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Original Contribution

TNF-induced mitochondrial damage: a link between mitochondrial complex I activity and left ventricular dysfunction

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ABSTRACT

Mitochondrial damage is implicated in the progression of cardiac disease. Considerable evidence suggests that proinflammatory cytokines induce oxidative stress and contribute to cardiac dysfunction. This study was conducted to determine whether a TNF-induced increase in superoxide ($O_2^{\cdot-}$) contributes to mitochondrial damage in the left ventricle (LV) by impairing respiratory complex I activity. We employed an electron paramagnetic resonance (EPR) method to measure $O_2^{\cdot-}$ and oxygen consumption in mitochondrial respiratory complexes, using an oxygen label. Adult male Sprague–Dawley rats were divided into four groups: control, TNF treatment (ip), TNF+ apocynin (APO; 200 μmol/kg bw, orally), and TNF+ Tempol (Temp; 300 μmol/kg bw, orally). TNF was injected daily for 5 days. Rats were sacrificed, LV tissue was collected, and mitochondria were isolated for EPR studies. Total LV ROS production was significantly higher in TNF animals than in controls; APO or Temp treatment ameliorated TNF-induced LV ROS production. Total mitochondrial ROS production was significantly higher in the TNF and TNF+ APO groups than in the control and TNF+ Temp groups. These findings suggest that TNF alters the cellular redox state, reduces the expression of four complex I subunits by increasing mitochondrial $O_2^{\cdot-}$ production and depleting ATP synthesis, and decreases oxygen consumption, thereby resulting in mitochondrial damage and leading to LV dysfunction.

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Neurohumoral mechanisms play important roles in the pathophysiology of cardiovascular disease. Current treatments aimed at blocking neurohormones such as angiotensin have considerably reduced mortality and morbidity; however, the progressive clinical course of heart disease emphasizes the need for innovative approaches to therapy. A growing body of evidence indicates that, along with neurohormones, proinflammatory cytokines (PICs) contribute to the progression of heart disease [1–3]. The PICs, including tumor necrosis factor (TNF), interleukin-1β (IL-1β), and IL-6, can induce oxidative stress and contribute to the pathophysiology of cardiovascular disease [4–7]. TNF is the most studied cytokine; it increases with the severity of heart disease and is of prognostic significance. TNF stimulates reactive oxygen species (ROS) in mitochondria by altering membrane permeability and by inhibiting the electron transport chain (ETC), thereby causing mitochondrial damage [8,9]. However, at the mitochondrial level, the potential implications of chronic TNF infusion in the progression of left ventricular (LV) dysfunction are not known.

Proper myocardial function depends on the energy produced by mitochondria, which is primarily generated by oxidative phosphorylation and fatty acid β-oxidation [10–12]. Damage to mitochondria results in the inability to generate energy in the form of ATP [13,14]. Overproduction of ROS also occurs as a result of this damage and contributes to cardiac dysfunction [15–17]. Complexes I and III of the mitochondrial ETC are potent sources of cellular superoxide; deficiencies in the ETC result in increased mitochondrial superoxide production, which is a major cause of cellular damage [18–20]. Complex I defects are some of the most frequent causes of ETC disorders. Mitochondrial complex I contains 46 distinct subunits; the number of subunits directly involved in electron transport is unknown. We explored the effects of TNF on mitochondrial complex I superoxide production in the LV and examined four specific protein subunits of complex I, the 17-, 20-, 30-, and 39-kDa subunits, which are known participants in oxidative phosphorylation and ATP production [21,22]. We also examined alterations in the protein levels of these subunits in response to TNF infusion. We used a gain-of-function strategy by blocking NAD(P)H oxidase using apocynin (APO) or by scavenging superoxide using Tempol (Temp).

We used electron paramagnetic resonance (EPR), the most sensitive and definitive method for quantification of oxygen consumption in mitochondria. EPR is superior to other free radical detection methods in that it allows for the direct measurement of specific free radicals using specific spin probes [23]. Under pathological conditions, increased oxidative stress itself can alter oxygen levels; this might affect mitochondrial oxygen consumption. To circumvent this problem, we developed an EPR method for measuring superoxide and oxygen consumption in mitochondrial respiratory

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complexes, using the oxygen label NOX-13.1-OS. By using a gas controller and newly synthesized nontoxic spin label, we were able to set up a physiological oxygen concentration of 20 mm Hg [24] and to follow the consumption of oxygen during detection of ROS. The merit of this method is that it allows us to measure superoxide production, complex activity, and oxygen consumption in parallel using the same incubation medium, temperature, and substrate concentration in each mitochondrial preparation.

Materials and methods

Chemicals and drugs

The spin probes 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) and 1-hydroxy-4-phosphono-oxy-2,2,6,6-tetramethylpiperidine (PPH), the metal chelators defferoxamine (DF) and diethyldithiocarbamate (DETC), Krebs–Hepes buffer (KHB), and the oxygen label NOX-13.1-OS were obtained from Noxygen Science Transfer and Diagnostics (Elzach, Germany). Recombinant rat TNF was obtained from Biosource (Camarillo, CA, USA). Bovine erythrocyte superoxide dismutase (SOD), polyethylene glycol-conjugated superoxide dismutase (PEG-SOD), APO, and Temp were obtained from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals and reagents used were of analytical grade and were purchased from Sigma–Aldrich unless otherwise specified.

Animals

Studies were conducted using adult male Sprague–Dawley rats (n= 8 in each group), weighing 325–350 g, obtained from Harlan (Indianapolis, IN, USA). Animals were housed in temperature-(23 ± 2°C) and light-controlled (12 h light/dark cycle) animal quarters; standard rat chow and water were provided ad libitum. All animal experimental protocols were approved by the Louisiana State University Institutional Animal Care and Use Committee.

Experimental protocol

Rats were treated for 5 days with TNF (40 μg/kg body wt (bw), intraperitoneally (ip)), TNF+ APO (200 μmol/kg bw, orally), TNF+ Temp (300 μmol/kg bw, orally), or vehicle. APO-only and Temp-only groups served as positive controls. Body weights were measured at baseline and on day 6 (study end). Tail plethysmography was performed daily as previously described [9]. On day 6, rats were injected with heparin (100 U/25 mg bw, ip) to prevent excessive blood clotting. Rats were then sacrificed, and LV were separated from hearts for mitochondrial isolation and tissue processing for EPR studies.

Echocardiography

Echocardiography was performed as previously described [25]. The Tei index (an indicator of diastolic dysfunction) was determined from Doppler recordings of LV inflow and outflow as described previously [26].

Isolation of LV mitochondria

LV mitochondria were isolated by differential centrifugation of heart homogenates as described previously [9]. Mitochondrial purity was assessed by transmission electron microscopy.

Electron spin resonance studies

Total LV ROS and $O_2^{\cdot-}$ production as well as mitochondrial total ROS and H_2O_2 production were measured by EPR using the spin probes CMH and PPH [27], respectively. Detection of ROS and oxygen concentrations was conducted under the following EPR settings: center field g= 2.002, field sweep 50 G, microwave power 20 mW, modulation amplitude 1.90 G, conversion time 10.24 ms, time constant 81.92 ms.

Preparation of LV tissue for EPR studies

Small portions (15–20 mg) of LV tissue from each animal were minced and placed into 4 wells of a 24-well plate with 20 mM KHB containing 25 μM DF and 5 μM DETC. Tissue pieces were then washed twice with the same buffer to remove any trace contamination and incubated at 37°C with specific spin probes for 30 min. The incubation of tissue was terminated by placing the plate on ice. All tissue EPR experiments measuring the concentration of oxidized $CM^{\bullet}$ and $PP^{\bullet}$ were conducted at 20°C in disposable capillary tubes as previously described [27].

Total LV tissue ROS production

Tissue pieces were incubated at 37°C with CMH (200 μM) for 30 min. Aliquots of the incubated probe medium were then taken into 50-μl glass capillary tubes (Noxygen Science Transfer and Diagnostics) for determination of total LV ROS production.

LV tissue $O_2^{\cdot-}$ production

Superoxide production was measured as previously described [27]. Briefly, tissue pieces were incubated at 37°C with PEG-SOD (50 U/ml) for 30 min, then the spin probe CMH (200 μM) was added for another 30-min incubation period. Aliquots of the incubated probe medium were then taken into 50-μl glass capillary tubes for determination of total LV superoxide production. Preincubation of tissue with PEG-SOD allows competitive inhibition of CMH by intracellular and extracellular released $O_2^{\cdot-}$. Because it is cell permeative, PEG-SOD can competitively inhibit the CMH/$O_2^{\cdot-}$ interaction in both the intracellular and the extracellular spaces, thus allowing accurate measurement of tissue $O_2^{\cdot-}$ production. For tissue $O_2^{\cdot-}$ production, the values obtained from incubation with PEG-SOD and CMH were subtracted from the values obtained from incubation with CMH only. For determination of LV $O_2^{\cdot-}$ production, the above-mentioned EPR settings were used.

Total mitochondrial ROS production

LV mitochondria from each rat (7–10 μg protein) were mixed with CMH. After addition of CMH, aliquots of the mitochondria were taken into 50-μl glass capillary tubes [11]. Total mitochondrial ROS production was detected using EPR under the following settings: center field g= 2.002, field sweep 9.000 G, microwave power 20 mW, modulation amplitude 1.90 G, conversion time 10.24 ms, time constant 81.92 ms, receiver gain 3.17×10^3. For the detection of ROS production, we used the Time Scan mode with the averaging of EPR amplitude every 10 scans over 10 min. Total mitochondrial ROS production experiments were performed at 37°C under 20 mm Hg of oxygen partial pressure. The setup of the oxygen concentration in KHB buffer was performed using the Gas-Controller NOX-E.4-GC (Noxygen Science Transfer and Diagnostics GmbH).

Mitochondrial $O_2^{\cdot-}$ and hydrogen peroxide (H_2O_2) production

Mitochondrial $O_2^{\cdot-}$ and H_2O_2 production was measured using PPH as the spin probe [11,28]. Aliquots of LV mitochondria (approximately 7–10 μg protein) were probed with PPH (500 μM) alone or PPH and SOD (50 U/ml) for quantification of $O_2^{\cdot-}$ production. Catalase (50 U/ml) was added to measure H_2O_2 formation. PPH allows the detection of extracellular and extramitochondrial production of $O_2^{\cdot-}$ [28]. PPH