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Increased cytochrome *c* in rat cerebrospinal fluid after cardiac arrest and its effects on hypoxic neuronal survival

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Abstract

Cerebrospinal fluid (CSF) proteins may be useful biomarkers of neuronal death and ultimate prognosis after hypoxic-ischemic brain injury. Cytochrome *c* has been identified in the CSF of children following traumatic brain injury. Cytochrome *c* is required for cellular respiration but it is also a central component of the intrinsic pathway of apoptosis. Thus, in addition to serving as a biomarker, cytochrome *c* release into CSF may have an effect upon survival of adjacent neurons. In this study, we use Western blot and ELISA to show that cytochrome *c* is elevated in CSF obtained from pediatric rats following resuscitation from cardiac arrest. Using biotinylated human cytochrome *c* in culture media we show that cytochrome *c* crosses the cell membrane and is incorporated into mitochondria of neurons exposed to anoxia. Lastly, we show that addition of human cytochrome *c* to primary neuronal culture exposed to anoxia improves survival. To our knowledge, this is the first study to show cytochrome *c* is elevated in CSF following hypoxic ischemic brain injury. Results from primary neuronal culture suggest that extracellular cytochrome *c* is able to cross the cell membrane of injured neurons, incorporate into mitochondria, and promote survival following anoxia.

Keywords

cerebrospinal fluid; brain ischemia; cardiac arrest; cytochrome *c*

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Disclosure/Conflict of Interest

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Introduction

Several protein biomarkers have been identified in cerebrospinal fluid (CSF) of humans and animals with brain injury. A partial list of these biomarkers includes the inflammatory mediators Interleukin-6 [1,2,3], Nerve Growth Factor [1], Tumor Necrosis Factors [4] and sFAS/Apo-1 [5] as well as the cytoskeletal structural proteins tau and α -spectrin [6,7]. Characterization of CSF biomarkers following brain injury is relevant to clinical medicine because biomarkers may 1) predict neurologic outcome, 2) allow stratification of injury for clinical studies, and 3) identify mechanisms of injury or repair that are important therapeutic targets.

Cytochrome *c* concentrations are increased in the CSF of infants with inflicted brain injury from child abuse [8]. Cytochrome *c* is of particular interest because it can participate in either life-sustaining cellular respiration or the intrinsic pathway of apoptotic cell death [9]. Under normal conditions cytochrome *c* is loosely bound to the inner mitochondrial membrane and functions as an essential component of the electron transport chain. Pathologic conditions can cause cytochrome *c* to translocate out of the mitochondria into the cytosol where it activates the apoptosome to trigger apoptotic cell death (the so-called intrinsic pathway of apoptosis) [10,11]. Translocation of cytochrome *c* from mitochondria into cytosol has been reported in the “selectively vulnerable” region of hippocampus in rodents following global ischemic brain injury [12,13]. Furthermore, hypothermia, the only clinically proven effective neuroprotective therapy, inhibits late cytochrome *c* translocation in rodents following global ischemia [14]. Serum levels of cytochrome *c* are increased in rodents following cardiac arrest and are associated with decreased survival [15].

Ahlmeier et al have reported that addition of cytochrome *c* to cell culture media supporting mixed neuronal culture caused cell death that could be blocked by an anti-cytochrome *c* antibody [16]. In addition, exogenous cytochrome *c* accelerated cell death in staurosporine-treated culture. Their data suggest that extracellular cytochrome *c* can cross the cell membrane into the cytoplasm and trigger apoptosis. In contrast, Piel et al showed that cytochrome *c* injected intravenously into mice with sepsis-induced myocardial dysfunction was taken up by cardiomyocytes, localized within the mitochondria, improved cellular respiration, improved myocardial contractility and improved survival [17,18]. These latter two studies suggest a potential benefit of extracellular cytochrome *c*.

CSF concentrations of cytochrome *c* following *in vivo* ischemic injury have not been described. Accordingly, we sought to determine if cytochrome *c* is elevated in CSF from rats following global ischemic brain injury caused by asphyxial cardiac arrest. We also sought to determine the effects of exogenous cytochrome *c* upon primary neuronal culture exposed to anoxia.

Materials and Methods

Animal studies were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Reagents and antibodies

Cytochrome *c* ELISA kit and human cytochrome *c*, isolated from heart tissue, were obtained from R&D systems (Minneapolis, MN). Mouse monoclonal anti-cytochrome *c* antibody was from Abcam (Cambridge, MA). CytoTox-Fluor Cytotoxicity Assay kit and CellTiter-Blue Cell Viability Assay kit were from Promega (Madison, WI). EZ-link Micro NHS-PEO4-Biotinylation kit and Mitochondrial Isolation kit for cultured cells, as well as StartingBlock blocking buffer, Horseradish Peroxidase (HRP)-Conjugated Streptavidin, M-PER

mammalian protein extraction reagents, Supersignal West Pico and Dura Chemiluminescent substrates were purchased from Pierce (Rockford, IL). Anti- β -actin antibody, equine heart cytochrome *c* and all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Asphyxial cardiac arrest (ACA)

Rats underwent ACA as described by Fink et al [19]. In brief, postnatal day 16–18 male Sprague-Dawley rats were randomized into three groups, ACA, sham surgery, or naive. Rats were anesthetized with 4% isoflurane/60% N₂O/balance oxygen in a Plexiglas chamber until unconscious then underwent tracheal intubation and were ventilated with 1.5–2% isoflurane/60% N₂O/balance oxygen for aseptic femoral arterial and venous cannulation, with a total anesthetic time of approximately 40 min. Rats were placed on a heating pad and a rectal thermometer was inserted with core temperature maintained at 37.5 $\pm$ 0.5 $^{\circ}$ C. Ten minutes before asphyxia, 1 mg/kg of vecuronium bromide was administered to the rats while they were still receiving anesthesia. Two minutes before asphyxia, isoflurane was discontinued and rats were exposed to 100% oxygen for 1 min to avoid the confounding cerebroprotective effects of inhaled anesthetics. All rats then received 21% oxygen for the final minute before asphyxia to avoid hyperoxia preinsult. Rats randomized to ACA were disconnected from the ventilator for 8 min. Cardiac arrest, defined as pulseless electrical activity with a mean arterial pressure of 0 mm Hg occurs within 90 seconds. At the end of 8 minutes asphyxia, cardiopulmonary resuscitation was initiated with chest compressions and by reconnecting the rats to the ventilator (100% oxygen), in addition to IV boluses of 0.005 mg/kg of epinephrine and 1 mEq/kg of sodium bicarbonate. Rats were extubated at 1 hour following return of spontaneous circulation then placed in a recovery chamber with 100% oxygen for 30 min. Sham rats underwent the same anesthesia and surgical procedures without ACA, and were extubated after anesthetic washout. Immediately prior sacrifice rats were anesthetized with 4% isoflurane/66% N₂O/balance oxygen and cerebral spinal fluid (CSF) was obtained by puncture between the occipital protuberance and spinal atlas into the cisterna magna with a 23 gauge needle. CSF was spun briefly to pellet red blood cells and the supernatant was frozen on dry ice. Rats were then sacrificed via decapitation and brain tissue was dissected and rapidly frozen until analysis.

Cytochrome *c* measurement

Cytochrome *c* was measured in rat CSF using both ELISA and Western blot. For ELISA, cytochrome *c* concentration in the rat CSF obtained during sacrifice at 1h, 24h, 48h and 7 days post ACA was measured using a Quantikine Rat/Mouse Cytochrome *c* Immunoassay solid phase ELISA kit following manufacturer's directions. Cytochrome *c* in the CSF was also detected by Western blot as described previously [20]. In brief, 8 μ l of each CSF sample was loaded onto 15% polyacrylamide gel before transferred to PVDF membrane. After blocking with 5% non-fat milk in 0.1%TBS-Tween 20, the blot was incubated with an anti-cytochrome *c* antibody (1:300) at 4 $^{\circ}$ C overnight and HRP-conjugated goat anti-mouse secondary antibody (1:5000) sequentially. Cytochrome *c* signal was visualized with Pierce Supersignal West Dura Chemiluminescent Substrate.

Cortical Primary neuronal culture and cytochrome *c* treatment

Cortical primary neuronal cultures were prepared from E17 fetal rats (Sprague-Dawley, Charles River and Wilmington, MA) as previously described [21] and used for experiments after 9 days. Cells were grown in serum-free Neurobasal medium (Invitrogen, Carlsbad, CA) supplemented with B27 and GlutaMAX (Invitrogen). Purified cytochrome *c* protein was dissolved in 1X PBS then diluted in culture medium to a final concentration of 15ng/ml or 150ng/ml. Culture medium was changed to medium containing cytochrome *c* immediately after hypoxia. Cell viability and cell death assays were performed 24h later. For the untreated group (UN), regular culture medium was also changed immediately after hypoxia.

***In vitro* hypoxia and cell death measurements**

Hypoxia was performed using a hypoxic glove box (Coy Laboratories, Grass Lake, MI) flushed with 92% argon, 5% CO₂ and 3% H₂ for 2-3 hours as described previously [22] resulting in ~40% cell death after 24h reperfusion. Staurosporin (20 μM, a 100% cell death internal standard) and 1 μM MK801 (a 100% cell survival internal standard) treatments were included in each cell death assay experiment. Cell death and cell viability were quantitatively assessed by CytoTox-Fluor Cytotoxicity assay (Promega, Madison, WI) and CellTiter-Blue Cell Viability Assay (Promega) respectively following manufacturer's instructions. Cell death was also measured by staining cells with 0.1 μg/ml propidium iodide (PI, a red fluorescent dye that permeates dead cells) and 0.1 μg/ml Hoechst (a blue fluorescent dye that stains chromatin in live and dead cells) and fixed with 2% formaldehyde in 1X phosphate buffered saline (PBS) 24 h after hypoxia. PI and Hoechst stained cells were photographed with a Leica DMIRB fluorescence microscope at 20X then counted by a blinded observer from 10 to 12 fields of at least three cultures. Data are expressed as percentage PI-stained cells.

Detection of biotinylated cytochrome *c* in post-hypoxia primary neuronal mitochondrial fractions

Cytochrome *c* was biotin labeled using EZ-Link Micro NHS-PEO4-Biotinylation Kit from Pierce following manufacturer's directions. Excess free biotin molecules were removed using a Zeba desalt spin column (Pierce). Biotin-labeled cytochrome *c* was dissolved in 1X PBS. Rat primary neurons were incubated with culture medium (control) or culture medium containing 15ng/ml or 150ng/ml biotinylated cytochrome *c* (Bt-Cyt C) just before hypoxia. Cell medium was not changed after hypoxia. 24h post-hypoxia, cells were rinsed 3 times with 1X PBS before harvesting. Cell mitochondrial and cytosolic fractions were isolated using a mitochondria isolation kit from Pierce following manufacturer's protocol. Purified mitochondria were then lysed with 2% CHAPS in Tris buffered saline and protein concentrations were measured by BCA protein assay. To detect Bt-Cyt C in each fraction, 18μg of cytosolic proteins and 45μg of mitochondrial proteins in each treatment group were separated by 15% SDS-PAGE. After transfer to PVDF membrane and blocking with Startingblock blocking buffers, biotinylated cytochrome *c* was visualized using HRP-conjugated streptavidin (1:1500) and SuperSignal West Dura chemiluminescence substrate (Pierce).

Statistical analysis

ANOVA was performed using SPSS software version 18.0 (Somers, NY). Levine's test of equality of error variance was performed. If variance was equal the Tukey post-hoc test was applied; if variance was unequal, Dunnett's T3 post-hoc test was applied. Results were considered to be significant when $p < 0.05$.

Results

Cytochrome *c* increases in CSF samples from rats after cardiac arrest

To detect cytochrome *c* concentration changes in pediatric rat CSF after cardiac arrest, rats were randomly divided into three groups: 1) Naïve group without any surgical procedures (n=5); 2) sham (n=5) and 3) PACA group (n=15) as described in the Materials and Methods. PACA CSF samples were collected during sacrifice at 24h, 48h and 7days after surgery and the concentration of cytochrome *c* was quantified by ELISA. Sham specimens were collected during sacrifice 24h after sham surgery. Because the cytochrome *c* levels from sham and naïve groups are very similar, these data were combined as control, increasing statistical power. As shown in Fig 1A, the mean value of cytochrome *c* concentration in CSF

of control group is 2.1ng/ml; 24h after PACA this concentration increases significantly to 14.1ng/ml ($p < 0.01$, vs. control). Cytochrome *c* concentration drops to 4.3 ng/ml at 48h after cardiac arrest and then to 2.0 ng/ml at 7days after ACA, however the sample size at these time points is small.

Cytochrome *c* content in CSF samples was further detected by Western blot using an anti-cytochrome *c* antibody. As shown in Fig.1C, a band at 13kD, representing endogenous cytochrome *c*, was detected in most of the CSF samples from the PACA group. Films were scanned and the density of the cytochrome *c* band was measured in each sample. Means were then normalized to sham. Although sample variability was too great to result in statistical significance between groups, there was a trend towards increased cytochrome *c* increase after ACA, peaking at 24h (Fig. 1B).

Taken together, both ELISA and Western blot results indicate increased cytochrome *c* release into the CSF after cardiac arrest, suggesting a possible role for cytochrome *c* in secondary neuronal injury after ischemia.

Cytochrome *c* is protective for the post-hypoxia primary neuronal culture

To test the effects of increased exogenous cytochrome *c* on neuronal cell viability, primary neurons were subjected to hypoxia or normoxia treatment followed by 24h incubation with cytochrome *c* at concentrations equivalent or greater to those found in the above ELISA experiments (15ng/ml and 150n/ml). Three complementary cell viability or toxicity assays (CellTiter-Blue Cell Viability Assay, CytoTox-Fluor Cytotoxicity Assay and PI/Hoechst staining) were applied to quantify the cytotoxic or protective effects of cytochrome *c* on neuronal cell survival. The CellTiter-Blue Cell Viability Assay is based on the ability of living cells to convert a redox dye (resazurin) into a fluorescent end product, while nonviable cells rapidly lose metabolic capacity and thus do not generate a fluorescent signal. It has been shown that this cell viability assay has excellent correlation to other ATP- and LDH- based cell viability or cytotoxicity methods. Meanwhile, The CytoTox-Fluor Cytotoxicity Assay used in this report was to measure the relative number of dead cells in cell populations. This assay measures a distinct protease activity associated with cytotoxicity by using a fluorogenic peptide substrate (bis-AAF-R110) to measure dead-cell protease activity, which is released from cells that have lost membrane integrity. The bis-AAF-R110 Substrate cannot cross the intact membrane of live cells and therefore gives no signal from live cells.

As shown in the Fig. 2, 24h incubation of 15ng/ml or 150ng/ml cytochrome *c* had no significant effect on cell death in the normoxia group as measured by the cytotoxicity assay. Using the cell viability assay, cytochrome *c* treatment at either concentration significantly increases neuronal metabolic capacity, suggesting an increase in cell viability as compared to the untreated group under normoxic conditions ($p < 0.01$). In hypoxic neurons, cytochrome *c* incubation has significant protective effects on cell survival as measured with both cell viability and cell death assays (Fig.2A lower panel). As shown in the cell viability assay, untreated primary neurons had significant decreased cell viability 24h after hypoxia compared with the 1 μ M MK801-treated group; 24h incubation with cytochrome *c* resulted in a dose-dependent and significant increase in cell viability. Consistent with the viability assay, the cytotoxicity assay also indicates that cytochrome *c* treatment significantly attenuates cell death in post-hypoxia neurons. To further confirm the above findings, PI and Hoechst staining and cell counting was performed on the post-hypoxia neurons treated with or without cytochrome *c*. There was a significant and dose-dependent decrease in the percentage of PI stained cells in cytochrome *c* treated cells as compared to untreated controls (Fig 2B), confirming the protective effects of cytochrome *c* on post-hypoxia neuronal survival.

Exogenous cytochrome *c* is taken up by mitochondria in post-hypoxia primary neurons

To detect whether exogenous cytochrome *c* in the culture medium is taken up by post-hypoxia neuronal cells, recombinant cytochrome *c* was labeled with biotin and dissolved in PBS. After purification to eliminate excess biotin molecules, biotinylated cytochrome *c* was then added into the culture media immediately after hypoxia. 24h later, primary neurons were carefully washed and harvested, and cytosolic and mitochondrial fractions were isolated. Biotinylated cytochrome *c* in each fraction was detected with immuno-blotting using HRP conjugated streptavidin. As shown in Fig.3, a 16kD band, representing biotinylated cytochrome *c*, was detected in the mitochondrial fraction only in biotinylated cytochrome *c*-treated post-hypoxia neurons, but not in the cytosolic fraction or in either fraction from control neurons without biotinylated cytochrome *c* treatment.

Discussion

This is the first study to report cytochrome *c* concentrations in CSF following hypoxic-ischemic brain injury in a pediatric rat model. We chose to focus on cytochrome *c* because elevated cytochrome *c* levels in CSF of children with traumatic brain injury (up to 63 ng/ml) have been reported [8]. Others have shown increased cytochrome *c* in CSF of patients with Alzheimer's disease [23], and influenza-associated encephalopathy [24]. Taken together, these studies suggest that cytochrome *c* is a biomarker of brain injury, presumably released into CSF during cell death or injury by leakage across the cell membrane or during cell lysis in apoptosis or necrosis [25].

Whether increased cytochrome *c* in CSF bathing injured neurons can exacerbate or attenuate cell death is an important question. Ahlmeyer et al found that addition of cytochrome *c* to culture media (25ng/ml) was toxic to both untreated and staurosporine-treated mixed-neuronal cultures derived from chicks or rats [16]. In contrast, we found 15 ng/ml (similar to the concentration detected in PACA CSF) and 150 ng/ml of cytochrome *c* to be protective in primary neuronal culture. This discordance may be caused by differences between our models. Staurosporine, a protein kinase inhibitor, causes apoptosis whereas anoxia causes both necrosis and apoptosis. It is possible that excess cytochrome *c* is more likely to form apoptosomes and trigger caspase-mediated apoptosis in an injury dominated by apoptosis compared with an injury dominated by necrosis. Another difference is that Ahlmeyer et al used mixed neuronal and astrocyte cultures whereas we used enriched primary neurons. Our findings are more consistent with Piel et al who showed that IV doses of cytochrome *c* can cross the cardiac cell membrane, migrate into mitochondria, and improve cardiac function in a model of sepsis-induced organ dysfunction [17,18]. Our results and the results of Piel et al suggest that cytochrome *c*, when taken up by injured cells, improves metabolic capacity and allows the cells to better withstand an injury [17,18]. Additional studies are necessary to confirm this hypothesis.

A limitation of this study is that cytochrome *c* levels are not correlated with histologic or neurologic outcomes. Similarly, a dose-response of ischemia time correlated with cytochrome *c* levels would be of interest.

In summary, this study demonstrates that cytochrome *c* is a potential biomarker of hypoxic-ischemic brain injury and extracellular cytochrome *c* may have a protective role.

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Figure 1.

Cytochrome *c* is increased in pediatric rat cerebral spinal fluid (CSF) after asphyxial cardiac arrest. Male PND 17-18D rats underwent asphyxial cardiac arrest (ACA) and CSF was collected 1h – 7D post ACA immediately prior to sacrifice. (A) Cytochrome *c* measured by ELISA. Control CSF is from sham and naïve rat. ** $P < 0.01$ vs 24h. $n = 2 - 11$ per group. (B) Cytochrome *c* measured by western blot and normalized to sham. $n = 2-4$ per group (3 at 1h, 3 at 24h, 2 at 72h, 2 at 7d, and 4 sham). (C) Representative western blot of cytochrome *c* in CSF at 24h after sham or ACA.

Figure 2.

Cytochrome *c* attenuates neuronal cell death after hypoxia. Rat primary neuronal cultures underwent normoxia or hypoxia and were treated with cytochrome *c* (Cyt C, 15 or 150 ng/ml) or untreated (UN) for 24h post hypoxia. **A.** Left panel: Cell viability; Right panel: cell death. MK801 (1 μ M) and staurosporine (20 μ M) were included as 100% live cell or 100% dead cell internal standards, respectively. $n = 12-65$ wells per group. **B.** Cell death 24h after hypoxia as measured by percent PI staining (left). Representative photos of PI and Hoechst-stained cells (right). $n = 10$ wells per group. RFU: relative fluorescence units; PI: propidium iodide; MK: MK801, SP: staurosporine. ** $P < 0.01$. Data are means $\pm$ SE.

Figure 3.

Cytochrome *c* enters neurons after anoxia. Rat primary neuronal culture underwent anoxia and was treated with medium (as control) or 15 ng/ml biotinylated cytochrome C (b-Cyt C) immediately after anoxia for 24h. Cells were harvested and the cytosolic (C, 18 μ g) and mitochondrial (M, 36 μ g) fractions were loaded for 15% SDS-PAGE. The biotin-conjugated cytochrome *c* signal was detected after incubation with HRP-conjugated streptavidin and enhanced chemiluminescence substrate.