Circulating levels of cytochrome *c* after resuscitation from cardiac arrest: a marker of mitochondrial injury and predictor of survival

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Ca2+ overload and reactive oxygen species can injure mitochondria during ischemia and reperfusion. We hypothesized that mitochondrial injury occurs during cardiac resuscitation, causing release of cytochrome *c* to the cytosol and bloodstream while activating apoptotic pathways. Plasma cytochrome *c* was measured using reverse-phase HPLC and Western immunoblotting in rats subjected to 4 or 8 min of untreated ventricular fibrillation and 8 min of closed-chest resuscitation followed by 240 min of postresuscitation hemodynamic observation. A sham group served as control. Plasma cytochrome *c* rose progressively to levels 10-fold higher than in sham rats 240 min after resuscitation (*P* < 0.01), despite reversal of whole body ischemia (decreases in arterial lactate). Cytochrome *c* levels were inversely correlated with left ventricular stroke work (*r* = −0.40, *P* = 0.02). Western immunoblotting of left ventricular tissue demonstrated increased levels of 17-kDa cleaved caspase-3 fragments in the cytosol. Plasma cytochrome *c* was then serially measured in 12 resuscitated rats until the rat died or cytochrome *c* returned to baseline. In three survivors, cytochrome *c* rose slightly to ≤2 μg/ml and returned to baseline within 96 h. In nine nonsurvivors, cytochrome *c* rose progressively to significantly higher maximal levels [4.6 (SD 2.0) vs. 1.6 (SD 0.3) μg/ml, *P* = 0.029] and at faster rates [0.7 (SD 0.5) vs. 0.1 (SD 0.1) μg·ml⁻¹·h⁻¹, *P* = 0.046] than in survivors. Plasma cytochrome *c* may represent a novel in vivo marker of mitochondrial injury after resuscitation from cardiac arrest that relates inversely with survival outcome.

apoptosis; biological markers; cardiopulmonary resuscitation; myocardial ischemia; ventricular fibrillation

CYTOCHROME *c*, a 14-kDa hemoprotein that normally resides in the outer surface of the inner mitochondrial membrane bound to cardiolipin (34), plays a key physiological role in oxidative phosphorylation as electron carrier between cytochrome *c* reductase (complex III) and cytochrome *c* oxidase (complex IV). However, cytochrome *c* can also translocate out of the mitochondria to the cytosol under various pathological conditions, such as ultraviolet irradiation (21), serum and glucose deprivation (9), exposure to H2O2 or superoxide anion (O2−) (44), Ca2+ overload (35), and hypoxia (16). In the cytosol, cytochrome *c* forms an oligomeric complex with 2-deoxy-ATP and apoptotic protease activating factor-1 (30). This complex recruits procaspase-9, forming what is known as the apopto-...
femoral artery into the abdominal aorta for pressure measurement and blood sampling. A thermocouple microprobe (model IT-18, Physitemp) was advanced through the right femoral artery into the thoracic aorta for thermodilution cardiac output measurement. A PE-50 catheter was advanced through the left external jugular vein into the right atrium and used exclusively for injection of thermal tracer. A 3-Fr catheter (model C-PUM-301J, Cook) was advanced through the right external jugular vein into the right atrium, and through its lumen a precurved guide wire was fed into the right ventricle for electrical induction of VF. Core temperature was maintained at 36.5–37.5°C with an infrared heating lamp.

**VF and Resuscitation Protocol**

VF was induced by delivery of a 60-Hz alternating current to the right ventricular endocardium (0.1–0.6 mA) for an uninterrupted interval of 3 min, the current was turned off, and VF was allowed to continue until completion of a predetermined interval (see below). Chest compression was then begun using an electronically controlled and pneumatically driven (50 psi) chest compressor (model CJ-80623, CJ Enterprises) set to deliver 0.39 ml/100 g body wt of 100% oxygen every 45 s. Positive-pressure ventilation was provided using an electronically controlled solenoid valve (model R-481, Clippard Instrument Laboratory) set to deliver 0.39 ml/100 g body wt of 100% oxygen every two compressions. Defibrillation was attempted after 8 min of chest compression by delivery of a maximum of two 2-J monophasic transthoracic shocks (LifePak 9R, Physio-Control). If VF persisted or an organized rhythm with a mean aortic pressure of ~25 mmHg ensued, chest compression was resumed for 30 s. The defibrillation-compression cycle was repeated up to three additional times, with an increase in the energy of individual shocks to 4 J if VF persisted and an increase to 8 J for the last two cycles. Successful resuscitation was defined as the return of an organized cardiac rhythm with a mean aortic pressure ≥60 mmHg for ≥5 min. Resuscitated rats were ventilated initially with 100% O2 for 15 min and with 50% O2 for the remaining postresuscitation interval.

**Experiments**

Two series of experiments were conducted. In **series 1**, changes in plasma cytochrome c were measured for up to 240 min after resuscitation in fully instrumented rats (see above). In **series 2**, changes in plasma cytochrome c were measured for up to 96 h after resuscitation in rats that were minimally instrumented to facilitate survival (see below).

**Series 1.** Three groups of four rats each were investigated. Two groups were subjected to 4 or 8 min of untreated VF followed by 8 min of closed-chest resuscitation before defibrillation was attempted and observed for ≤240 min after resuscitation. One group, which received identical treatment, except for VF and closed-chest resuscitation, served as the sham group. Rats were randomized to the experimental assignment immediately after completion of surgical preparation.

**Series 2.** The animal preparation for **series 2** differed from that for **series 1**, in that hemodynamic measurements were limited to right atrial and aortic pressures by advancing PE-50 tubing from the left jugular vein into the right atrium and from the left carotid artery into the descending thoracic aorta. Additional surgical manipulation was avoided, except for the 3-Fr catheter (model C-PUM-301J, Cook) and guide wire required for induction of VF. Core temperature was measured through a rectal thermometer. During closed-chest resuscitation, rats were ventilated using a volume-controlled ventilator (model 683, Harvard Apparatus) set to deliver 6 ml/kg at 25 breaths/min unsynchronized to chest compression. For defibrillation, a biphasic waveform defibrillator (Heartstream XL, Philips Medical Systems) was used to deliver 3, 5, and 7 J as needed according to **series 1** protocol. These changes reflected implementation of new developments in cardiac resuscitation. After 240 min of resuscitation, rats were allowed to recover from anesthesia. The left carotid PE-50 tubing was replaced with soft PE-50 tubing primed with heparinized glycerol. The proximal end was sealed with a metal clip that could be removed for blood sampling and tunneled under the skin to the interscapular region, where a skin incision held closed by a removable metal clip allowed intermittent access. For blood sampling, rats were anesthetized using isoflurane (0.5–1.0%) and 100% oxygen, and arterial blood (200 μl) was collected into a heparinized syringe. A total of 12 rats were successfully resuscitated after 8 min of untreated VF and 8 min of closed-chest resuscitation. Cytochrome c was serially measured until the rat died or cytochrome c returned to baseline levels.

Additional experiments were performed after documentation of prominent increases in postresuscitation plasma cytochrome c levels to determine 1) in vitro, whether cytochrome c could induce apoptosis in circulating leukocytes and 2) in vivo, whether plasma cytochrome c could reflect increased apoptotic activity of leukocytes, notwithstanding evidence suggesting that, in other acute settings such as acute respiratory distress syndrome (31), sepsis (33), and burn injury (12), apoptosis is suppressed in neutrophils. Apoptosis was investigated using flow cytometry to detect exteriorization of phosphatidylserine in total leukocytes, neutrophils, and lymphocytes (43).

For in vitro studies, 5 ml of blood were collected through PE-50 tubing advanced from the left carotid artery into the descending thoracic aorta in adult male Sprague-Dawley rats. Blood was heparinized (30 U/ml), and 300-μl aliquots were incubated with rat heart cytochrome c at 0, 2 and 10 μg/ml at 37°C for 4 h in an incubator shaker (model I2400, New Brunswick Scientific). For in vivo studies, rats were subjected to 8 min of untreated VF followed by closed-chest resuscitation as described for **series 2** and monitored until they died. Arterial blood was collected when the mean aortic pressure decreased below 40 mmHg. Blood samples were processed for flow cytometry as described below.

**Materials**

Acetonitrile (ACN), EDTA, HEPES, mannitol, propidium iodide (PI), rat heart cytochrome c, sucrose, trifluoroacetic acid (TFA), and mouse monoclonal anti-β-actin antibody were purchased from Sigma; 1-mm-thick 12% and 14% Novex Tris-glycine polyacrylamide gels from Invitrogen; polyvinylidene difluoride membranes from Roche Applied Science; rabbit polyclonal anti-cytochrome c and rabbit polyclonal anti-caspase-3 antibodies from Cell Signaling Technology; mouse monoclonal anti-prohibitin antibody from Calbiochem; annexin V conjugated with fluorescein isothiocyanate (FITC) and mouse monoclonal anti-cytochrome c antibody (clone 7H8.2C12) from BD Biosciences PharMingen; Immunolyse (red blood cell lysis agent) from Coulter; and goat polyclonal anti-rabbit IgG and goat polyclonal anti-mouse IgG horseradish peroxidase (HRP)-conjugated antibodies, bicinchoninic acid protein concentration assay kit, and West femto maximum sensitivity chemiluminescent detection kit from Pierce Biotechnology.

**Hemodynamic Measurements**

Continuous physiologic measurements were transduced, conditioned (BIOPAC Systems), and digitized at 250 scans/s using a 16-bit data acquisition board (model AT-MIO-16XE-50, National Instruments). Systemic and left ventricular pressures were obtained through fluid-filled systems attached to disposable pressure transducers (Maxim Medical) zeroed to midchest level. Cardiac output was measured by thermodilution after a right atrial bolus injection of 200 μl of 0.9% NaCl at room temperature and curve analysis using custom-developed LabVIEW-based software. Cardiac index (CI) was calculated by normalization of cardiac output (in ml/min) to body weight (in kg).
The stroke volume index was calculated by dividing CI by heart rate. Left ventricular stroke work index (LVSWI) was calculated by multiplying the stroke volume index by the difference between left ventricular systolic and diastolic pressures.

**Plasma Cytochrome c**

Arterial blood samples (200 µl) were collected into heparinized syringes and centrifuged at 5,000 rpm (2,320 g) for 10 min at 4°C (Sorvall Biofuge Stratos, Heraeus). The supernatant (plasma) was frozen at −80°C for later analysis using reverse-phase high-performance liquid chromatography (HPLC) and Western immunoblotting in series 1 but used immediately for analysis by HPLC in series 2. HPLC. A reverse-phase HPLC technique previously used to measure cytochrome c in mitochondrial suspension and cytosol (15, 36) was adapted for measurement of cytochrome c in plasma. Samples were first treated with 1:1 (vol/vol) 50% ACN solution-0.1% TFA (ACN-TFA) and then centrifuged at 5,000 rpm (2,320 g) for 10 min to precipitate high-molecular-mass plasma proteins. Cytochrome c was measured in the supernatant using a Beckman HPLC system equipped with a Jupiter C-4 reverse-phase analytic column (150 × 4.6 mm, 5 µm; Phenomenex) preceded by a guard column [4.0 × 3.0 mm, widepore C-4 (butyl) column, SecurityGuard, Phenomenex], an ultraviolet-visible light spectroscopic detector, and Beckman System Gold software. After the mobile phase was programmed to provide a constant-flow (1.0 ml/min) linear gradient starting at 20% ACN-TFA and increasing to 60% ACN-TFA over a 15-min interval, the sample was washed for 5 min in 60% ACN-TFA and allowed to equilibrate for 5 min in 20% ACN-TFA. Each sample (20 µl) was loaded in an injection loop and automatically delivered into the mobile phase at the start of the linear gradient. Absorbance was measured at 393 nm. For quantification of plasma cytochrome c levels, standard curves (0.2–20 µg/ml) were prepared using rat heart cytochrome c dissolved in plasma from pentobarbital sodium-anesthetized rats not subjected to surgical manipulation (Fig. 1A). In vivo increases in cytochrome c were confirmed by serial plasma measurements after injection of 5 mg/kg of rat heart cytochrome c during spontaneous circulation (Fig. 1B).

**Western immunoblotting.** Western immunoblotting was performed as previously described by Ausubel et al. (5) and Harlow and Lane (23). Plasma samples were thawed on ice, and 8 µl were resolved in 1-mm-thick 12% Novex Tris-glycine polyacrylamide gel. After electrophoresis, a wet electroblotting apparatus (Hoefer TE22, Amersham Pharmacia Biosciences) was used to transfer the fractionated proteins to a polyvinylidene difluoride membrane. After transfer, the blots were blocked and incubated at 4°C overnight with rabbit polyclonal anti-cytochrome c antibody (1:2,000 dilution) and then with goat polyclonal anti-rabbit IgG HRP-conjugated antibody (1:1,000 dilution) for 1 h at room temperature. Chemiluminescence was documented in X-ray film. Protein molecular mass markers were run simultaneously in each gel. The specificity of the cytochrome c band was based on molecular mass along with positive and negative controls.

**Western Immunoblotting of Left Ventricular Tissue**

Hearts from series 1 experiments were harvested 240 min after resuscitation or earlier if hemodynamic deterioration occurred (i.e., a decrease in mean aortic pressure below 40 mmHg in 2 instances). The right ventricle and atria were removed, and the left ventricle was frozen in liquid N2 and stored at −80°C until analysis. For assessment of cytochrome c release, subcellular fractions were separated by the technique of Ott et al. (34) with minor modifications and subjected to Western immunoblotting. Briefly, left ventricular tissue (~100 mg) was homogenized (Dounce homogenizer) in 2 ml of MSH buffer [210 mM mannitol, 70 mM sucrose, 5 mM HEPES (pH 7.4), and 1 mM EDTA (pH 8)] and centrifuged at 1,000 g for 10 min to pellet nuclei and debris. The resulting supernatant was centrifuged at 10,000 g for 30 min. The pellet containing mitochondria was washed and resuspended in MSH buffer. The supernatant was further centrifuged at 33,000 g for 3.3 h, and the resulting supernatant, representing the cytosolic fraction, was collected. Both fractions were stored at −80°C until analysis. The protein concentration in each fraction was determined by the bicinchoninic acid kit. Mitochondrial and cytosolic fractions (20 µg each) were resolved in 1-mm-thick 12% Novex Tris-glycine polyacrylamide gel and immunoblotted as described above for plasma cytochrome c. Cytochrome c was probed using a mouse monoclonal anti-cytochrome c antibody (1:4,000 dilution). Prohibitin, a 30-kDa inner mitochondrial membrane protein, and β-actin were selected as loading controls for mitochondrial and cytosolic fraction, was collected. Both fractions were stored at 33,000 g for 30 min. The pellet containing mitochondria was washed and resuspended in MSH buffer. 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cytosolic fractions, respectively. Prohibitin was probed using a mouse monoclonal anti-prohibitin antibody (1:1,000 dilution). β-Actin was probed using a mouse monoclonal anti-β-actin antibody (1:2,500 dilution). The blots were incubated with the primary antibodies for 3 h at room temperature and then with HRP-conjugated goat polyclonal anti-mouse IgG (1:1,000 dilution) for 1 h at room temperature and documented by chemiluminescence.

For assessment of caspase-3 activation, 100 mg of left ventricular tissue were homogenized in 0.4 ml of MSH buffer with a homogenizer (Mixer Mill, model MM200, Retsch). The cytosolic fraction (100 μg) was separated by subcellular fractionation as described above and subjected to 14% gel electrophoresis and Western immunoblotting using rabbit polyclonal anti-caspase-3 antibody (1:1,000 dilution), which recognizes 35-kDa procaspase-3 and its 19- and 17-kDa cleaved fragments (32). For loading controls, β-actin was used as described above.

Detection of Leukocyte Apoptosis by Flow Cytometry

During apoptosis, phosphatidylserine, which normally resides in the cytoplasmic side of cell membranes, translocates to the extracellular side (20). This step is characteristic of early apoptosis (preceding DNA fragmentation) and is used to detect apoptosis on the basis of annexin V binding to phosphatidylserine (43). Annexin V, a 36-kDa Ca²⁺-dependent phospholipid-binding protein with high affinity for phospholipid bilayers containing phosphatidylserine (4), is used in various apoptosis assays, commonly in conjunction with the vital dye PI, to concomitantly assess disruption of plasma membrane. In the present studies, blood was incubated with annexin V-FITC and PI and then subjected to flow cytometry to detect cells that bind annexin V and those that bind PI (43).

Blood labeling. Labeling with annexin V-FITC and PI was performed according to Van Oostveldt et al. (41) with modifications. Briefly, blood samples (100 μl) were washed first with PBS and then with annexin V binding buffer [10 mM HEPES, 140 mM NaCl, and 2.5 mM CaCl₂ (pH 7.4)]. The buffers were removed by centrifugation at 370 g for 5 min, and the pellets were suspended in 100 μl of annexin V binding buffer. Annexin V-FITC (5 μl) was added, and the samples were protected from light and incubated for 15 min at room temperature. The samples were then washed with PBS containing 2.5 mM CaCl₂ for removal of unbound annexin V-FITC. Red blood cells were lysed using 1 ml of Immunolyse diluted in PBS (1:24 dilution). The samples were then centrifuged at 370 g for 5 min, and the pellets were resuspended in 400 μl of PBS containing 2.5 mM CaCl₂. PI was added to a final concentration of 2.5 ng/μl, and the sample was incubated on ice for 5–10 min before immediate analysis. All other samples were analyzed within 1 h. Unstained, single-stained (annexin V-FITC or PI), and double-stained (annexin V-FITC and PI) samples were used to adjust compensation and to set horizontal and vertical cursors on the two-parameter dot-plot.

Flow cytometry. A FACSCalibur cytometer (Becton Dickinson) equipped with a 488-nm argon ion laser was used for flow cytometry. The total leukocyte population was gated on the basis of cell size (forward scatter) and granularity (side scatter), identifying two separate gates corresponding to neutrophils and lymphocytes. The FITC and PI fluorescence signals were detected using 530- and 585-nm band-pass filters, respectively. Two-dimensional fluorescence dot-plot profiles representing 10,000 gated events for total leukocytes and proportionally fewer events for the subpopulations of neutrophils and lymphocytes were generated. The data were analyzed using CellQuest Pro software (Becton Dickinson).

Statistical Analysis

Differences among groups for continuous variables were analyzed using one-way ANOVA and Holm-Sidak’s test for multiple comparisons. Alternative nonparametric tests were used if the data failed tests for normality or equal variance. The strength of association between variables was analyzed using Pearson’s product-moment correlation test. Values are means (SD) unless otherwise stated. Two-tailed P < 0.05 was considered significant.

RESULTS

Series 1

Baseline hemodynamic measurements were comparable among groups. After resuscitation, rats subjected to VF developed characteristic postresuscitation myocardial dysfunction, evidenced by persistently low mean aortic pressure and progressive reductions in LVSWI and CI without measurable differences related to the duration of untreated VF (Fig. 2). Myocardial dysfunction accounted for early death of one rat subjected to 4 min of untreated VF (at 150 min) and one rat subjected to 8 min of untreated VF (at 155 min). As previously reported, cardiac arrest and resuscitation were associated with anaerobic metabolism, evidenced by prominent increases in arterial lactate and reductions in arterial bicarbonate. Arterial lactate was maximal after return of spontaneous circulation and gradually decreased toward baseline during the postresuscitation interval (Fig. 3).

Plasma cytochrome c rose progressively during the postresuscitation interval in rats subjected to VF, with a statistically significant difference from sham rats beginning 30 min after resuscitation (Fig. 3). Western immunoblotting confirmed elevated levels of cytochrome c in plasma during the postresuscitation interval (Fig. 4). The plasma levels of cytochrome c were inversely correlated with LVSWI (r = –0.40, P = 0.02, for 38 measurements obtained at 5 postresuscitation events in 8 resuscitated rats).

In left ventricular tissue, higher values for cytochrome c were observed in cytosolic and mitochondrial fractions proportional to the injury, but the differences were not statistically significant (Figs. 5A and 6). The level of 17-kDa caspase-3

![Fig. 2. Hemodynamic and left ventricular measurements at baseline and after resuscitation in rats randomized to 4 min (shaded circles, n = 4) or 8 min (filled circles, n = 4) of untreated VF or to sham intervention (open circles, n = 4). Numbers in brackets indicate number of survivors. LVSWI, left ventricular stroke work index; CI, cardiac index; MAP, mean aortic pressure. Shaded horizontal bars represent 95% confidence interval of the aggregate baseline values. Values are means ± SE. *P < 0.05; †P < 0.01; and ‡P < 0.001 vs. sham (by 1-way ANOVA and Holm-Sidak’s test for multiple comparisons).](http://ajpheart.physiology.org/ by 10.22032/246 on September 21, 2017)
fragments measured in the cytosolic fraction, however, was significantly increased in rats resuscitated from VF (Figs. 5B and 6).

Series 2

Of 12 successfully resuscitated rats, 3 survived for >24 h and were euthanized according to the protocol after 70–96 h. In this group, cytochrome c rose gradually to ≤2 μg/ml and then returned to baseline levels (Fig. 7). The remaining nine rats died 1.3–32.5 h after resuscitation, with a progressive rise in plasma cytochrome c (Figs. 1C and 7) to higher maximal levels [4.6 (SD 2.0) vs. 1.6 (SD 0.3) μg/ml, P = 0.029] and faster rates [0.7 (SD 0.5) vs. 0.1 (SD 0.05) μg·ml⁻¹·h⁻¹, P = 0.046] in nonsurvivor than in survivor rats. Comparison of survivors and nonsurvivors demonstrated similar coronary perfusion pressures during chest compression but numerically higher energy requirements for successful defibrillation in nonsurvivors (Table 1). After resuscitation, mean aortic pressure was numerically lower in nonsurvivor than in survivor rats at 60 min [78 (SD 26) vs. 98 (SD 2) mmHg] and at 120 min [85 (SD 18) vs. 100 (SD 4) mmHg].

Leukocyte Apoptosis

Flow cytometry scattergrams of total leukocytes, neutrophils, and lymphocytes at baseline and after resuscitation are shown in Fig. 8. The aggregate data from the in vitro and in vivo studies are shown in Table 2. The in vitro studies show that incubation with increasing concentrations of cytochrome c (≤10 μg/ml) did not induce apoptosis in total leukocytes, neutrophils, or lymphocytes, as evidenced by a comparably low percentage of annexin V+/PI⁻ and annexin V+/PI+ cells. The in vivo studies showed a decreased percentage of annexin V+/PI⁻ neutrophils after resuscitation, but not in lymphocytes, in which the level of apoptosis was already low. The plasma cytochrome c levels measured by HPLC in these rats corresponded to 0.13 μg/ml (SD 0.18) at baseline and 2.36 μg/ml (SD 0.74) after resuscitation.

DISCUSSION

The present study demonstrates for the first time in a rat model of VF and closed-chest resuscitation that cytochrome c is released into the bloodstream after resuscitation from cardiac arrest. Release of cytochrome c was associated with activation of the executioner caspase-3, impaired left ventricular function,
and decreased survival. Accordingly, measurement of cytochrome c in plasma may serve as an in vivo marker of mitochondrial injury that inversely relates to survival outcome.

**Cytochrome c Release to the Cytosol**

Release of cytochrome c to the cytosol is an important pathogenic event that signals activation of the intrinsic (or mitochondrial) apoptotic pathway, with subsequent activation of executioner caspases. In the present studies, we documented activation of the executioner caspase-3 in the cytosol of left ventricular tissue by detection of 17-kDa cleaved fragments. Caspase-3 activation was associated with numerical increases in cytosolic and mitochondrial cytochrome c. Additional work is underway in our laboratory to validate these tissue findings, explore possible participation of the extrinsic apoptotic pathway, and examine the downstream effect of caspase activation, bearing in mind that apoptotic death is one of many other possible cell effects, some of which may not be lethal and may explain reversible dysfunction (13, 40). The possibility of increased mitochondrial cytochrome c after resuscitation is intriguing but consistent with recent studies reporting upregulation of genes encoding for proteins of the respiratory chain during apoptosis (11).

Various mechanisms have been proposed to explain how cytochrome c is released from the mitochondria. One mechanism involves opening of a high-conductance megachannel. This channel, known as the mitochondrial permeability transition pore (MPTP) (14), forms by apposition of at least three

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Values are means (SD); n, number of rats; CPP, coronary perfusion pressure averaged from minute 2 to minute 8 of chest compression; DF, defibrillation; VF, ventricular fibrillation. No statistical differences were observed between groups.
cytochrome

reperfusion trigger processes leading to a progressive rise in whole body ischemia. This would suggest that ischemia and after return of spontaneous circulation, despite reversal of related to survival outcome. Cytochrome

membrane (1, 6, 7, 37). Regardless of the mechanisms, our larger molecules, such as lactate dehydrogenase, which is the extracellular space and appears in the bloodstream is not

blood flow. The specific organs that contributed to circulating origin of MPTP opening, but not outer mitochondrial membrane permeabilization of the outer mitochondrial membrane without MPTP opening. Proapoptotic and antiapoptotic proteins of the β-cell leukemia/lymphoma-2 (Bcl-2) family play a critical role, given their ability to form channel-like structures when proapoptotic members translocate to the outer mitochondrial membrane (19, 28). Cytochrome c release is facilitated by concurrent peroxidation of cardiolipin by reactive oxygen species. Peroxidation of cardiolipin decreases its binding affinity for cytochrome c, creating a soluble pool that can be released to the cytosol (34).

The specific mechanism(s) responsible for cytochrome c release in our experimental model is not known. It is conceivable that a particular mechanism of cytochrome c release may relate to the type and severity of tissue injury. For instance, MPTP opening, but not outer mitochondrial membrane permeabilization, causes collapse of the mitochondrial voltage gradient, leading to uncoupling of oxidative phosphorylation and cessation of ATP production (17).

Cytochrome c Release to the Bloodstream

The mechanism by which cytosolic cytochrome c reaches the extracellular space and appears in the bloodstream is not well understood. Several studies have shown that cytochrome c release outside the cell occurs without concomitant release of larger molecules, such as lactate dehydrogenase, which is considered a marker of cell necrosis, with disruption of the cell membrane (1, 6, 7, 37). Regardless of the mechanisms, our study demonstrated a progressive rise in plasma cytochrome c during the postsresuscitation period to levels that were inversely related to survival outcome. Cytochrome c levels increased after return of spontaneous circulation, despite reversal of whole body ischemia. This would suggest that ischemia and reperfusion trigger processes leading to a progressive rise in plasma cytochrome c after return of metabolically adequate blood flow. The specific organs that contributed to circulating cytochrome c were not determined in this study. Organs rich in

mitochondria, with a high metabolic rate and a propensity to ischemia-and-reperfusion injury, such as the heart and brain, are likely candidates. However, without knowing the specific mechanisms of cytochrome c release, especially after return of spontaneous circulation, we cannot exclude other mitochondria-rich organs, such as the liver, kidney, pancreas, and skeletal muscle. The possibility that plasma cytochrome c was contributed by circulating blood cells was considered. Circulating red blood cells contain a considerable amount of caspase-3; however, they lack nuclei, mitochondria, and the machinery for activation of apoptotic pathways (8). Neutrophils, on the other hand, can undergo apoptosis. However, studies have shown that apoptosis in neutrophils is actually delayed under conditions such as acute respiratory distress syndrome (31), sepsis (33), and burn injury (12). Huda et al. (27) demonstrated no signs that apoptosis is activated in neutrophils after tourniquet-induced ischemia in human skeletal muscle 4 h after reperfusion. Acute stress seems to suppress the mitochondrial apoptotic pathway of neutrophils consequent to downregulation of proapoptotic Bcl-2 proteins (26). Similarly, we found in the present studies that the percentage of neutrophils undergoing apoptosis is reduced after resuscitation from cardiac arrest and that increased levels of cytochrome c are not likely to induce apoptosis in leukocytes. Accordingly, the increased plasma levels of cytochrome c in the present studies most likely originated from organs that suffered ischemia and reperfusion injury during cardiac arrest and resuscitation.

Significance

Our studies suggest that levels of circulating cytochrome c could serve as an in vivo marker of mitochondrial injury and organ damage (i.e., left ventricular dysfunction) and prognosticate survival after resuscitation from cardiac arrest. Recently, similar observations were made in patients with a wide variety of critical conditions. For example, Adachi et al. (1) reported a rapid rise in serum cytochrome c in survivors of systemic inflammatory response syndrome and multiorgan dysfunction syndrome, with higher levels in patients who did not survive. Similarly, levels of cytochrome c were significantly higher in nonsurvivors of influenza-associated encephalopathy than in survivors (25). A similar negative predictive value of serum cytochrome c has been reported in patients presenting with fulminant hepatitis (38).
We conclude that cardiac arrest and resuscitation trigger events leading to progressive and prominent increases in circulating cytochrome c. We propose that plasma cytochrome c may represent a novel in vivo marker of mitochondrial injury during resuscitation from cardiac arrest that could serve to quantitate the severity of injury, prognosticate survival outcomes, and act as a surrogate measurement for gauging the effects of therapeutic interventions.

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GRANTS

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