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Anesthetic Preconditioning Improves Adenosine Triphosphate Synthesis and Reduces Reactive Oxygen Species Formation in Mitochondria after Ischemia by a Redox Dependent Mechanism

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Background: **Mitochondrial changes that characterize the heart after anesthetic preconditioning (APC) or the mechanisms by which mitochondrial triggering factors lead to protection are unknown. This study hypothesized that generation of reactive oxygen species (ROS) during APC is required to initiate the mitochondrial protective effects, and that APC leads to improved mitochondrial electron transport chain function and cardiac function during reperfusion.**

Methods: **Isolated guinea pig hearts were subject to 30 min ischemia and 120 min reperfusion. Prior to ischemia hearts were either untreated (I/R), or treated with sevoflurane (APC), in the presence or absence of the ROS scavenger tiron (TIR), or the superoxide dismutase mimetic MnTBAP (TBAP). Intracellular ROS were measured by spectrofluorometry using the fluorescent probe dihydroethidium (DHE). In another series of experiments, using the same protocol, hearts were reperfused for only 5 min and removed for measurement of adenosine triphosphate (ATP) synthesis by luciferin–luciferase luminometry and ROS generation by dichlorohydro-fluorescein (DCF) fluorescence in isolated mitochondria.**

Results: **The APC improved cardiac function and reduced infarction. Tiron or MnTBAP abrogated the protection afforded by APC. Mitochondrial ATP synthesis was decreased by 70 ± 3% after IR alone, by only 7 ± 3% after APC, by 69 ± 2% after APC+TIR, and by 71 ± 3% after APC + TBAP. Mitochondrial ROS formation (DCF) increased by 48 ± 3% after IR alone, by 0 ± 2% after APC, by 43 ± 4% after APC + TIR, and by 46 ± 3% after APC + TBAP. ROS generation (DHE) was increased in I/R group at 5 and 120 min reperfusion. This was attenuated by APC but this protective effect was abrogated in APC + TIR and APC + TBAP groups.**

Conclusions: **The results indicate that ROS are central both in triggering and mediating APC, and that the mitochondrion is the target for these changes.**

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ANESTHETIC preconditioning (APC) is a phenomenon whereby transient exposure of the heart to a volatile anesthetic leads to a state of increased resistance to the deleterious contractile and vascular effects of ischemia and reperfusion (IR). This resistance is evidenced on reperfusion by reduced myocardial stunning, dysrrhythmias, calcium loading, and infarct size.[1–3] Much investigative effort has focused on the phosphorylation signaling pathways involved in the triggering of APC. Release of reactive oxygen species (ROS)[4,5] and opening of adenosine triphosphate-sensitive potassium (K_{ATP}) channels[1,2] appear be involved in this sequence. However, little is known about the specific mitochondrial changes that characterize the preconditioned heart, or about the mitochondrial-mediated mechanism by which known triggering factors lead to myocardial protection.

The importance of mitochondrial dysfunction in IR-related injury is well known. It is accepted that, during ischemia, mitochondrial oxidative phosphorylation rapidly declines, and inadequate ATP concentrations, intracellular acidification, and depleted purine stores contribute to myocardial dysfunction at reperfusion.[6] In addition, large quantities of ROS, most likely from a mitochondrial source,[7] cause widespread oxidative damage to lipids and proteins, that leads to myocardial stunning and cell death.[8] In addition to sarcolemmal K_{ATP} channels,[1] mitochondrial K_{ATP}[9–12] channels may be involved in initiating and/or mediating APC, which suggests an important role for the mitochondrion in APC. Our recent findings that APC modulates nicotinamide adenine dinucleotide (NADH)[11,13] levels and mitochondrial $[Ca^{2+}]$,[12] loading during ischemia and on reperfusion, also point to the mitochondrion as a central target for both triggering and mediating the protective effects of APC.

In this study, we examined the impact of APC on ATP synthesis and ROS formation in mitochondria isolated upon initial reperfusion from intact hearts subject to preconditioning by sevoflurane before global ischemia. We hypothesized that APC protects hearts against IR injury, in part, by initiating a sequence of events involving ROS generation that culminates in preservation of mitochondrial bioenergetics as assessed by reduced ROS generation and improved oxidative phosphorylation.

Materials and Methods

Langendorff Heart Preparation

The investigation conformed to the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health No. 85–23, revised 1996) and was approved by the institutional review board. The preparation has been described in detail previously.[2–4] Hearts from guinea pig (n = 100) were perfused at 37°C, at constant pressure (55 mmHg) with an oxygenated Krebs–Ringer's solution of the following composition (in mM): Na^+ 138, K^+ 4.5, Mg^{2+} 1.2, Ca^{2+} 2.5, Cl^- 134, HCO_3^- 14.5, $H_2PO_4^-$ 1.2, glucose 11.5, pyruvate 2, mannitol 16, probenecid 0.1, EDTA 0.05, and insulin 5 U/L.

Left ventricular pressure (LVP) was measured isovolumetrically using a transducer connected by stiff saline-filled tubing to a latex balloon placed in the left ventricle through an incision in the left atrium. Measured characteristics of LVP were systolic and diastolic LVP; calculated characteristics were developed (systolic–diastolic) LVP and maximal and minimal first derivative of LVP ($dLVP/dt_{max}$ and $dLVP/dt_{min}$, respectively). Coronary inflow (CF) was measured by an ultrasonic flowmeter (Transonic T106X, Ithaca, NY). Atrial and ventricular bipolar leads were used to measure spontaneous heart rate. Coronary inflow (a) and coronary venous (v) Na^+, K^+, Ca^{2+}, Po_2, pH, and Pco_2 were measured off-line with an intermittently self-calibrating analyzer (Radiometer ABL 505, Copenhagen, Denmark). Coronary sinus Po_2 tension (PvO_2) was also measured continuously on-line with a Clark electrode (model 203B, Instech; Plymouth Meeting, PA). Myocardial O_2 consumption (Mvo_2) was calculated as CF/heart weight (g) · (Pao_2–PvO_2) · 24 ml O_2/mL at 760 mmHg.

Global ischemia was achieved by clamping the aortic inflow line. If ventricular fibrillation occurred on reperfusion and did not convert within 30 s a bolus of lidocaine (250 μg) was given. At the end of 120 min reperfusion hearts were removed, cut into six transverse sections and stained with 1% 2,3,5-triphenyltetrazolium chloride in 0.1 M KH_2PO_4 buffer (pH 7.4, 38°C) for 10 min as described previously.[3,4] Infarct size was expressed as a percentage of total heart weight.

Detection of ROS in Intact Hearts

The ROS formation was measured by the fluorescent dye DHE (Molecular Probes, Eugene, OR). The DHE enters cells and upon oxidation by ROS, with relative selectivity for superoxide[14] ($O_2^{\cdot-}$), is converted to ethidium bromide; in this form it intercalates with DNA and causes the nucleus to exhibit a right shift in fluorescence. To measure fluorescent intensity of oxidized DHE, each experiment was carried out in a light-blocking Faraday cage, as we have described in detail previously, for measurements of cytosolic Ca^{2+},[3,15] mitochondrial Ca^{2+},[12,15] Na^+,[15] and NADH[13,15] in isolated beating hearts. Briefly, the distal end of a trifurcated fiberoptic cable (optical surface area 3.85 mm^2) was placed against the LV free wall through a hole in the tissue bath. Netting was applied around the heart for optimal contact without impeding relaxation. The fiberoptic cable was connected to a modified spectrophotofluorometer (SLM Aminco-Bowman II; Spectronic Instruments, Urbana, IL). Fluorescent emissions (λ_{em}) at 590 nm (bandwidth 4 nm) were amplified by a photomultiplier tube (700 V) and recorded following excitation (λ_{ex}) with a 150 W xenon arc lamp filtered through a 540 nm monochromator (bandwidth 4 nm). The excitation wavelength penetrates, with decreasing intensity, through the whole 4 mm of the guinea pig left ventricular wall.

In preliminary experiments (n = 4) background autofluorescence (no DHE) was determined after initial perfusion and equilibration, and for each experimental protocol. All subsequently recorded values of DHE fluorescent intensity were adjusted for the minimal change in background fluorescence with any maneuver or drug. DHE was first dissolved in 1 ml dimethyl sulfoxide containing 16% (weight/vol) Pluronic I-127 (Sigma Chemical, St. Louis, MO). The perfusate contained probenecid (100 mM) to retard cellular leakage of DHE. Hearts were loaded with 10 μM DHE in Krebs-Ringer's solution for 25 min followed by washout of residual DHE with standard perfusate for 15 min. Loading of DHE increased diastolic LVP approximately 8% and increased coronary flow approximately 10%; the effect on diastolic LVP was nearly reversed and flow returned to baseline values on washout. DHE loading increased fluorescence intensity from 0.03 ± 0.01 before loading to 2.7 ± 0.2 arbitrary units (a.u.) after washout. Each washout value was adjusted to 0 a.u. to normalize fluorescence intensity values for each experiment.

Isolation of Cardiac Mitochondria

Mitochondrial ATP synthesis and ROS formation were determined in cardiac mitochondria in a parallel series of experiments using the same treatment protocols except that reperfusion function and infarct size were not determined. Briefly, mitochondria were isolated by differential centrifugation as described by Solem and Wallace.[16] Mitochondria prepared by this methodology have been shown to be metabolically active with respiratory control ratios of 3.5–5.0 with the substrate succinate and 8.0–10.0 with substrates glutamate/malate giving corresponding ratios of ATP to oxygen of 1.5–1.7 and 2.5–2.7.[16] Treated hearts were removed at 5 min reperfusion and immediately placed in buffered physiologic solution at 4°C. Next the atria and right ventricle were trimmed away and the left ventricle was dissected, weighed, and minced into 1-mm pieces in 1 ml of 4°C isolation buffer (200 mM mannitol, 50 mM sucrose, 5 mM KH_2PO_4, 1 mM

EGTA, 5 mM 3-(n-morpholino)propanesulfonic acid, and 0.1% bovine serum albumin; pH 7.15 adjusted with potassium hydroxide). The tissue was rinsed clear of blood and light debris with isolation buffer and transferred to a glass Potter–Elvehjem homogenizing vessel placed on ice. Immediately after adding 2.5 mg protease in 2.5 ml isolation buffer, the tissue was gently homogenized on ice with a Teflon (DuPont, Wilmington, DE) pestle for 30 s. Then 17.5 ml cold isolation buffer was added to dilute the protease and homogenization was continued for 60 s. Exposure to the concentrated protease was limited to 30 s to maintain mitochondrial integrity and yield. Untreated control hearts (Sham) were similarly isolated.

The tissue suspension was centrifuged at 8,000 *g* for 10 min to remove protease. The pellet was resuspended in 3.5 ml isolation buffer, and an additional 25 ml isolation buffer was added and the suspension centrifuged (700 *g* for 10 min) to remove cellular debris. The supernatant containing the mitochondrial fraction was further centrifuged (8,000 *g* for 10 min), and the pellet was washed twice by resuspension in 3.5 ml isolation buffer without EGTA and centrifuged at 8,000 *g* for 10 min. The final mitochondrial pellet was resuspended to 1 g of the original tissue (weight/ml) in cold isolation buffer without EGTA. All procedures were performed at 4°C, and total protein concentration was determined using the dye binding method of Bradford[17] with bovine serum albumin as a standard.

Mitochondrial ATP Synthesis and Tissue ATP Concentration

Mitochondria (10 μg mitochondrial protein/ml) were preincubated for 10 min in a medium containing 180 mM sucrose, 45 mM KH_2PO_4, 10 mM Mg-acetate, 1 mM EDTA, 1 mM pyrophosphate, 1 g/l bovine serum albumin, and 150 mM ATP in a 25°C water bath. ATP synthesis was initiated by adding respiratory substrates (1 mM pyruvate + 1 mM malate) to drive electron flow through complex I of the electron transport chain (ETC). Aliquots of 50 ml were taken from the incubations at 4 min intervals for 12 min, and reactions were halted by adding the samples to 500 ml 2.5% (vol/vol) trichloroacetic acid.[18] Samples were neutralized with 100 ml of 1 mM Tris base. The neutralized supernatant was assayed for ATP by luciferin–luciferase luminometry using a modification of the luminescence method of Strehler[19] and components of the Sigma Bioluminescent Somatic Cell Assay Kit (catalog no. FL-AA, Sigma Chemical Co., St. Louis, MO). Mitochondrial protein concentration was determined using the Bradford assay,[17] and the rate of mitochondrial ATP synthesis was calculated and expressed as μmol ATP · min^{-1} · mg mitochondrial $protein^{-1}$.

Tissue ATP concentration was measured in segments of the left ventricle. These were rapidly dissected, frozen in liquid nitrogen, and stored at −70°C for later analysis. Frozen tissue samples (20 mg) were homogenized in 500 ml 2.5% trichloroacetic acid. After centrifugation, 400 ml aliquots of supernatant were neutralized with 80 ml 1 M Tris.[18–20] The ATP concentration was expressed as nmol ATP · mg mitochondrial $protein^{-1}$.

Detection of ROS in Mitochondria

Mitochondrial ROS was measured from the same mitochondrial pellet used to measure ATP synthesis. Determination of ROS was accomplished by a slight modification of the procedure of Garcia-Ruiz *et al.*[21] Briefly, production of ROS was monitored with a fluorescent probe, dichlorohydro-fluorescein diacetate (DCFDA), which is oxidized in the presence of hydrogen peroxide (H_2O_2) to DCF.[22] Guinea pig heart mitochondria (0.25–0.5 mg/ml) were incubated at 25°C with 2 μM DCFDA (Molecular Probes, Eugene, OR) in the absence or presence of 100 μM antimycin A (Sigma Chemical) to stimulate maximal ROS. Antimycin A profoundly enhances mitochondrial superoxide production by inhibiting at the ubiquinone site of complex III of the ETC.[21] Superoxide is rapidly converted to hydrogen peroxide by mitochondrial MnSOD. The mitochondrial suspension was gently agitated and incubated at room temperature for measurements taken at 0, 30, 60, and 120 min. Fluorescence was determined at λ_{em} 529 nm (bandwidth 10 nm) and λ_{ex} 503 nm (bandwidth 5 nm), according to the spectral characteristics of DCF. The ROS formation was expressed as arbitrary units of fluorescence intensity of DCF.

Protocol

Scavengers selected to block triggering by ROS during sevoflurane exposure were tiron (4,5-dihydroxy-1,3-benzene-disulphonic acid, 100 μM), and MnTBAP (Mn(III) tetrakis (4-benzoic acid) porphyrin chloride, 40 μM). There were six identical treatment groups for both isolated heart and isolated mitochondrial experiments (fig. 1). These were ischemia/reperfusion alone (I/R), sevoflurane before I/R (APC), with or without the ROS scavengers tiron (APC + TIR, TIR) or MnTBAP (APC + TBAP, TBAP). In addition, there was an isolated heart group in which hearts were perfused for 200 min but not subjected to ischemia or treatments (Time Control group; n = 4, not shown) and a Sham group (n = 24, 4 hearts/group) in which mitochondria were isolated from hearts neither subjected to treatments nor to IR injury. Isolated heart studies began 30 min after equilibration. Hearts were assigned randomly to each of the seven groups. Parallel experiments were performed for each experimental group, each on separate hearts: (1) isolated hearts for global cardiac function and ROS formation, and for infarct size measured at 120 min reperfusion (n = 48, 8 hearts/group); (2) isolated hearts as

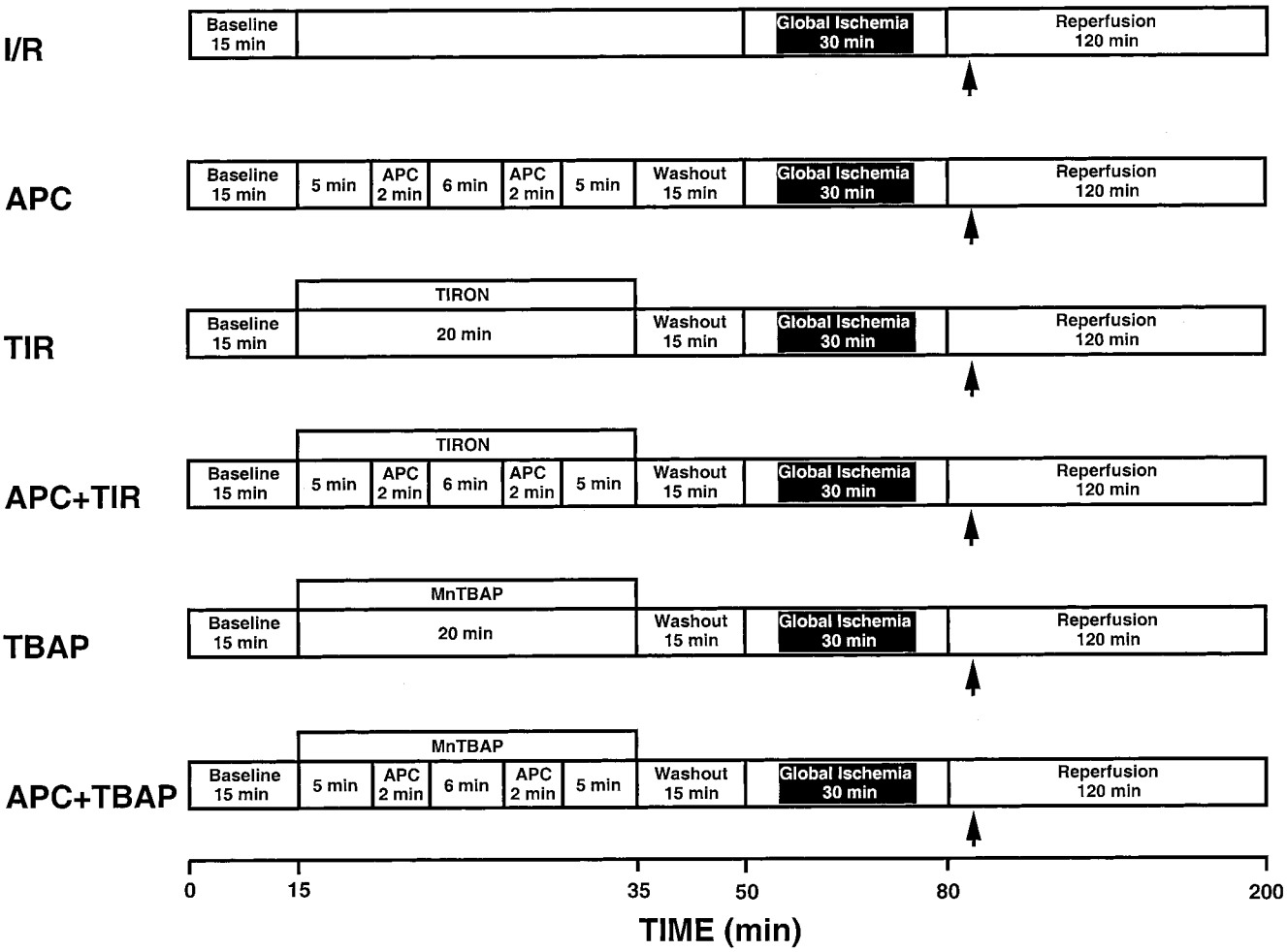

Fig. 1. Schema for protocols used in six randomized groups of guinea pig hearts. Anesthetic preconditioning (APC) pulses were elicited by exposing hearts to 2.98% sevoflurane at a normal cardiac perfusion pressure for two 2-min periods. Sevoflurane was not detectable in the effluent at the end of the 15 min washout period before ischemia. ROS scavengers given during sevoflurane exposure were tiron (4,5-dihydroxy-1,3-benzene-disulphonic acid, 100 μM), and MnTBAP (Mn(III) tetrakis (4-benzoic acid) porphyrin chloride, 40 μM). Each scavenger of ROS was given beginning 5 min before sevoflurane, during sevoflurane exposures, and for 5 min after the second sevoflurane exposure. Arrows indicate time of removal of the hearts (5 min reperfusion) for isolation of mitochondria (ATP and ROS assays).

above but with removal of the heart at 5 min reperfusion to isolate cardiac mitochondria for ATP and ROS assays (n = 24, 4 hearts/group). A 5-min reperfusion point was selected to examine for maximal differences in mitochondrial function shortly after ischemia and to rule out any effects on these measures by nonviable cells. We have reported that ROS generated during IR and measured in coronary effluent are no longer detectable after 5 min reperfusion.[4]

The APC consisted of two 2-min periods of sevoflurane exposure delivered by vaporizer (3.2 vol%); these periods were separated by a 5 min-period of perfusion without sevoflurane. Sevoflurane was detected by gas chromatography (GC-8AIF, Shimadzu, Kyoto, Japan) as described previously.[2,4] Inflow sevoflurane concentration was 0.42 ± 0.09 mM, which is equivalent to 2.98 ± 0.35 vol% for a minimal alveolar concentration (MAC) of approximately 1.42 ± 0.09. Sevoflurane was not detectable in the effluent at the end of the 15 min washout period before ischemia. Each scavenger of ROS was given beginning 5 min before sevoflurane, during sevoflurane exposures, and for 5 min after the second sevoflurane exposure. The scavengers were administered alone (without APC) to rule out any direct effects of these drugs.

Statistical Analysis

All data were expressed as mean ± SE. Within group (time effect) for a given variable were compared to a baseline control (at 15 min) by Student-Newman-Keul test whenever univariate analysis of variance for repeated measures was significant (Super ANOVA 1.11 software for Macintosh from Abacus Concepts, Berkeley, CA). $P < 0.05$ (two tailed) was considered statistically significant.

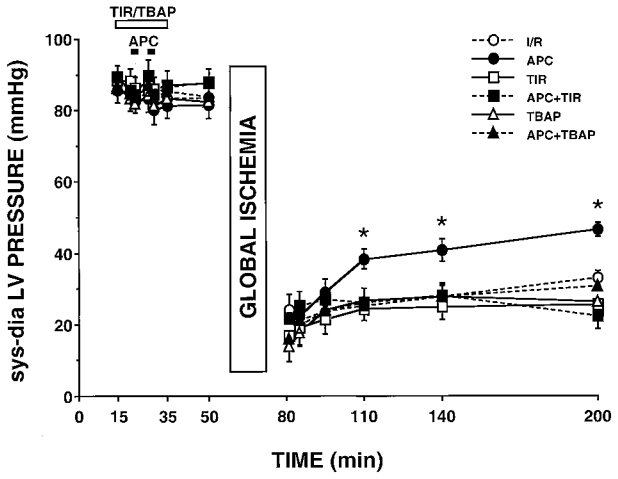

Fig. 2. Time course for developed left ventricular pressure (LVP) before, during, and after global ischemia in ischemia–reperfusion alone (I/R), anesthetic preconditioning (APC), with or without the ROS scavengers tiron (APC + TIR, TIR) or MnTBAP (APC + TBAP, TBAP). Developed LVP was least reduced in APC-treated groups on reperfusion. *$P < 0.05$ *versus* I/R. Other details in text.

Results

Cardiac Function, ROS Formation, and Infarct Size in Intact Hearts

There were no differences among groups in baseline functional variables: systolic or diastolic left ventricular pressure (LVP), $dLVP/dt_{max}$, $dLVP/dt_{min}$, coronary flow, heart rate, and myocardial oxygen consumption. This was true for hearts destined for functional studies and those destined for mitochondrial studies. In the time control group, no measured mechanical or metabolic variables changed over the 200 min of continuous perfusion (data not shown).

At 120 min reperfusion (200 min), developed LVP was reduced by 47 ± 2% compared to baseline in the APC group; this was significantly less than the decrease in the I/R group (62 ± 4%) (fig. 2). The improvement in developed LVP was due mostly to improved diastolic LVP as shown in the Table. This protection was completely abolished by either tiron or MnTBAP pretreatment during the preconditioning pulses. The Table also shows that $dLVP/dt_{max}$ and $dLVP/dt_{min}$ were improved in the APC group and that tiron or MnTBAP returned these levels to levels similar to those observed in the I/R group. For each of these variables, tiron or MnTBAP given without APC before ischemia had no effect different from those of the I/R group.

Both coronary flow and myocardial oxygen consumption improved significantly in the APC group on reperfusion and these effects were again abrogated by bracketing APC with either tiron or MnTBAP (Table). For each of these variables, tiron or MnTBAP given without APC before ischemia had no effect different from those of the I/R group.

DHE fluorescence intensity at 5 and 120 min reperfusion, respectively, was increased by 44 ± 5% and by 25 ± 6% above the baseline control in the I/R group, and by only 20 ± 3% and 4 ± 3% in the APC group. The non-APC groups did not differ significantly from the I/R group. Infarct size after 120 min reperfusion was decreased in the APC group compared to all other groups ($P < 0.05$) and there was no difference among the non-APC groups (fig. 3).

ATP Synthesis and ROS Formation in Isolated Mitochondria

ATP synthesis and ROS formation were assessed in mitochondria isolated at 5 min reperfusion. Figure 4 shows that mitochondrial ATP synthesis rate in Sham hearts, at the equivalent time-point as the standard (Sham, 11.2 ± 2.6 μmol ATP · min^{-1} · mg mitochondrial $protein^{-1}$, 100%), was reduced by 71 ± 3% ($P < 0.05$) in the I/R group, whereas APC returned ATP synthesis to near the Sham group values. Tiron or MnTBAP completely abrogated the effect of APC to restore ATP synthesis and neither tiron nor MnTBAP alone had any direct effect to alter ATP synthesis compared to the I/R controls. ATP concentration in the Sham group (6.7 nmol ATP · mg mitochondrial $protein^{-1}$) was not significantly different compared to the other groups.

Figure 5 shows that ROS formation was increased by 48 ± 3% ($P < 0.05$) in the I/R group compared to the Sham group (a.u., 100%), but was not increased above the Sham group in the APC group. Tiron or MnTBAP given in the presence of APC completely abrogated the effect of APC on reducing ROS formation.

Discussion

APC is manifested by attenuated cardiac injury consequent to IR in several animal models,[1,2,23] and there is evidence of its occurrence in humans.[24] Although APC is well known to improve global cardiac structure and function after ischemia, our experiments in isolated mitochondria demonstrate two mechanisms by which APC preserves mitochondrial energy production and mitochondrial function during early reperfusion after global ischemia. The APC phenomenon was evidenced on initial reperfusion not only by nearly normalized oxidative phosphorylation, but also by decreased ROS formation, to levels found in nonischemic hearts.

These results confirm and extend our previous observation in intact hearts that APC results in reduced coronary effluent ROS concentrations during initial reperfusion after ischemia.[4] These studies, and our associated studies in intact hearts on the effects of APC on mitochondrial NADH and Ca^{2+},[11–13] together indicate that APC leads to improved mitochondrial bioenergetics during IR. This suggests two key roles of mitochondrial–

Table 1. $dLVP/dt_{max}$, $dLVP/dt_{min}$, Diastolic LVP, Coronary Flow, and MVO_2 in I/R, APC, TIR, APC + TIR, MnTBAP, and APC + MnTBAP Groups before, during, and after Sevoflurane Exposure and on Reperfusion (RP) after Ischemia

Time	Baseline (15 min)	APC pulses (25 min)	Washout (50 min)	RP 60 min (140 min)	RP 120 min (200 min)
$dLVP/dt_{max}$ (mmHg/s)	—	—	—	—	—
I/R	2373 ± 130	2482 ± 243	2348 ± 244	878 ± 69	895 ± 98
APC	2588 ± 96	1885 ± 193*	2355 ± 201	1150 ± 130*	1538 ± 130*
TIR	2481 ± 131	2181 ± 131	2209 ± 110	852 ± 41	834 ± 67
APC + TIR	2200 ± 155	1984 ± 255	2360 ± 66	821 ± 42	946 ± 36
MnTBAP	2180 ± 64	2273 ± 171	2246 ± 83	899 ± 51	940 ± 40
APC + MnTBAP	2270 ± 134	2156 ± 134	2133 ± 119	874 ± 88	926 ± 67
$dLVP/dt_{min}$ (mmHg/s)	—	—	—	—	—
I/R	−2353 ± 150	−2140 ± 153	−2185 ± 167	−630 ± 156	−900 ± 52
APC	−2422 ± 189	−1785 ± 92*	−2225 ± 135	−1013 ± 54*	−1368 ± 185*
TIR	−2277 ± 170	−2294 ± 90	−2116 ± 54	−814 ± 93	−903 ± 170
APC + TIR	−2115 ± 186	−2196 ± 155	−2141 ± 186	−814 ± 86	−926 ± 186
MnTBAP	−2227 ± 51	−2280 ± 66	−2187 ± 116	−740 ± 74	−966 ± 68
APC + MnTBAP	−2098 ± 131	−2196 ± 73	−2141 ± 75	−814 ± 102	−948 ± 56
Diastolic LVP (mmHg)	—	—	—	—	—
I/R	2 ± 1	2 ± 1	2 ± 1	21 ± 2	19 ± 2
APC	2 ± 1	2 ± 2	2 ± 2	11 ± 1*	10 ± 2*
TIR	2 ± 1	1 ± 1	1 ± 2	21 ± 1	16 ± 2
APC + TIR	2 ± 1	1 ± 2	2 ± 2	20 ± 2	16 ± 2
MnTBAP	2 ± 1	1 ± 1	1 ± 2	21 ± 1	18 ± 2
APC + MnTBAP	2 ± 1	1 ± 2	2 ± 2	20 ± 2	17 ± 2
Coronary Flow ($ml \cdot min^{-1} \cdot g^{-1}$)	—	—	—	—	—
I/R	7.6 ± 0.3	7.7 ± 0.4	7.7 ± 0.3	4.1 ± 0.4	4.2 ± 0.5
APC	7.7 ± 0.2	7.9 ± 0.4	7.4 ± 0.4	5.6 ± 0.4*	5.9 ± 0.4*
TIR	8.1 ± 0.3	7.8 ± 0.3	7.8 ± 0.3	3.6 ± 0.3	4.0 ± 0.4
APC + TIR	8.1 ± 0.2	7.9 ± 0.4	7.6 ± 0.3	3.5 ± 0.4	3.8 ± 0.5
MnTBAP	8.0 ± 0.3	7.8 ± 0.5	7.7 ± 0.3	3.7 ± 0.3	4.1 ± 0.4
APC + MnTBAP	7.9 ± 0.3	7.8 ± 0.4	7.5 ± 0.4	3.6 ± 0.4	3.9 ± 0.5
MVO_2 ($\mu l \cdot min^{-1} \cdot g^{-1}$)	—	—	—	—	—
I/R	91 ± 3	89 ± 3	90 ± 4	47 ± 5	48 ± 5
APC	90 ± 4	91 ± 3	89 ± 3	66 ± 5*	66 ± 4*
TIR	92 ± 3	88 ± 3	88 ± 3	45 ± 4	46 ± 5
APC + TIR	89 ± 3	89 ± 3	89 ± 3	50 ± 5	51 ± 5
MnTBAP	90 ± 3	88 ± 3	88 ± 3	45 ± 4	46 ± 5
APC + MnTBAP	88 ± 3	90 ± 3	89 ± 3	50 ± 5	51 ± 5

Values are mean ± SEM; n = 8/group.

* $P < 0.05$ *vs.* I/R.

APC = anesthetic preconditioning; I/R = ischemia/reperfusion; LVP = left ventricular pressure; MVO_2 = myocardial oxygen consumption; RP = reperfusion; APC + TIR/TIR = APC with/without the ROS scavenger tiron; APC + TBAP/TBAP = APC with/without the ROS scavenger MnTBAP.

induced ROS formation in APC: 1) ROS are generated by mitochondria during anesthetic exposure leading to triggering of APC; 2) protection afforded by APC occurs, at least in part, because of a decrease in mitochondrial ROS formation during subsequent IR. Thus, the early component of ROS generation (likely a minute level) is involved in triggering the signaling pathways that induce APC. This subsequently leads to a decrease in the later component (the large excess of ROS normally formed during reperfusion after ischemia), effecting structural and functional preservation.

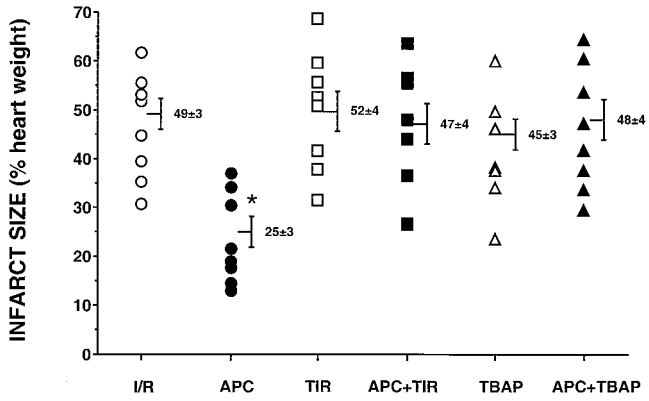

Fig. 3. Myocardial infarct size expressed as a percentage of total heart weight in guinea pig hearts receiving either the ROS scavenger tiron or MnTBAP in the presence or absence of preconditioning by sevoflurane (APC). Note the marked protection against infarction by APC compared to other groups. *$P < 0.05$ *versus* I/R.

Amelioration of Ischemia/Reperfusion Injury to Mitochondria by APC

IR injury profoundly disrupts mitochondrial energy metabolism. It is well known that mitochondria isolated from hearts subject to IR exhibit decreased mitochondrial membrane potential ($\Delta\psi_m$), ETC dysfunction, and

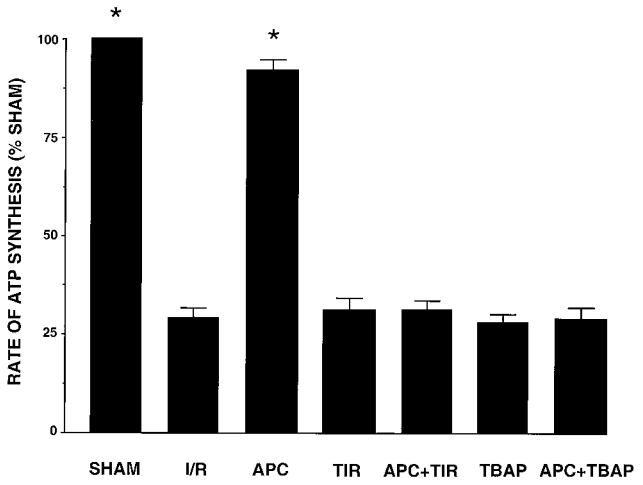

Fig. 4. Rate of ATP synthesis in mitochondria isolated from hearts exposed to ischemia/reperfusion alone (I/R), anesthetic preconditioning (APC), with or without the ROS scavengers tiron (APC + TIR, TIR) or MnTBAP (APC + TBAP, TBAP). In addition, there was a Sham group in which mitochondria were isolated from hearts not subjected to ischemia or treatment. Note that of ATP synthesis rate in mitochondria isolated from Sham and APC groups were significantly greater compared to other groups. *$P < 0.05$ *versus* I/R. Other details in text.

ATP synthesis.[25,26] IPC has been shown to improve mitochondrial electron transport function[27,28] and ATP synthesis on reperfusion after ischemia, and it is suggested to open K_{ATP} channels.[20,29] In contrast, there is little information on the effect of APC on mitochondrial bioenergetics, although alterations in ROS generation, NADH concentrations and mCa^{2+} have been documented.[4,12,13]

We postulate that there are common mitochondrial

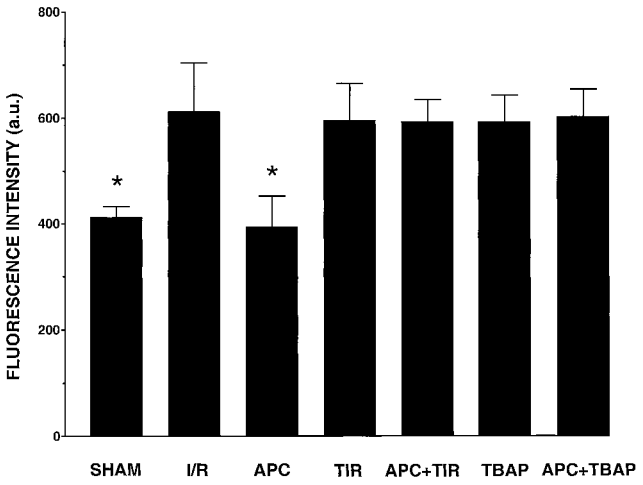

Fig. 5. Fluorescence intensity in arbitrary units (a.u.) of the oxidation production of dichlorohydro-fluorescein diacetate (DCFDA) to dichlorohydro-fluorescein (DCF) in proportion with generation of H_2O_2 to DCF. DCF oxidation was measured spectrofluorometrically (λ_{ex} 529 nm and λ_{em} 503 nm) in isolated mitochondria after global ischemia in ischemia/reperfusion alone (I/R), anesthetic preconditioning (APC), with or without the ROS scavengers tiron (APC + TIR, TIR) or MnTBAP (APC + TBAP, TBAP). In addition, there was a Sham group in which mitochondria were isolated from hearts not subjected to ischemia or treatment. *$P < 0.05$ *versus* I/R.

triggering mechanisms induced by IPC and by APC that lead to improved mitochondrial bioenergetics as an important factor in augmenting myocardial function after ischemia. We observed that a profoundly attenuated rate of ATP synthesis in mitochondria isolated shortly after global ischemia in nonpreconditioned hearts was effectively reversed by APC. We also observed that ATP synthesis rates in APC hearts were comparable to ATP synthesis rates in nonischemic hearts. Moreover, since we observed a significant reduction in ROS production in mitochondria isolated from preconditioned hearts, it is likely that APC also acts to attenuate the postischemic generation of mitochondrial ROS. Although we cannot exclude the possibility that APC also influences extramitochondrial ROS generating systems, the demonstrated role of the ETC in oxidant stress during ischemia,[30] and our previous finding of normalized NADH[11] and mitochondrial $[Ca^{2+}]$[12] in sevoflurane-treated hearts, strongly suggests that the mitochondria is a key target for these protective effects. In support of this, it was evidenced nearly 30 yr ago that volatile anesthetics can inhibit NADH oxidoreductase.[31]

Our results suggest that reduced ROS formation is involved in mediating mitochondrial protection after global ischemia. Interestingly, our results also demonstrate a crucial role of ROS in triggering APC before global ischemia. This was demonstrated by the effects of two ROS scavengers given only briefly before, during and after APC, to abrogate, not only the protective effects on global function and infarct size, but also to reverse APC-induced mitochondrial protection. This was evidenced by return of ROS generation and depression of ATP synthesis to levels observed in nonpreconditioned hearts.

Role of ROS in Triggering and Mediating APC

There is ample evidence that ROS are involved in triggering preconditioning. Exogenous administration of $O_2^{\cdot-}$[32] and H_2O_2[33] induces preconditioning. Furthermore, scavengers of ROS are known to inhibit preconditioning induced by various stimuli including brief ischemia[32] and opioids.[34] Our finding that the triggering of APC was inhibited by ROS scavengers is in agreement with our previous results[4] and those of Mullenheim *et al.*,[5] although the latter study was based indirectly on a ROS scavenging effect since ROS formation was not assessed.

We propose that $O_2^{\cdot-}$ is generated from a mitochondrial ETC source during sevoflurane exposure, although one or more of its downstream products (*e.g.*, H_2O_2 or hydroxyl radical) could be responsible for triggering the eventual preconditioning effect. We deliberately used two individual agents with highly different structures and ROS-depleting mechanisms. MnTBAP is a manganese containing porphyrin that acts as a superoxide dismutase mimetic, converting $O_2^{\cdot-}$ to H_2O_2 and H_2O; however

there is also evidence that it can act directly on H_2O_2[35] and peroxynitrite,[36] both of which may have preconditioning effects. Tiron is a cell permeable, nonenzymatic ROS scavenger, also with a high specificity for $O_2^{\cdot-}$.[37] Although it is an acceptable antioxidant, tiron does chelate a variety of metal ions and may interfere with metal ion binding of several intracellular proteins.[38,39] We specifically utilized two mechanistically dissimilar but potent antioxidants with relative $O_2^{\cdot-}$ selectivity to exclude possible non-$O_2^{\cdot-}$ depleting effects of these agents that could result in APC attenuation or interference with ATP production. Either agent, when given with sevoflurane, had comparable effects on ROS production in mitochondria isolated during early reperfusion. Neither agent alone had direct effects on LVP, coronary flow, or infarction in agreement with previous reports that the drugs were washed out before the global ischemia.[40,41] Indeed, our findings with MnTBAP and tiron support our earlier study that IR after APC led to reduced dityrosine fluorescence, an indicator of peroxynitrite which is primarily a product of $O_2^{\cdot-}$ and nitric oxide (NO^+).[4]

The mechanism by which ROS formed during the preconditioning phase leads to improved ATP synthesis on reperfusion is unclear. ROS are ubiquitous intracellular messengers that can initiate changes in mitochondrial function, or conversely, they can be formed as a result of altered mitochondrial function. During ischemia ROS,[42] NADH and mitochondrial Ca^{2+} accumulate[12,15,43] and $\Delta\psi_m$ decreases.[44] The rise in mitochondrial Ca^{2+} is likely secondary to a rise in cytosolic Ca^{2+} consequent to inhibition of sarcolemmal Na^+ and Ca^{2+} pumps, to Na^+ and Ca^{2+} influx due to Na-H^+ exchange, and to reversed or slowed Na^+-Ca^{2+}- exchange. The decrease in $\Delta\psi_m$ favors influx of Ca^{2+} from the cytosol to the mitochondrial matrix; this could increase the probability of opening the membrane permeability transition pore that disables the mitochondrion by dissipating $\Delta\psi_m$.[45]

Exogenous ROS (H_2O_2) are known to activate mitochondrial K_{ATP} channels,[46] possibly through activation of a specific isoform of protein kinase C (ϵPKC).[47] This action could lead to a small depolarization of $\Delta\psi_m$ and a decrease in the electrochemical gradient for Ca^{2+} entry.[48] In support of this hypothesis is a reported role for the membrane permeability transition pore in preconditioning[49]; preconditioning was shown to inhibit mitochondrial Ca^{2+} loading and to inhibit the pore opening, and the putative mitochondrial K_{ATP} channel opener diazoxide (a known preconditioning agent) was shown to induce a low conductance through the membrane permeability transition.[50,51]

Possible limitations of this study are that our Langendorff preparation is crystalloid perfused, and the presence of neutrophils and physiologic antioxidants normally present in blood might modify some of the responses we observed. Furthermore, our isolated heart preparation lacks autonomic influences, which could modify ischemia/reperfusion injury.

In conclusion, brief sevoflurane exposure improved mitochondrial bioenergetics and decreased mitochondrial ROS formation. This was associated with improved global cardiac function and reduced infarct size after ischemia. We demonstrated that APC leads to improved ETC function during IR, so that ATP availability is increased and ROS production is decreased, and it is tempting to speculate that it is these changes in mitochondria that underlie the global cardiac structural and functional preservation that is seen after reperfusion. The protective effects of APC were fully reversible by ROS scavengers given only during sevoflurane exposure. Our results support a model in which sevoflurane first acts on the mitochondrion *via* a redox signaling mechanism, which may then elicit an effect to open mitochondrial K_{ATP} channels by a ROS dependent mechanism. Knowledge of the mechanism of APC may help us to modify or mimic useful cardiac protective effects of anesthetics in the clinical setting.

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