ONLINE DATA SUPPLEMENT

Polyethylene Glycol-Superoxide Dismutase Prevents Cardiac Dysfunction in Endotoxin Treated Rats

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Measurement of Mitochondrial Hydrogen Peroxide Generation

Mitochondrial isolate hydrogen peroxide generation was measured using the Amplex red assay technique according to manufacturer's instructions (Molecular Probes, Eugene, OR). In brief, aliquots of freshly prepared mitochondrial suspensions were incubated with either malate/pyruvate (i.e. a Complex I substrate mix) or succinate/antimycin (a mixture using a Complex II substrate with an inhibitor of the distal electron transport chain as described in reference E1) in assay buffer. Amplex red was added to initiate reactions: this indicator reacts with hydrogen peroxide to generate a fluorescent signal with an excitation frequency of 540 nm and an emission frequency of 590 nm. Hydrogen peroxide standards, supplied with the Amplex red kit, were used to calibrate assays. Assay results are reported in pmole of hydrogen peroxide per mg protein.

Assessment of Mitochondrial Respiration

Mitochondria oxygen consumption was assessed using a Clark-type electrode (E2, E3). Hearts were first placed cold isolation buffer (180 mM KCl, 5 mM MOPS, 2 mM EGTA, pH 7.25 at 4^{o}C). After mincing finely with scissors, muscle pieces were homogenized for two, 7 second periods using a Polytron homogenizer set at ½ speed. A portion of this homogenate was assayed for protein content; the remaining homogenate was centrifuged at 600 x g for 7.5 minutes at 4^{o}C, and the pellet discarded. The supernatant was centrifuged at 5000 x g for 10 minutes at 4^{o}C, and the resulting mitochondrial pellet was resuspended in isolation buffer. State 3 and State 4 mitochondrial oxygen respiration rates were then measured according to established

procedures (E2, E3) using a Clark-type oxygen electrode (Instech, Plymouth Meeting, PA). For these assays, mitochondria were diluted to a protein concentration of 0.5 mg/ml in buffer (120 mM KCl, 5 mM KH_2PO_4, 5 mM MOPS, 1 mM EDTA, pH 7.25). State 2 respiration was initiated by adding pyruvate (10 mM)/malate (2.5 mM) and ADP (0.5 mM) was added to initiate State 3.

Electron Transport Chain NADH Oxidase Activity Assay

The electron transport chain NADH oxidase assay was used to assess the respiration rate of isolated, uncoupled mitochondria in response to addition of exogenous NADH; a reduction in this rate indicates inhibition of electron transport by the mitochondrial proteins (E4). Mitochondrial isolates were placed in hypotonic respiration solution (20 mM KH_2PO_4, 0.1 mM EDTA, pH 7.25) to induce mitochondrial swelling (permitting access of exogenous NADH to the electron transport chain). We then added cytochrome c to a final concentration of 700 μM to compensate for any potential loss of cytochrome c from the intermembrane space. NADH (20 mM) was added to initiate respiration and we measured NADH-stimulated oxygen consumption rates.

Measurement of Protein Carbonyl levels

Protein carbonyl content was assessed as previously described (E5). For determination of protein carbonyl sidegroup content (E5), paired mitochondrial samples were first precipitated with trichloroacetic acid on ice. After centrifugation, one sample from each pair was resuspended using 2 N HCl containing 0.1% DNP (2,4 dinitrophenylhydrazine) and the second sample was resuspended in HCl without DNP.

Assays were then incubated at room temperature for 60 min, and proteins reprecipitated with trichloroacetic acid. After washing precipitates three times with an ethanol-ethyl acetate mixture (1:1), samples were then dissolved in 6 M guanidine-HCl. The absorbance of the DNP derived sample, minus the absorbance of the nonderived protein (wavelength 370 nm), was then taken as an index of protein carbonyl group content, using a molar extinction coefficient of 21000.

Measurement of Lipid Aldehyde Formation

Mitochondrial lipid peroxidation was assessed using the Calbiochem Lipid Peroxidation kit assay (Calbiochem, San Diego, CA). This assay measures a combination of both malondialdehyde and 4-hydroxynonenal (two aldehydes) using an enzymatic technique that is more specific than the traditional thiobarbituric acid method. Because of the small amounts of mitochondrial samples available for assay, we did not have sufficient tissue to further determine the relative contributions of malondialdehyde versus 4-hydroxynonenal to these measurements. For these assays, resuspended mitochondrial samples (200 μl) were added to N-methyl-2-phenylindole in acetonitrile (10.3 mM in 650 μl). Methanesulfonic (15.4 mM) was then added (150 μl) and the reaction mixture was incubated at 45^{o} C. for 40 minutes. Samples were cooled on ice, absorbance measured at 586 nm, and lipid aldehyde content was determined by comparison to a standard curve generated using known concentrations of malondialdehyde and 4-hydroxynonenal (this test has equal molar extinction coefficients for both substances). Results are expressed per mg of protein in mitochondrial samples.

Nitrotyrosine and Oxyblot Protein Modification Determinations

SDS-PAGE protein gels were run for Western blot analysis of specific proteins (i.e. nitrotyrosine levels, protein carbonyl sidegroup formation). Prior to SDS-PAGE gel analysis, samples used for oxyblot determinations were prepared as per the manufacturer's instructions (Oxyblot^{TM} Kit, Serologicals Corp., Norcross, GA). Derived protein samples for oxyblot determinations and non-derived mitochondrial protein samples for nitrotyrosine determinations were diluted with an equal volume of a loading buffer (126 mM Tris-HCL, 20% glycerol, 4% SDS, 1.0% 2-mercaptoethanol, 0.005% bromophenyl blue, pH 6.8). Samples were then loaded onto 12% Tris Glycine polyacrylamide gels (60 μg of protein per lane for oxyblots, 100 μg per lane for nitrotyrosine determinations) and proteins separated by electrophoresis (Novex Minicell II, Carlsbad, CA). Standard molecular weight markers were also loaded onto gels to permit approximation of protein band molecular weights. After electrophoresis, proteins were transferred to polyvinylidene fluoride membranes and incubated over night at 4^{o}C with antibodies to targeted proteins (anti-nitrotyrosine antibody, Cayman Chemical, Ann Arbor, MI, and anti-DNP antibody for Oxyblot determination, Serological Corporation, Norcross, GA). Membranes were then incubated with HRP conjugated secondary antibodies and antibody binding was detected using enhanced chemiluminescence (NEN Life Science Products, Boston, MA). Gel densitometry was performed using a Microtek scanner (Carson, CA) and UN-SCAN-IT software (Silk Scientific, Orem, UT).

Assessment of Cardiac Pressure Generating Capacity

Pressure generating capacity of hearts was assessed using the Langendorf technique. In brief, after pentobarbital anesthesia (50 mg/kg), the chest was opened and

the heart removed with associated ascending aorta. The aortic root was quickly cannulated and the cannulated heart attached to a constant pressure temperature controlled (37°C) Langendorf perfusion circuit (Radnoti Glass Technology, Monrovia, DA). Aortic root pressure was adjusted to 80 mm Hg and perfused with Krebs-Henselheit solution (pH 7.40, NaCl 135 mM, KCl 5 mM, dextrose 11.1 mM, $CaCl_2$ 2.5 mM, $MgSO_4$ 1 mM, $NaHCO_3$ 14.9 mM, $NaHPO_4$ 1 mM, insulin 50 units/L, gassed with 95%O_2/5%CO_2). A thin walled balloon was inserted into the left ventricle via the left atrium and attached to a pressure transducer. After a 15 minute equilibration period, stepwise increments and decrements in left ventricular balloon diastolic pressure were made by infusing small amounts of saline into the balloon circuit. Developed systolic pressures were recorded over a range of diastolic pressure from 10 to 30 mm Hg. using a Gould strip chart recorder (Gould Electronics, Cleveland, OH).

References

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