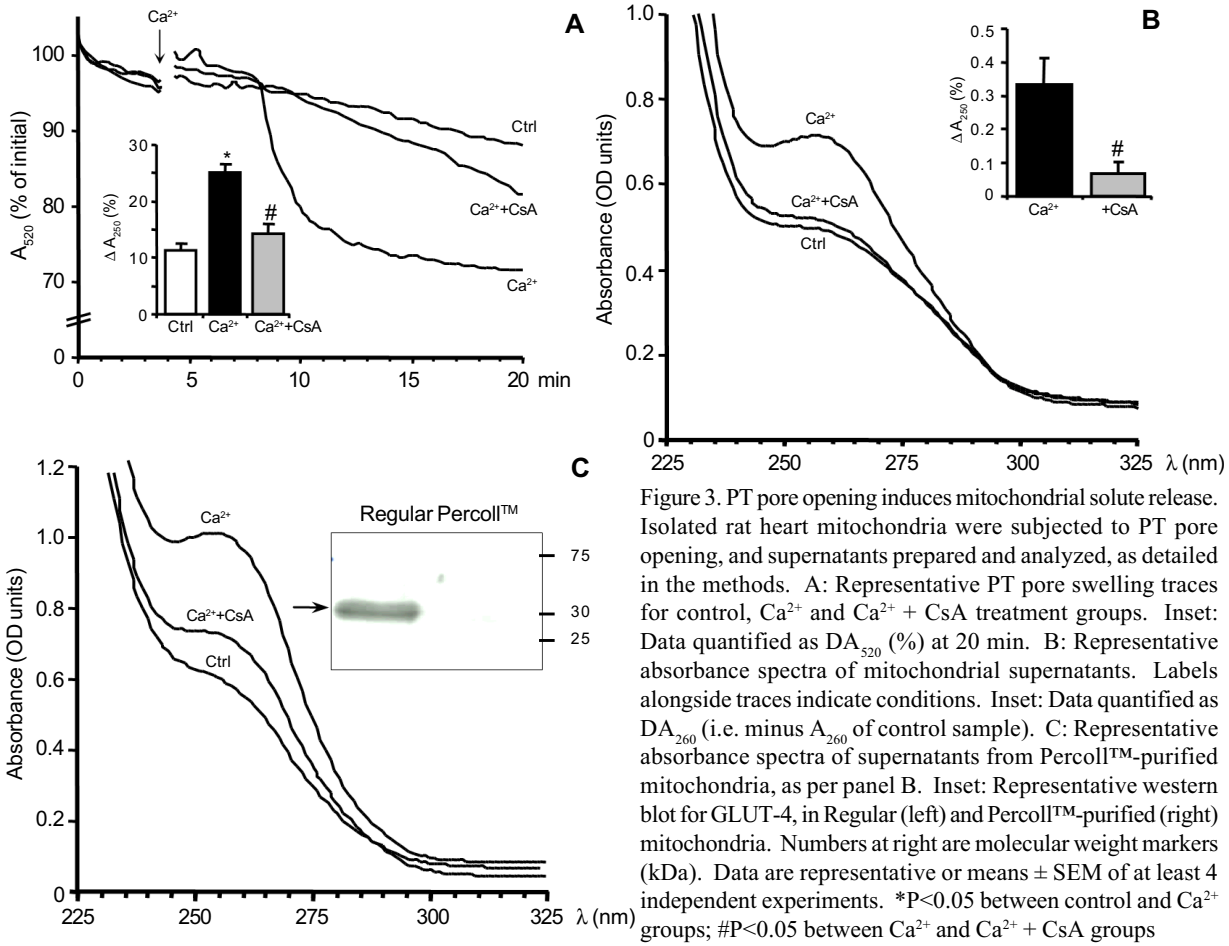


Figure 3. PT pore opening induces mitochondrial solute release. Isolated rat heart mitochondria were subjected to PT pore opening, and supernatants prepared and analyzed, as detailed in the methods. A: Representative PT pore swelling traces for control, Ca^{2+} and Ca^{2+} + CsA treatment groups. Inset: Data quantified as DA_{520} (%) at 20 min. B: Representative absorbance spectra of mitochondrial supernatants. Labels alongside traces indicate conditions. Inset: Data quantified as DA_{260} (i.e. minus A_{260} of control sample). C: Representative absorbance spectra of supernatants from Percoll™-purified mitochondria, as per panel B. Inset: Representative western blot for GLUT-4, in Regular (left) and Percoll™-purified (right) mitochondria. Numbers at right are molecular weight markers (kDa). Data are representative or means ± SEM of at least 4 independent experiments. *P<0.05 between control and Ca^{2+} groups; #P<0.05 between Ca^{2+} and Ca^{2+} + CsA groups

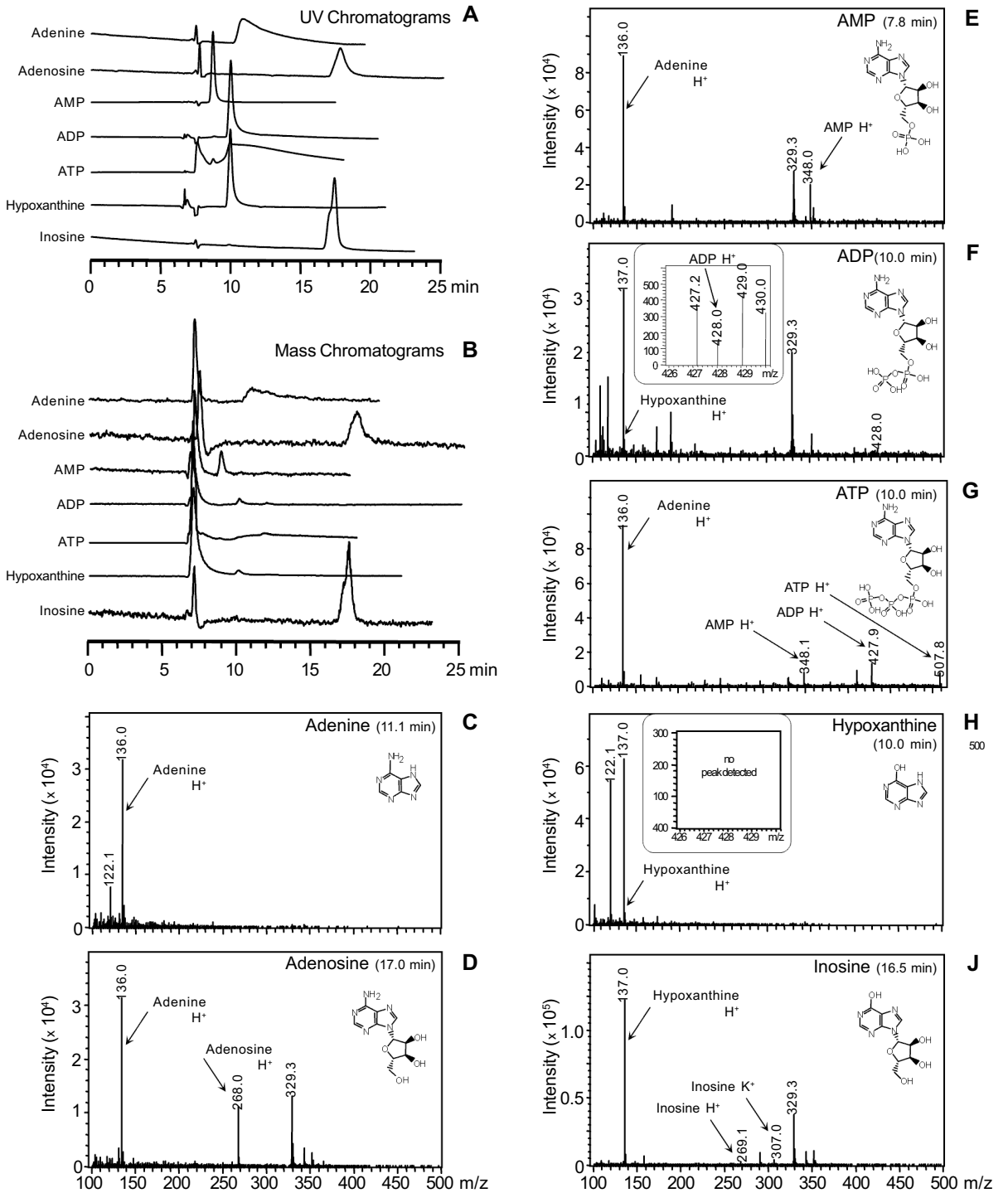
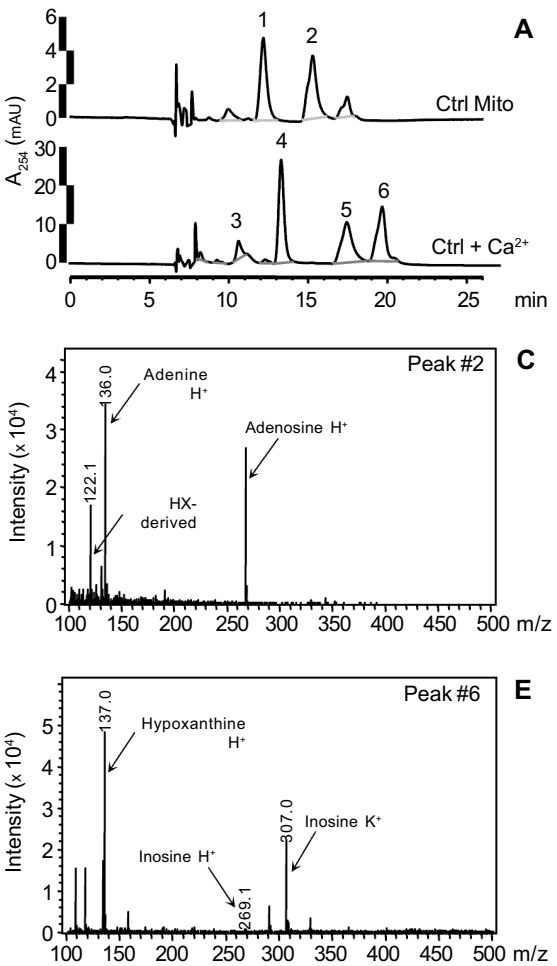


Figure 4. LC-MS analysis of purines. Commercially available purine standards were analyzed by LC-MS as detailed in the methods. A: Combined UV chromatograms (y-axis: A_{254}) for all 7 standards. B: Combined mass chromatograms (y-axis: abundance) for all 7 standards. Traces in A and B are all to the same relative scale. C-J: Positive ion mass spectra of the main peak for each standard, with major ions and fragmentation products identified. The retention time is listed in the upper right corner of each mass spectrum. C: Adenine. D: Adenosine. E: AMP. F: ADP. G: ATP. H: Hypoxanthine. J: Inosine. The unidentified species at m/z 329.3 in several spectra was assigned as a buffer contaminant

[2,4,17,20,25,41] also originate from mitochondrial PTP opening. This is supported by a quantitation of total purines released by mitochondria vs. hearts. Based on extinction coefficients of adenosine and inosine, mitochondrial purine release upon PTP opening (Figure 3) is ~15 nmol/mg protein. Rat myocardium contains ~35 mg mitochondrial protein/gram tissue (assuming 50% yield in typical isolations), so the mitochondria in a 1 g heart can release ~525 nmol purines. Integrating cardiac purine release over the first 5 min of reperfusion (Figure 2) gives ~1100 nmol from a 1 g heart. Thus, purines from the PTP could account for half of cardiac purine release, and consistent with this the PTP inhibitor CsA inhibits cardiac purine release by half (Figure 2). Some cardiac purine release may not come directly from the mitochondrial PTP, but may nevertheless ultimately depend on PTP opening for their origin. For example, cellular deenergization by PTP opening, or adenylate kinase released from mitochondria [12], are known to enhance cytosolic nucleotide degradation [3].

Cardioprotective PTP signaling: Cardioprotection by purines is well established [1,14,20,25,32,35], and despite a potential role for adenosine and inosine in IPC, the upstream signals regulating their release are poorly understood. In addition, while PTP opening is essential for IPC [23], the downstream signals mediating this effect are unknown. Herein we propose that purines are a missing link between PTP opening and IPC-mediated cardioprotection. Notably, the dephosphorylation



Peak	RT	Area	Identity
1	12.2	149	Hypoxanthine-Derived
2	15.3	144	Adenosine
3	10.7	96	3-RUAMP + ADP
4	13.4	647	3-RUAMP
5	17.5	507	Adenosine
6	19.7	533	Inosine

B

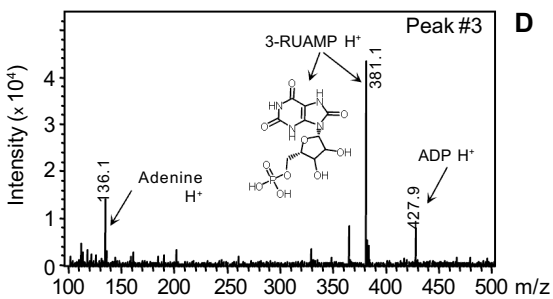


Figure 5. LC-MS analysis of mitochondrial supernatants. Supernatants from Percoll™ purified heart mitochondria (Figure 3C) were analyzed by LC-MS as detailed in the methods. A: UV chromatograms of supernatants from control (upper trace) and Ca^{2+} treated (lower trace) mitochondria. Note y-axes are not the same. B: Retention times, areas, and identities of the major numbered peaks from traces in panel A. Identities were based on retention times in UV and mass chromatograms, and m/z patterns relative to standards in Figure 4. C-E: Mass spectra of peaks 2, 3 and 5 from panel A. Note the appearance in D of a species putatively identified as 3-N-ribosyluric acid 5'-monophosphate (3-RUAMP). To conserve space, spectra for all peaks are not shown. All data are representative of at least 3 independent experiments

product of 3-RUAMP (3-N-ribosyluric acid) is a strong antioxidant [37], agreeing with a potential cardioprotective role for mitochondrial PTP-derived solutes.

The current data also have diagnostic and therapeutic implications. For example, measurement of CsA/SfA-sensitive increases in A_{260} of isolated mitochondrial supernatants represents a rapid and sensitive in-vitro PTP assay. In addition, while inosine is not a unique marker for PTP opening, it may be useful as a plasma biomarker for myocardial injury [17], as an adjunct to existing markers such as creatine kinase and Troponin-I [31]. Furthermore, the current data suggest that therapeutic use of PTP inhibitors such as CsA or SfA may diminish any protective effects of PTP-derived purines. This could be overcomed by supplementation with inosine or adenosine.

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PURINE RELEASE: A PROTECTIVE SIGNALING MECHANISM OF THE MITOCHONDRIAL PERMEABILITY TRANSITION PORE IN ISCHEMIA

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Both mitochondrial permeability transition pore (PTP) opening and purine signaling are implicated in cardioprotection via ischemic preconditioning (IPC). The PTP opening is accomponied by release of intramitochondrial solutes, and therefore we hypothesized that purine release from mitochondria during PTP opening may by required for IPC signaling. Herein we show that upon PTP opening, isolated mitochondria release adenosine, inosine and 3'-ribosyl uric acid monophosphate (3-RUAMP), and that perfused hearts subject to IPC release

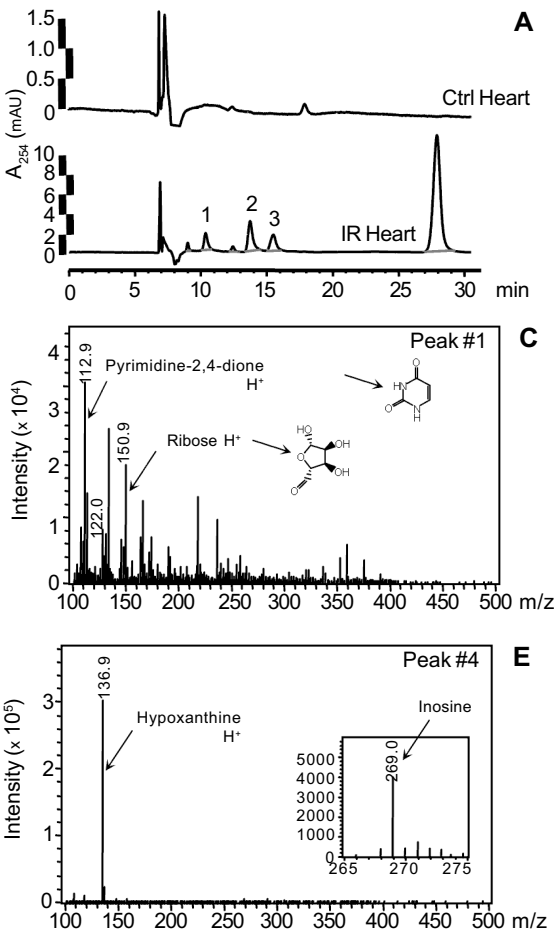

Figure 6. LC-MS analysis of cardiac effluents. Hearts were subject to IR and cardiac effluents (Figure 2) analyzed by LC-MS as detailed in the methods. A: UV chromatograms of effluent from control (upper trace) and IR (lower trace) hearts. Note y-axes are not the same. B: Retention times, areas, and identities of the major numbered peaks from traces in panel A. Identities were based on retention times in UV and mass chromatograms, and m/z patterns relative to standards in Figure 4. C-E: Mass spectra of peaks 1, 3 and 4 from panel A. Note the appearance in C and D of species identified as metabolites of 3-RUAMP (Figure 5D). To conserve space, spectra for all peaks are not shown. All data are representative of at least 3 independent experiments

inosine and 3-RUAMP derivatives. Both these events were inhibited by the PTP blockers cyclosporin A and sanglifehrin A. Implications for cardioprotective signaling by purines and the PTP are discussed.

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