Intrinsic Apoptotic Pathway

Effects of Calcium on Murine Cytochrome C Release in Brain and Liver Mitochondria

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Acceptance of Senior Honors Thesis

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Abstract

A cell may use one of three main apoptotic pathways leading to programmed cell death: the extrinsic pathway, the perforin/granzyme pathway and the intrinsic pathway. The most pertinent to this discussion is the intrinsic pathway, which utilizes the mitochondria as an essential intermediary. Mitochondria's primary function in relation to this pathway is the subsequent release of pro-apoptotic factors including cytochrome c, which activate a caspase cascade leading to the death of the cell. Cytochrome c is released partly due to an increase in cytosolic calcium levels. Two methods of the release of cytochrome c have been proposed. The first is through permeability transition pores (PTP) in the inner mitochondrial membrane, which result in the rupturing of the mitochondria and the subsequent release of inner membrane space proteins. The second method is through mitochondrial apoptosis-induced channels (MAC) in the outer membrane, which maintains the integrity of the mitochondria. Murine brain and liver mitochondria were incubated with various concentrations of calcium and a subsequent western blot for cytochrome c was performed. It appears that an increase in calcium concentration induces a higher level of cytochrome c release in liver mitochondria providing stronger evidence for the MAC pathway. Brain mitochondria did not express a calcium dosage response providing evidence for the PTP pathway.

Introduction

"It is normal to give away a little of one's life

In order to not lose it all"
—Albert Camus (1913-1960)

Self-preservation is a notion that has been ingrained into the genetic makeup of all living things. From single-celled bacterial cells forced to adapt to new environments and stresses to carnivorous animals fighting over a final scrap of food, the fight for survival is an extending natural tendency. However, along with that comes the notion for self-preservation; the sacrifice of part of one's self is inevitable.

The human body, as well as all other multi-cellular organisms, undergoes this process every day. There are over 100 trillion cells comprising the human body. It has been estimated that due to the high rate of cellular apoptosis that the human body will replace every cell within a seven year time span. This is a vitally important process to defend again viruses and antigens, eliminate DNA damage and maintain effective cellular signaling. For this to occur efficiently the body requires an extensive process to regulate the health of each cell and effectively cause cells to kill themselves when replacements are necessary; this process is known as apoptosis.

The emphasis of this research paper will focus on an independent study taking an in-depth investigation into one of the steps involved in the apoptotic pathway. However, before this research can be discussed, a thorough knowledge of the steps involved in apoptosis must be established.

Background

Extrinsic Pathway

The extrinsic apoptotic pathway accomplishes the same end goal as the intrinsic path, which is activation of a serine enzyme known as caspase-3 and eventual programmed cell death. However, the way in which activation of caspase-3 is achieved varies greatly between the two pathways.

The extrinsic apoptotic pathway begins with proteins located in the interstitial fluid surrounding the cell. Although the extrinsic pathway has a similar form between species and cell lines, there are a vast number of models used between these organisms. The most common human extrinsic models are characterized by the tumor necrosis factor (TNF) receptor gene superfamily (*1*). Members of the TNF receptor family all contain large cysteine-rich extracellular residues, which bind apoptotic and anti-apoptotic ligands. The intracellular surface of TNF receptors are comprised of an approximately 80 amino acid domain appropriately tagged as the 'death domain' (*2*). There are several main models of apoptotic ligands and their respective receptors in mammals, including ApoDL/DR4, ApoDL/DR5, Apo3/DR3, TNF-α/TNFR1, and FasL/FasR (*3*). These 'death domains' play a pivotal role in the signal transduction from the extracellular environment to the intracellular environment. For the purposes of explanation, the FASL/FASR model will be used.

The pathway begins with the trimeric ligand binding a clustering of Fas receptors on the extracellular membrane. This binding causes a conformation shift in FASR1, which activates and recruits the intracellular adapter protein FADD (*4*). The recruitment of FADD results in the dimerization of two 'death domains' of adjacent FASR1

molecules which further recruit procaspase-8 (*5*). As a result, a death-inducing signal complex (DISC) is formed which causes the autocatalytic activation of procaspase-8 to caspase-8 (*3, 6*). The activated caspase-8 then cleaves the zymogen procaspase-3 to caspase-3 which is the commonality point between the extrinsic and intrinsic apoptotic pathways (*7*). Caspase-8 is also able to cleave Bid to tBid, which is able to bind to receptors in the mitochondrial membrane and activate the release of cytochrome c and other proapoptic factors, similar to the intrinsic apoptotic pathway (*8, 9*). It is with the release of these factors that the extrinsic pathway joins with the intrinsic apoptotic pathway.

Perforin/granzyme Pathway

A second major pathway leading to programmed cell death apart from the extrinsic and intrinsic apoptotic pathway is the perforin/granzyme pathway. It is one largely involved in the immune response of an organism and thus occurs only in multi-cellular organisms. In humans this pathway is best characterized through the function of cytotoxic T lymphocytes (CTLs) (*3, 10*). *In vivo,* these CTLs recognize and destroy antigen-presenting cells primarily through the extrinsic apoptotic pathway. However, if required they are able to kill antigen-bearing cells in the absence of the extrinsic pathway.

The granzyme/perforin pathway involves CTLs recognizing antigen-bearing cells and secreting the molecule perforin. Perforin has the ability to bind the extracellular membrane of the target cell and initiate a pore-forming complex resulting in the release of cytoplasmic granules into the target cell (*11*). Of these granules released, the two that are most important in this pathway are granzyme A and granzyme B (*12*). Granzyme A aids in the caspase-independent apoptotic pathway through inducing DNA damage, and

destruction of the nuclear envelope as well as the rapid loss of cellular membrane integrity (*13*). Granzyme A also inhibits the function of the protein complex Ape1 (*13*), which is vitally responsible for the cellular response to DNA damage (*14*). Granzyme B is a serine protease that can cleave several downstream proteins in the apoptotic pathway. It can cleave procaspase-10, as well as the protein complex ICAD resulting in the activation of several DNases (*15*). This activity combined with the inhibition of Ape1 from granzyme A, leads to eventual cell death.

Specifically important to the area of research contained in this paper is that the release of granzyme B can also have a direct effect on the intrinsic (mitochondrial-dependent) apoptotic pathway. It has been suggested in recent studies that granzyme B can perform a specific aspartate cleavage of the Bid protein complex. This cleavage appears to allow an increase in the rate of cytochrome c passage through the outer mitochondrial membrane thus inducing the latter half of the intrinsic apoptotic pathway(*16*). This relationship and precise process of cytochrome c release will be described in greater detail later.

Intrinsic Pathway

The intrinsic apoptotic pathway consists of a diverse series of intracellular mediators resulting in the activation of the caspase pathway, eventually leading to the execution phase of apoptosis and cell death (*17*). The mitochondria play a very significant role in the execution of this pathway leading to it being commonly referred to as simply the mitochondrial apoptotic pathway (*18*). The specific path a cell uses to mediate apoptosis is dependent upon the organism in which it is contained and the specific tissue type, as well as the environmental stresses leading to the need of cellular

death. In mammals, the most common method is through the mitochondrial pathway, an overview of which is demonstrated below:

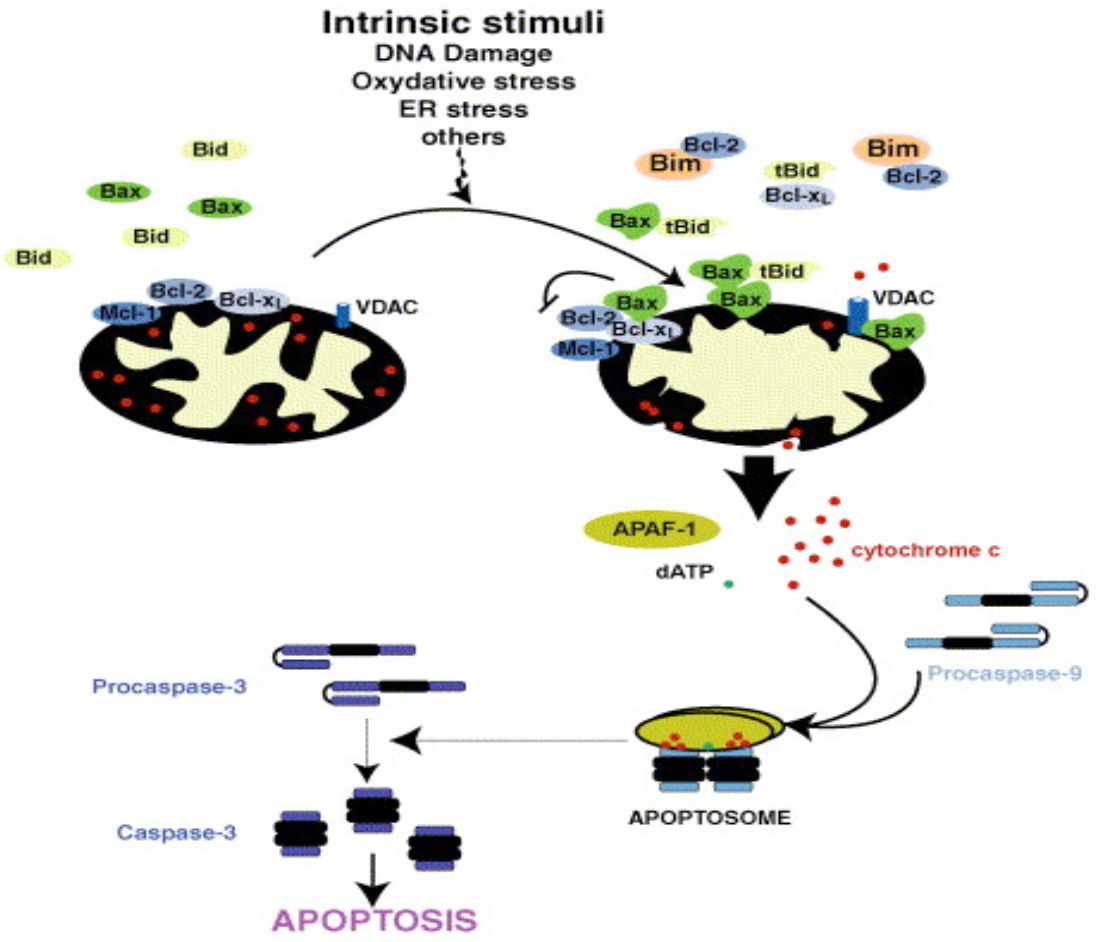


Figure 1. A depiction of the intrinsic apoptotic pathway occurrence in humans. The intrinsic apoptotic pathway is stimulated by a variety of stresses. These stimuli lead to the localization and oligomerization of proteins Bax and Bak at the mitochondria. These proteins result in the release of pro-apoptotic factors from the inter-membrane space, which activate a caspase pathway resulting in mediated cellular death(*19*).

Initiation. DNA damage, serum starvation, chemotherapeutic agents, and UV radiation primarily initiate the intrinsic apoptotic pathway (*20-22*). These events can initiate a variety of intracellular signals, which can act to either induce or repress the

intrinsic apoptotic pathway. These intracellular signals all act on several families of proteins that are localized in the outer mitochondrial membrane. The largest family of these proteins are Blc-2 homologs (*23, 24*), which are further divided into pro-apoptotic proteins and anti-apoptotic proteins.

Apoptosis can be triggered via the intrinsic pathway through either the repression of anti-apoptotic proteins, or the induction and activation of pro-apoptotic proteins. Pro-apoptotic proteins are those that are localized in the mitochondrial membrane and act as repressors on anti-apoptotic proteins. Anti-apoptotic proteins include but are not limited to Bcl-XL, A1 and Mcl-1 (*23*). These anti-apoptotic proteins are responsible for preventing the apoptotic pathway from killing the cell unnecessarily. They act as guardians of the cell; however, with the correct stimuli, they lose their inhibitory effect and voluntary cell death is triggered. The process through which this occurs is the activation of pro-apoptotic factors including signs of DNA damage, free radicals, toxins, and radiation.

Primary Stage. One mechanism for the activation of the primary stage of the intrinsic apoptotic pathway involves the proteins Bax, and Bak (*25*). In a typical functioning cell, Bax exists as a monomer in the cytoplasm. However, in the event of DNA damage, toxins or other apoptotic indicators, Bax is directed to the mitochondria (*26, 27*). Upon arrival, Bax monomers link together to form an oligomer of 5 to 7 residues in length (*28, 29*). Bak does not need to translocate to the mitochondria because that is its usual place of residence. Upon activation due to apoptotic signaling, Bak also oligomerizes through several conformational changes (*30*). It is the oligomerization of these two proteins that induces the increased permeability of the mitochondrial

membrane (*31, 32*).

Another mechanism resulting in the activation of this stage of apoptosis, of which the research in the later portion of this paper is concerned, involves the concentration of several cytosolic divalent cations with the most pertinent being calcium. It has been suggested that calcium acts as an activator in the increase of the mitochondrial membrane permeability. Regardless of the cause of increased permeability of the mitochondria, it ultimately results in the release of several inter-membrane space (IMS) proteins, which are essential for the trigger of the next stage of apoptosis.

Release of Inner Mitochondrial Space Proteins. The specific process of how these IMS proteins are released is still unknown, however there are two main theories: permeability transition pores, and mitochondrial apoptosis-induced channels

Permeability Transition Pore. The first theory is that the increased membrane permeability is due to the opening of a permeability transition pore (PTP) located in the inner mitochondrial membrane (*33, 34*). It has been proposed that the opening of this pore to allow solute molecules results in the swelling of the mitochondrial matrix and the eventual rupturing of the outer mitochondrial membrane (*33, 35*). Upon this rupturing, IMS proteins are released into the cytosol and trigger the execution phase of apoptosis. Studies have shown that there may be several receptors on the pore which regulate its opening and closing (*36*). They have demonstrated that there are several cytosolic molecules that actively compete for receptor sites on the permeability transition pore. These molecules are both pro-apoptotic and anti-apoptotic in nature; the relative concentration of each of these molecules is dependent upon environmental factors such as stress, DNA damage or immune action. The most pertinent of these molecules is the

divalent cation calcium. The endoplasmic reticulum has high stores of calcium, which upon apoptotic signaling can be released at high levels. This high level of calcium may cause the formation of the PTP, which can either be prolonged or transient. It has been proposed that the Km for this calcium activation is 10 μM (*37*). One main goal of this research is to investigate this value and determine its validity.

Mitochondrial Apoptosis-Induced Channels. The second proposed mechanism for the release of IMS proteins into the cytosol during apoptotic signaling involves channels in the outer mitochondrial membrane known as mitochondrial apoptosis-induced channels (MAC)(*38*). MAC can be defined as a voltage independent and high conductance channel in the mitochondrial membrane (*39*). The PTP theory holds that IMS proteins are released through the subsequent rupture of the outer mitochondrial membrane and the loss of mitochondrial integrity. The MAC theory differs in that channels are opened in the outer mitochondrial membrane, releasing IMS proteins while keeping the integrity of the outer mitochondrial membrane (*40*).

The evidence for the MACs acting as the cause of IMS protein release has been mounting over the past years. Studies have suggested that the release of IMS proteins, specifically cytochrome c, has directly corresponded to increased activity of MACs during apoptotic signaling (*41*). As stated in a previous section, the primary stage of the intrinsic apoptotic pathway begins with translocation of Bax to the mitochondria and its subsequent oligomerization (*26, 27*). Bax channels in the outer mitochondrial membrane have been known to exist for several years and have been proven to be involved with the release of cytochrome c. However, new studies suggest that these Bax channels may be the same as MACs (*42*).

Researchers investigating these MACs discovered several pieces of key evidence that led to the hypothesis of these channels being directly involved in the release of cytochrome c as well as other pro-paptotic factors. The first piece of evidence is that cytochrome c itself modifies the behavior of these channels leading to the conclusion that cytochrome c is released through them (*40*). Secondly, cytochrome c has an approximate size of 3 nm, which would easily fit through the 5 nm diameter of MACs (*42*). Bax channels also respond to the same stimuli as MACs, such as cytochrome c, as well as have similar behaviors. One of these stimuli is an increase in cytosolic calcium levels. Finally, cells were incubated with Bax antibodies preventing their apoptotic oligomerization. These cells had complete immune-depletion of MAC activity when put upon apoptotic initiation (*42*). All of these results point to the fact that Bax channels and MACs are one and the same.

Cytochrome C. As stated previously, several IMS proteins are released through either PTPs or MACs and are considered pro-apoptotic. These proteins include Smac/DIABLO, the serine protease HtrA2/Omi, AIF, and cytochrome c (*43*). These proteins are released from the mitochondria and activate a caspase pathway leading to the execution phase of apoptosis. The release of cytochrome c, which the research presented here is based upon, leads to the activation of Apaf-1 and the formation of an apoptosome. The apoptosome is able to bind and activate procaspase-9, which is the initiator caspase that begins the execution phase of apoptosis. Cytochrome c binds the closed inactive form of Apaf-1, forcing it into its open active conformation. This frees the N-terminus of Apaf-1, which was normally bound and inhibited by WD-40, allowing Apaf-1 to bind and activate procaspase-9 (*44, 45*). This activation of pro-caspase-9 is the committal step

of the mitochondrial activated caspase pathway.

Execution Phase. The execution phase of apoptosis is the final stage of voluntary cell death. It is here that both the intrinsic and extrinsic pathways converge through the activation of procaspases. All procaspases contain two essential domains: an N terminal pro-domain as well as a protease domain. Following proteolytic processing by a subsequent caspase, the protease domains combine to form a heterodimer and a eventually a tetramer, which is the active form known as a caspase (*46*). The execution phase is initiated when caspase-9 binds to and activates procaspase-3 (*44*). Caspase-9 is able to bind to and activate procaspase-6 and procaspase-7 as well (*47*). Caspase-3, which is considered to be the most important execution caspase, is responsible for activation of the endonuclease caspase-activated DNase (CAD). CAD is responsible for the degradation of DNA, and chromatin degradation (*15*). Caspase-3 induces the formation of apoptotic bodies through cytoskeletal reorganization as well as the activation of other apoptotic factors (*15*).

Purpose

As shown through the extensive background information, apoptosis is an essential aspect of normal cellular biology. There are a variety of factors and reasons that lead to cellular apoptosis and the cell has several pathways through which it can mediate this process. The main pathway through which mammalian cells undergo apoptosis is the intrinsic pathway known as the mitochondrial-dependent pathway. One of the key steps in this pathway is the release of cytochrome c from the mitochondrial inter-membrane space. There are several molecules that can lead to the release of cytochrome c, including calcium. This study looks at the effect of various concentrations of calcium on the release

of cytochrome c.

As stated previously, the actual mechanism through which cytochrome c is released from the mitochondria is still being debated. Some believe that cytochrome c is released as a result of PTPs in the inner mitochondrial membrane leading to the swelling of the matrix and subsequent rupture of the outer mitochondrial membrane and release of cytochrome c. The second theory is that calcium activates the opening of MACs in the outer mitochondrial membrane, releasing cytochrome c while maintaining the integrity of the mitochondrial membrane. This study will attempt to provide evidence for one or both of these mechanisms.

Results

Similar Protein Quantities Obtained from Liver and Brain Mitochondria

The initial starting mass of both the brain and liver was equal to allow accurate comparison between the two samples. The initial step in this process was the homogenization of the tissue and accompanying buffer. By nature, this procedure is very rough on the cellular structure and the organelles contained within. The entire homogenizing process was done on ice, using cold equipment, and performed as rapidly as possible. This was done to minimize the adverse effects on the cell structure, specifically pertaining to the viability of intact mitochondria. The effectiveness of this procedure was determined through collecting a sample of the initial homogenate (IH) and performing a cytochrome c oxidase assay.

The 11K samples were indicative of the total amount of mitochondria lost through the centrifugation process. Following the 600 x g spin, the pellet was removed as it contained large un-homogenized tissue chunks and heavier organelles, specifically

nuclei. The supernatant was spun at a much higher speed (11 000 x g) causing whole mitochondria to pellet, leaving buffer, lighter organelles, and broken mitochondria in the supernatant, which was kept as the 11K sample. A protein assay was performed utilizing a Bradford BSA standard curve.

Following the protein assay the specific protein concentrations of the various samples were determined. The total protein concentrations of the brain and liver IH were very similar at 25.16 mg/mL and 24.55 mg/mL respectively (Table 1). This corresponds with what was expected, as the exact same mass of starting tissue was used in each case. This demonstrates the effectiveness of the preparation procedure. The protein concentrations of the IH, 11K and final pellet (FP) samples were also obtained and are contained in Table 1.

Mitochondria More Viable from Liver Cells than Brain Cells

Cytochrome c oxidase is an enzyme embedded in the inner mitochondrial membrane. It catalyzes the oxidation of cytochrome c, producing a molecule that absorbs light in the visible range. Thus, this reaction can be visualized by measuring the absorbance change at 500 nm. This allowed for the integrity of the outer mitochondrial membrane to be calculated. The substrate ferrocytochrome c was incubated with the samples and if non-intact mitochondria were present, the enzyme would react with its substrate and be visualized through changing absorbance readings.

The final pellet samples were subjected to an enzyme assay without using a detergent to determine the percent of intact mitochondria. In a parallel assay, n-Dodecyl β-D-Maltoside was used to rupture the mitochondrial membrane and thus both broken and intact mitochondria contributed to the activity. By comparing the activity found with

and without detergent the amount of intact mitochondria was determined.

The total activity of the IH from the brain sample was 0.2491 units (Table 1). This signifies that there were a high number of mitochondria contained in the 300 mg tissue sample. The IH of the liver sample had nearly a two-fold increase in the total activity to 0.4893 units. This shows that there was a much higher amount of mitochondria in liver tissue compared to the brain tissue. Various calculations were performed describing the activity of the samples in various degrees. These results are contained in Table 1. The fold purification was calculated for each sample by comparing its specific activity to the specific activity of the IH sample from the identical tissue. The fold purifications obtained from the other samples are contained in Table 1.

The percent yield was determined through comparing the total activity of each sample to the total activity of the IH from the same tissue. The percent yields obtained in the IH, 11K and Final Pellet samples are contained in Table 1.

The percent of intact mitochondria from the brain sample was measured to be 67.44 percent, and the liver sample had 62.78 percent intact. This shows that the integrity of the liver mitochondria was more easily preserved in comparison to the brain mitochondria.

Liver

Sample	Protein Conc. (mg/mL)	Volume (mL)	Total Protein (mg)	Activity (units/mL)	Total Activity (units)	Specific Activity (units/mg)	Fold Purification	Percent Yield (%)
IH	11.86	2.070	24.55	0.2364	0.4893	0.0199	1.000	100
11K	10.54	1.920	20.24	0.0618	0.1187	0.0059	0.2965	21.00
FP	30.62	.1500	4.590	2.048	0.3072	0.0669	3.36	62.78

Brain

Sample	Protein Conc. (mg/mL)	Volume (mL)	Total Protein (mg)	Activity (units/mL)	Total Activity (units)	Specific Activity (units/mg)	Fold Purification	Percent Yield (%)
IH	12.39	2.030	25.16	0.1227	0.2491	0.0099	1.000	100
11K	5.930	1.850	12.51	0.0483	0.0894	0.0071	0.7172	35.88
FP	16.71	.1420	2.380	1.183	0.1680	0.0706	7.131	67.44

Table 1. This table displays the results obtained through the protein and enzyme assays for both the liver sample (1a) and brain sample (1b).

Cytochrome C Release is Calcium Dosage Dependent in Liver and Independent in Brain

A western blot was performed using antibodies to specifically target cytochrome c. The samples were run on an SDS-PAGE gel and transferred to a PVDF membrane for antibody incubation. Secondary antibody was added and the blot was developed using horseradish peroxidase with luminol enhancing solution.

Triton X-100 was used as a positive control as it ruptures the outer mitochondrial membrane resulting in the maximum amount of cytochrome c release. A pure sample cytochrome c was also as a control. The blots were analyzed using ImageJ software. The

intensity of the pure cytochrome c band was used to provide a relative quantification of the cytochrome c released through every sample.

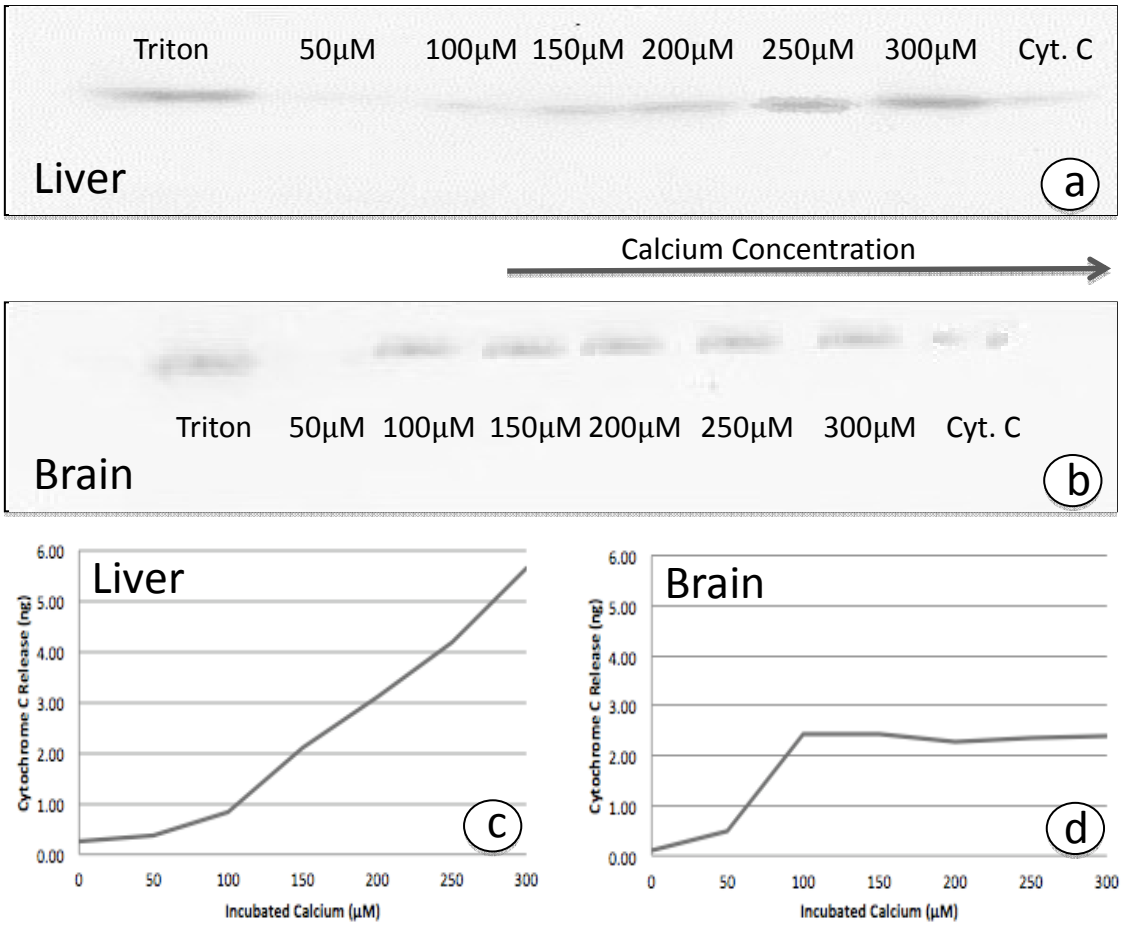


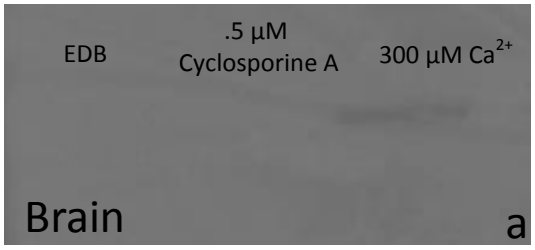
Fig 2. Liver mitochondria exhibit calcium dosage dependent cytochrome c release while brain mitochondria do not. Pure mitochondria from murine liver and brain were incubated with increasing concentrations of calcium and a subsequent western blot targeting cytochrome c was performed. In both western blots, liver (a) and brain (b), lanes 1 and 2 represent a negative control and positive control utilizing Triton X-100. Lanes 3-8 represent steady increasing calcium concentrations from 50 µM to 300 µM. Liver mitochondria demonstrated an 11 fold increase in cytochrome c release across the calcium gradient (c). This demonstrates a calcium dosage dependent response pointing to mitochondrial apoptosis-induced channels as the method for release. Brain mitochondria demonstrated no increase in cytochrome c release in response to increasing calcium levels beyond 100 µM. The cytochrome c released from the 100 µM to 300 µM samples averaged 48% of the total cytochrome c released from the liver sample.

The liver western blot (Figure 2a) shows that the increase in cytochrome c was established with an increase in calcium concentration. Using ImageJ software a relative quantification of cytochrome c levels was established. The maximal calcium

concentration used (300 µM) resulted in a band with an intensity of 94% of the positive Triton X-100 sample. This demonstrated the threshold calcium concentration for full cytochrome c release. The brain western blot demonstrated a lack of calcium dosage dependence beyond a concentration of 100 µM. The Triton X-100 sample was used to demonstrate maximal cytochrome c release. The 300 µM calcium sample produced a band with a 99% pixel density in comparison to the Triton X-100 sample, demonstrating maximal cytochrome c release. The 50 µM calcium band had an intensity of only 18%, compared to 96% band intensity in the 100 µM calcium sample. This demonstrates the calcium threshold concentration in brain mitochondria to be between 50 µM and 100 µM.

A small amount of both the liver and brain supernatants, following the incubation in 300 µM calcium were used in a cytochrome c oxidase assay. The percentage of intact mitochondria following calcium incubation was compared to the percent of intact mitochondria before incubation. The liver sample was shown to have a decrease from 62.78 percent to 55.23 percent intact mitochondria following the incubation with calcium. The brain sample demonstrated a more substantial decrease from 67.44 percent to 35.11 percent intact mitochondria following the incubation with calcium.

Liver and brain mitochondria samples were also incubated in the presence of cyclosporine A, which inhibits the formation of permeability transition pores. Following incubation, a western blot was performed on the samples targeting cytochrome c shown in Figure 3. Brain mitochondria did not release cytochrome in the presence of calcium and cyclosporine A, demonstrating a PTP dependent mode of release. Liver mitochondria released equivalent amounts of cytochrome c in the presence and absence of cyclosporine a demonstrating a PTP independent mode of cytochrome c release.



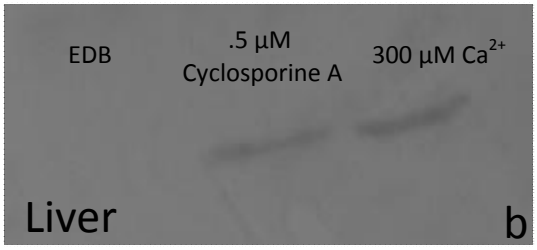


Figure 3. Cyclosporine A inhibits cytochrome c release in brain mitochondria but not liver mitochondria. Pure brain and liver mitochondria were incubated with 300 µM calcium, 0.5 µM cyclosporine A with 300 µM calcium and enzyme dilution buffer acting as a negative control and a subsequent western blot targeting cytochrome c was performed. In the brain sample (a) cyclosporine A blocked complete cytochrome c release demonstrating a permeability transition pore (mPTP) dependent mechanism.

Discussion

The activities of the 11K sample for both the brain and liver samples were similar at 0.0894 units and 0.1187 units respectively (Table 1). Both were significantly smaller values than the total activity found in their IH samples showing that relatively low amounts of mitochondria were lost through the centrifugation process.

The total activity for the brain sample final pellet was 0.1680 units, which was very similar to the 0.3072 units of activity found in the liver final pellet. The difference could be a result of the structures of the outer mitochondrial membranes, however more extensive research in this field would have to be obtained.

The liver western blot (Figure 2a) displays a linear increase in intensity of the bands moving from the 50 µM calcium sample to the 300 µM calcium sample as demonstrated in Figure 2c. This proves that an increase in calcium caused an increase in the amount of cytochrome c released. This provides evidence in support of the MAC theory opposed to PTPs. If cytochrome c was to be released through PTPs and the rupturing of the mitochondria, then the 50 µM should release similar cytochrome c levels as higher

calcium concentrations.

Following the incubation with calcium, the liver mitochondria demonstrated only a small decrease in percentage of intact mitochondria. This demonstrates liver mitochondria utilize a non-PTP dependent pathway, as the PTP pathway results in the swelling and subsequent rupturing of the mitochondria. Furthermore cytochrome c release was not affected in the presence of cyclosporine A. Cyclosporine A inhibits the formation of mPTPs, demonstrating an mPTP independent mode of release, lending further evidence to MACs.

When comparing the 300 µM calcium liver sample to the positive control of Triton X-100, equivalent amounts of cytochrome c was released in each. This signifies that 300 µM calcium is sufficient to bind and open all of the mitochondrial channels, resulting in the release of maximal cytochrome c. All samples were incubated for exactly one hour. Through past experience this has been shown to be a sufficient time for calcium to be able to bind to its receptors and release the maximal amount of cytochrome c.

The brain mitochondria responded to calcium incubation resulting in the release of cytochrome c. There was no change in the amount of cytochrome c released in calcium concentrations greater than 100 µM. However the 50 µM calcium sample showed no significant cytochrome c released in comparison to other samples. It is apparent that an increase in cytochrome c does not cause an increase in cytochrome c release. Therefore it appears that brain mitochondria utilize the PTP pathway to release cytochrome c.

Following the incubation with calcium, brain mitochondria demonstrated a 32.33 percent decrease in level of intact mitochondria following incubation with 300 µM calcium. This provides further evidence to a PTP dependent pathway utilized in the brain

mitochondria. Furthermore, cytochrome c release was inhibited through the use of cyclosporine A. Cyclosporine A inhibits the formation of mPTPs providing direct evidence of an mPTP dependent mode of release in brain mitochondria.

It is important to note that not all mitochondria from the brain sample were ruptured through the incubation of calcium. This could be due to a synergistic mechanism involving both PTPs and MACs working in conjunction.

Each band showed a similar level of cytochrome c release to that of the Triton X-100 sample demonstrating a complete release of cytochrome c through the rupturing of the mitochondrial membrane. It is also apparent that the threshold calcium concentration is between 50 µM and 100 µM as the 50 µM calcium was the only sample that did not release cytochrome c.

Liver mitochondria produced a much brighter band in the Triton X-100 positive control compared to the brain mitochondria. Based on the differences in their apoptotic mechanisms it can be suggested that brain mitochondria have a lower level of cytochrome c than liver mitochondria. If the levels were equal between tissues, the positive controls would also be equal.

All of this evidence appears to point to MACs as the primary mechanism for cytochrome c release in liver and mPTPs as the primary mechanism in the brain. However, based on this research cohesive activity of MACs and mPTPs in either tissue cannot be ruled out. It is possible that MACs and mPTPs work in conjunction to cause the release of cytochrome c. It is evident however that the MACs act as the primary release mechanism in liver mitochondria, with mPTPs being the primary mechanism in brain mitochondria.

Methods

The primary methods for the procedure were obtained from *Exploring Cell Biology* (*48*).

Preparation of Tissue Homogenate

Lab mice were primarily asphyxiated using carbon dioxide and decapitated. The liver and brain were then quickly removed. A 300 mg portion of brain tissue and a 300 mg portion of the left lateral lobe of the liver were separated and prepared separately but in parallel for the remainder of the experiment. The tissue samples were immediately washed in Extraction Buffer A [Sigma] (10 mM hydroxyethyl piperazineethanesulfonic acid pH 7.5, 200 mM mannitol, 70 mM sucrose, 1 mM ethylenebis(oxyethylenetrinitrilo)-tetraacetic acid). The tissues were diced over ice and added to ten times the volume of ice cold Extraction Buffer A containing 2 mg/mL BSA [Biorad]. They were homogenized using ten fluid strokes in a cold glass dounce. A 50 µL aliquot was removed before performing the differential centrifugation and labeled Initial Homogenate (IH).

Differential Centrifugation

The homogenate was centrifuged at 4°C for ten minutes at 600 x g to separate the supernataint from the pellet. The pellet contained whole cells and nuclei, while the supernatant contained unbroken mitochondria and other light organelles. The supernatant was decanted and re-centrifuged at 4°C for fifteen minutes at 11 000 x g. The supernatant was removed and kept on ice labeled 11K. The pellet was re-suspended in ten volumes of cold Extraction Buffer A, and re-centrifuged at 4°C for fifteen minutes at 11 000 x g. The resulting final pellet (FP) was re-suspended in 120 µL of Storage Buffer [Sigma] (10 mM HEPES pH 7.4, containing 250 mM sucrose, 1mM ATP, 0.08 mM ADP, 5mM sodium succinate, 2 mM dipotassium phosphate, 1 mM dithiothreitol) and labeled final pellet

(FP).

Protein Assay

To determine mitochondrial content of the samples, a protein assay was performed. A standard curve was generated using diluted Bradford reagent (1:5) and various concentrations of BSA. The absorbance of the samples was measured at 595 nm using a Genesys 5 UV-Vis spectrophotometer.

Cytochrome C Oxidase Assay

Two parallel samples of the IH, 11K and final pellet were prepared for the enzyme assay from both the liver and the brain. One sample from each was diluted to 0.2 mg/mL with Enzyme Dilution Buffer [Sigma] (10 mM Trizma-hydrochloric acid pH 7.0, 250 mM sucrose), and the other diluted to 0.2 mg/mL with Enzyme Dilution Buffer containing 1mM n-Dodecyl β-D-Maltoside [Sigma]. The samples were incubated for ten minutes at 4°C. Following incubation the absorbance of the samples were measured at 550 nm. The incubated samples containing Assay Buffer [Sigma] (10 mM Trizma-hydrochloric acid pH 7.0, 120 mM KCl), Enzyme Dilution Buffer, and Ferrocytochrome c Substrate Solution [Sigma] (0.22 mM ferrocyctochrome c, .5 µM DTT) in a ratio of 1:19:1:1 were mixed and the absorbance measured in ten second intervals.

Calcium Incubation

Several mitochondria samples were prepared from the FP sample for incubation with calcium. Aliquots of 100 µg of mitochondrial were added to 50 µM, 100 µM, 150 µM, 200 µM, 250 µM, and 300 µM calcium concentrations in 100 µL final volume. A negative control substituting Enzyme Dilution Buffer for the calcium was created. A positive control was created by substituting the calcium with Enzyme Dilution Buffer

containing 0.2% Triton X-100 [Sigma]. The various samples were incubated at 27°C for sixty minutes and centrifuged at 4000 x g for five minutes. The supernatants of the samples were removed and analyzed for western blotting.

Western Blot for Cytochrome C

The following precipitation procedure was taken directly from the Edman Degradation Sample Preparation Protocols (*49*). The supernatants collected from the calcium incubation were placed in fresh tubes with 400 µL of cold methanol and vortexed well. Then 100 µL of chloroform was added and the tubes were re-vortexed. To the tubes 300 µL of DI water was added and the samples were again vortexed and centrifuged for one minute at 14 000 x g. The top aqueous layer was discarded, 400 µL of methanol was added, vortexed well and centrifuged for two minutes at 14 000 x g. The supernatant was carefully decanted and allowed to air dry at 24°C for approximately fifteen minutes until visibly dry. The pellets were re-suspended in 20 µL of 2X sample buffer [Biorad] and 2 µL of β-mercapto-ethanol and incubated for 2 minutes at 100°C. A SDS-PAGE 4-20% gradient pre-cast gel [Biorad] was prepared with 1 liter of electrode buffer [Biorad] (25 mM Trizma-hydrochloric acid pH 7.0, 192 mM glycine, 0.1 % sodium dodecyl sulfate). The gel was run at a constant 150 mV for fifty minutes. The completed SDS-PAGE gel was transferred to a PVDF membrane at 80 mV for ninety minutes in transfer buffer [Biorad] (25 mM Tris pH 8.3, 192 mM glycine, 20 % methanol, 0.1 % SDS).

The PVDF membrane was wet in methanol and placed in a blocking solution of Tris-buffered-saline with 5% nonfat dry milk at 24°C for 45 minutes with constant agitation. Purified mouse anti-cytochrome c antibody [BD Pharmingen] at a dilution of

1:400 was placed on the membrane and incubated overnight at 24°C. Goat anti-mouse secondary antibody [Biorad] at a dilution of 1:5000 was added to the membrane and incubated with constant agitation at 24°C. Washes using tris-buffered saline with Triton X-100 (TTBS) and tris-buffered saline (TBS) were then performed. Development was achieved using equal amounts of 0.75 mg/mL diaminobenzinide [Biorad] in TBS and hydrogen peroxide [Biorad] in Tris-buffered-saline for five minutes. Images were obtained with a camera using UVP Bio-imaging EpiChemi3 Darkroom and analyzed using ImageJ software.

Conclusion

Apoptosis is a natural and beneficial action of multi-cellular organisms. During times of stress and development, affected cells must be destroyed in order to protect the organism as a whole. The intrinsic apoptotic pathway involves non-receptor mediated signaling leading to the activation of pro-caspases that together exhibit the actions killing the cell. Mitochondria are key players in this pathway releasing pro-apoptotic factors, including cytochrome c, from their inter-membrane space in response to appropriate stimuli. One of these stimuli is a high concentration of cytosolic calcium. Through the independent research study conducted it is apparent that the mechanism of cytochrome c release differs between liver and brain mitochondria. It is apparent that liver mitochondria utilize the mitochondrial apoptosis induced channels as their primary mode of release. The brain mitochondria differ, as they did not respond to calcium dosage. As a result it is clear that they utilize permeability transition pores as their primary mode of release of cytochrome c and other pro-apoptotic factors.

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