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Investigating the Stability of Energy Metabolism in Cultured Neurons: Do Cerebellar GranuleNeurons Maintain A Metabolic Steady State in the Early Stages of Apoptosis?

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INVESTIGATING THE STABILITY OF ENERGY METABOLISM IN CULTURED NEURONS: DO CEREBELLAR GRANULE NEURONS MAINTAIN A METABOLIC STEADY STATE IN THE EARLY STAGES OF APOPTOSIS?

by
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ABSTRACT

ADRIENNE WEBB: Investigating the Stability of Energy Metabolism in Cultured Neurons: Do Cerebellar Granule Neurons Maintain a Metabolic Steady State in the Early Stages of Apoptosis?

(Under the direction of Dr. Mika Jekabsons)

Apoptosis is a process that occurs naturally during development of an organism and is a specific type of programmed cell death that characterized by its distinct morphological features. However, inappropriate inhibition or activation can result in various pathological conditions. For example, studies have shown links between inactivation of some pro-apoptotic proteins and certain cancers. Also, overstimulation of apoptosis has been linked to many neurodegenerative disorders. Therefore, a clearer understanding of the process of apoptosis could provide new avenues for the treatment of these diseases that have been linked to it. The signaling involved in triggering apoptosis involves many proteins that ultimately lead to activation of proteolytic enzymes which destroy the cell. One possible signaling route that may facilitate this process is a change in energy metabolism. One of the goals of Dr. Jekabsons' research is to identify the reactions in catalyzing glucose oxidation whose kinetics are directly affected by apoptotic stimuli. A prerequisite to identifying these reactions is the simultaneous measurements of multiple reaction fluxes within the cell, which is experimentally possible provided the neurons maintain a metabolic steady state. The goal of this project was to determine if healthy and apoptotic cerebellar granule neurons maintained a steady state. The ability of these cultured neurons to sustain a steady-state was assessed by determining the rates of

glucose consumption and lactate production, and the intracellular concentrations of ATP, ADP, and glucose-6-phosphate.

In both experiments, the neurons were exposed to either high or low extracellular K^+, mimicking the healthy and apoptotic conditions, respectively. The levels of the specified metabolic intermediates were calculated from measurements taken by a spectrophotomete, fluorometer, and luminometer. Results show a steady decrease in extracellular glucose, a steady increase in extracellular lactate, and a maintained concentration of ATP, ADP and G6P in both healthy and apoptotic neurons. Thus, it can be concluded that both healthy and apoptotic neurons maintain a metabolic steady state over the course of time required.

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Introduction

Apoptosis is a specific type of programmed cell death and is characterized by such morphological features as nuclear condensation, DNA fragmentation, membrane blebbing, and cell shrinkage. It is a developmentally important process in establishing the morphology and function of organs. For example, the webbed digits of vertebrates are "sculpted" through apoptosis of the unwanted cells between the digits. It also plays a critical role in establishing the function of the mature nervous system since a large number of neurons undergo apoptosis during development. Apoptosis also plays a key role in the immune system, in which cytotoxic lymphocytes initiate cell death in target cells[1].

The pathway of programmed cell death is a highly complex process and requires the collaboration of multiple factors in order to trigger a cell to die. The members of the BCL-2 family of proteins play major roles in the apoptotic pathway. These proteins can be either anti-apoptotic, such as BCL-2 and BCL-xL, or pro-apoptotic, such as BH123 proteins and BH3-only proteins. Previous studies have shown that BH3-only proteins are regulated by different signals according to each specific protein and are considered the primary sensors of cellular stress[5]. For example, one BH3-only protein, Bad, is inhibited by the kinase activity of Akt, therefore blocking the apoptotic pathway[6]. When activated, these BH3-only proteins activate BH123 proteins, such as BAX and BAK. The activation of these proteins has been shown to cause the permeabilization of the mitochondrial outer membrane, allowing proteins that normally reside in the

intermembrane space in the mitochondria to be released into the cytosol. One of these proteins is cytochrome c, which is a cofactor for apoptotic protease-activating factor-1 (APAF-1). APAF-1 triggers the formation of apoptosome and the activation of initiator caspases[5]. The initiator caspases activate effector caspases, which then cause such events in the cell as mitochondrial damage, nuclear membrane breakdown, DNA fragmentation, chromatin condensation, and, therefore, cell death[7]. There is some evidence that some pro-apoptotic proteins also target cell metabolism. For example, the BH3-only protein, Bad, associates with the glycolytic enzyme, glucokinase, during hepatocyte apoptosis. It is suggested that dephosphorylated Bad inhibits the activity of glucokinase, thus causing a decline in cellular metabolism[8]. This evidence links pro-apoptotic proteins with cell metabolism and suggests that inhibition of metabolism could be a signal that contributes to apoptosis. However, there have been no studies indicating that such signaling occurs in neurons.

Although apoptosis occurs naturally and is essential for the organism, it can also be triggered inappropriately, resulting in pathological conditions such as neurodegenerative diseases. Parkinson's, Alzheimer's, and Huntington's diseases are neurological disorders caused by the death of neurons in the mature brain, and at least some of this death is by apoptosis[2]. One of the goals of the research projects conducted by Dr. Jekabsons is to identify metabolic reactions in CGNs whose kinetics are directly affected by apoptotic stimuli, as this could indicate that pro-apoptotic proteins target neuronal metabolism. Since cell metabolism is a highly complex chain of processes, it is necessary to simplify the reactions that are taking place. As shown in Figure 1, these reactions can be grouped into blocks that are connected by a few key intermediates. It has

been shown in previous research projects that the mitochondrial respiration rate decreases in apoptotic cerebellar granule neurons (CGNs)[4]. This decreased respiration rate indicates that the kinetics of at least one of the blocks in Figure 1 is directly affected by the apoptotic stimulus.

The reaction fluxes must be determined in order to identify which blocks are directly affected. Most of these fluxes can be calculated by measuring (1) glucose consumption rate, (2) lactate production rate, and (3) mitochondrial respiration rate. However, two criteria must be met in order to calculate the fluxes. First, the stoichiometries between the reactions must be known; and secondly, the cell must maintain a metabolic steady state during the measurement period. The reaction stoichiometries among the blocks shown in Figure 1 are known with reasonable certainty. The goal of this project was to determine if both healthy and early-stage apoptotic CGNs maintain a metabolic steady state over the time required to measure glucose consumption, lactate production, and respiration rate. Criteria for a steady-state condition include constant flux rates as well as constant concentrations of the explicit intermediates that connect the reaction blocks. The ability of both healthy and apoptotic neurons to maintain a steady state was accomplished by measuring the glucose consumption rate and lactate production rate, as well as the intracellular concentrations of glucose-6-phosphate (G6P), ATP, and ADP over a five-hour period.

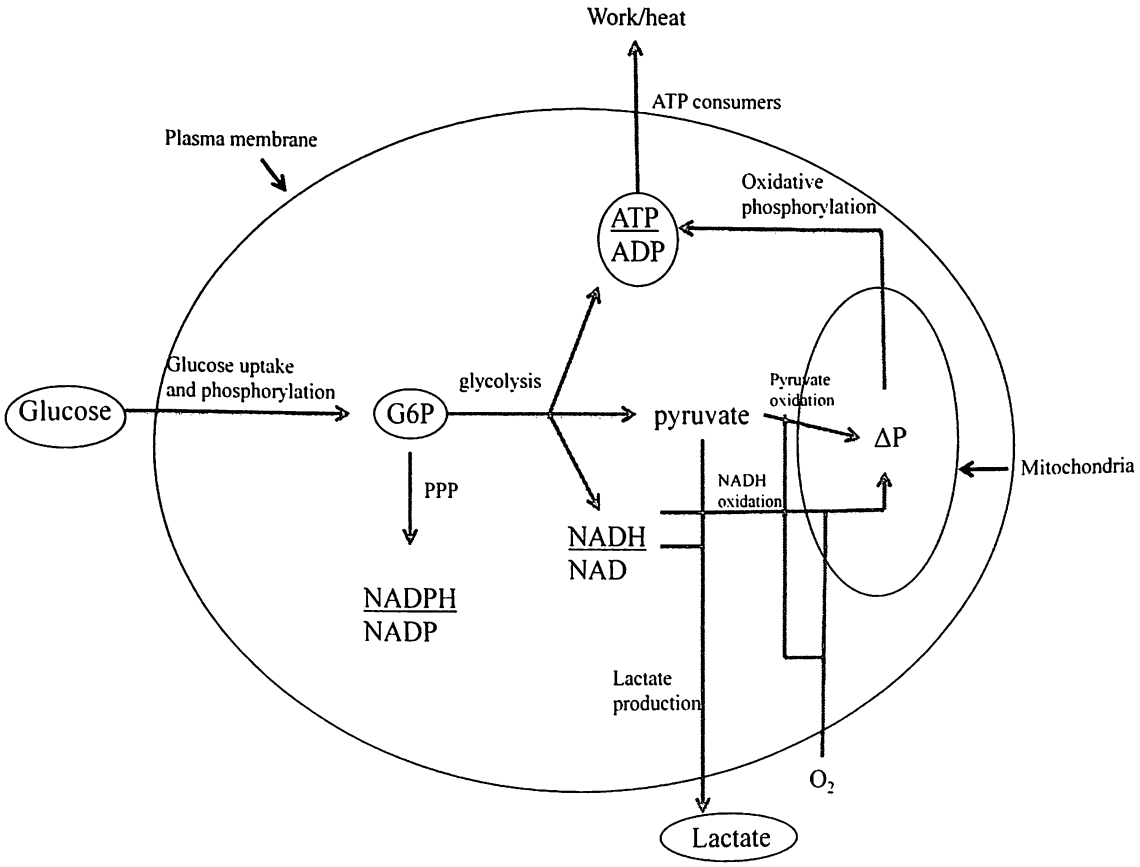

Figure 1 Simplified schematic of reactions involved in neuronal cell metabolism. Glucose catabolism has been simplified into eight blocks, designated by arrows, linked through five explicit intermediates. In the neuron, the cell takes up glucose and converts it to glucose-6-phosphate (G6P). G6P can then enter either the pentose phosphate pathway (PPP) or glycolysis, generating ATP, NADH, and pyruvate. The cell can then consume ATP. Some of the pyruvate, along with NADH, is reduced to produce lactate, which is exported from the cell. Some of the pyruvate and NADH is oxidized by the mitochodria via the citric acid cycle and respiratory chain, creating a mitochondrial membrane potential (ΔP). Oxidative phosphorylation in the mitochondria produces ATP, which is then exported to the cytoplasm where it can be used by ATP requiring reactions. The parameters and intermediates in these pathways that are studied in this project are circled[2].

Granule neurons in the developing cerebellum undergo apoptosis if they fail to establish an excitatory synaptic connection with axon terminals from brain stem neurons, and/or if they do not receive an adequate supply of growth factors. These *in vivo* apoptotic triggers can be mimicked *in vitro* by manipulating the availability of K^+ and serum growth factors in the culture media. High (25mM) external K^+ causes partial depolarization of the plasma membrane, an effect which occurs *in vivo* by release of an excitatory neurotransmitter by a presynaptic neuron. In a normal neuron *in vivo*, the resting membrane potential is around -75mV with closed voltage-gated Ca^{2+} channels. If the membrane is depolarized sufficiently by a neurotransmitter, it will cause the opening of the Ca^{2+} channels, which allows Ca^{2+} to enter the cell from the cytoplasm and signal survival kinases such as Akt to suppress apoptosis. This depolarized condition can be simulated *in vitro* by exposing CGNs to high extracellular K^+ by incubating them in media containing 25mM K^+ and growth factors (supplied by supplemental media with fetal bovine serum, FBS) that promote the survival and maturation of immature CGNs. The high K^+ outside the cell depolarizes the membrane, yielding a membrane potential of -35mV and thus opens the voltage-gated Ca^{2+} channels. This condition is denoted as the "healthy" condition since it mimics a healthy neuron's response to depolarization of the plasma membrane[2].

Low (3.5mM) external K^+ results in a maintained hyperpolarized membrane potential, which would occur *in vivo* if granule neurons failed to form an excitatory synapse. The apoptotic condition can be simulated *in vitro* by exposing CGNs to low extracellular K^+ in a media containing bovine serum albumin (BSA). Replacing the growth factors with albumin would thus mimic the *in vivo* condition where CGNs have

inadequate access to growth factors. The combined deprivation of extracellular K^+ and growth factors initiates several mechanisms within the cell that are involved in apoptosis, one of which may be reduced glucose catabolism[4]. The decrease in cytoplasmic calcium occurs because of the low concentration of K^+ outside of the neuron. The membrane is not sufficiently depolarized, and the voltage-gated Ca^{2+} channels remain closed. Therefore, Ca^{2+} cannot enter the neuron through these channels. This decreased amount cytoplasmic calcium and deprivation of growth factors causes a significant decrease in cell metabolism and help to induce apoptosis. This condition is referred to as the "apoptotic" condition because apoptosis is induced in the exposed neurons[2].

Assays have been developed in order to determine the glucose consumption rate, lactate production rate, and concentrations of intermediates involved in the reactions associated with cell metabolism. The glucose consumption rate can be measured by measuring the amount of glucose present in the high K^+ and low K^+ buffer at one-hour intervals for five hours. These measurements are taken through the use of a spectrophotometer. The lactate production rate can be measured in a similar manner except through the use of a fluorometer. The concentration of ATP, ADP, and G6P in cell extracts could be measured by measuring the luminescence of perchloric acid cell extracts from high and low K^+ samples taken at two and four hours. The enzyme luciferase is used in these assays to produce light that can be measured by a luminometer and can be obtained from photobacterium.

The main goal of this project is to determine whether healthy and apoptotic neurons maintain steady-state metabolism. If these neurons do maintain a steady state, the rate of carbon flux in the metabolic pathway of neurons under both conditions can be

calculated. It is important to measure these fluxes for comparison between healthy and apoptotic neurons in order to determine if neurons that are induced to enter the apoptotic pathway exhibit defects or impairment in metabolism. The hypothesis of this project is that neurons maintain a metabolic steady state in both healthy and apoptotic conditions.

Materials and Methods

A. Preparation of cells

CGNs were collected from 5-7 day old rat pups and prepared according to the procedure in the article published by Jekabsons and Nicholls 2006. Approximately $7x10^6$ CGNs were contained in each two-well chamber. These cells remained incubated at 37°C in culture for 6-9 days before use in experiments[4].

B. Experiments to determine rates of glucose consumption and lactate production

Each well in the chamber was incubated for one hour at 37°C in 2mL of either 25K FBS or 3.5K BSA (contents listed in Table 1). After one hour, the cells were washed once with buffer solution. The cells were then incubated in 350uL buffer for five one-hour intervals. At the end of each hour, the buffer was collected from each well and centrifuged for two minutes at 4°C (18,000 x *g*). The supernatant was saved and stored at -20°C for use analysis of glucose and lactate concentrations. The cells were washed once and 350uL of fresh buffer added for the next one-hour interval. A chamber without cells containing only either 25K FBS or 3.5K BSA in each well was also run in parallel to serve as a control and account for the evaporation rate (Figure 2). The control wells served both to account for the concentration changes caused by evaporation of each one-hour period and to accurately assess the final volume so that total amounts of glucose consumed and lactate produced by the cells could be determined.

Table 1: FBS and BSA buffer solutions (pH 7.35 and equilibrated to 37°C)

25K FBS	3.5K BSA
116mM NaCl	137.5mM NaCl
25mM KCl	3.5mM KCl
20 mM TES buffer	20mM TES buffer
1.3mM $MgCl_2$	1.3mM $MgCl_2$
1.3mM $CaCl_2$	1.3mM $CaCl_2$
1.2 mM Na_2SO_4	1.2mM Na_2SO_4
0.4mM KH_2PO_4	0.4mM KH_2PO_4
0.2mM $NaHCO_3$	0.2mM $NaHCO_3$
9.6mM glucose	9.6mM glucose
10% FBS	0.3% BSA

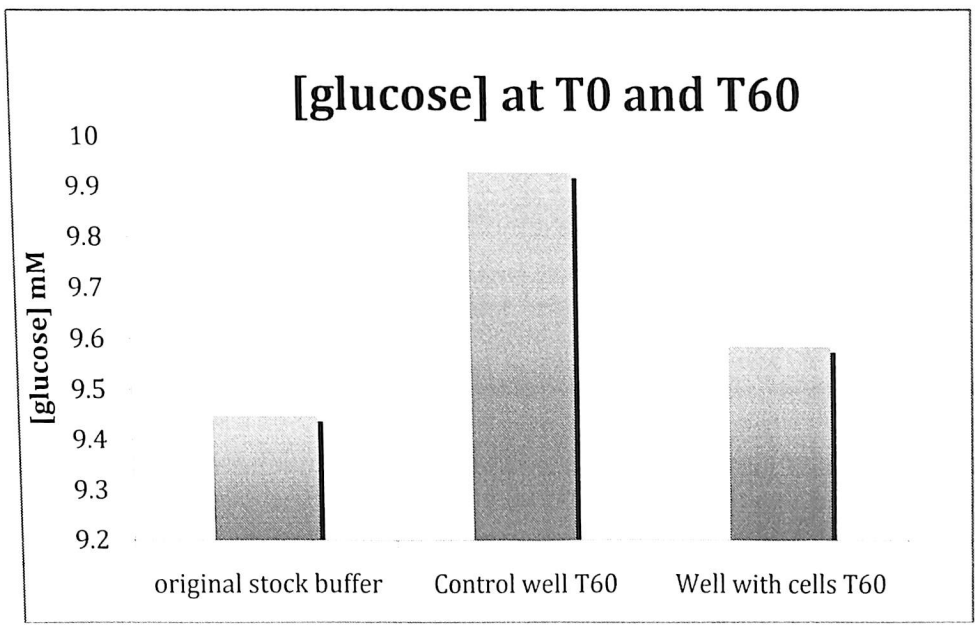

Figure 2 Volume and evaporation rate calculation for glucose and lactate assays. The concentration of glucose was used to determine the volume after one hour of incubation to account for evaporation of supernatant from cells on 19 February 09. The concentration of glucose in the control well after one hour is shown here to reflect the evaporation of the water because [glucose] is much increased from [glucose] in the original stock buffer. The average [glucose] of all time points (T60-T300) for the control wells was then divided into the amount of glucose (umol) added to each well. This value then gave the final volume used to calculate the amount of glucose and lactate present in the supernatant. Each [glucose] in the wells containing cells was multiplied by this volume to calculate the amount of glucose present in the supernatant

C. Experiments to determine the intracellular concentrations of G6P, ATP, and ADP

For determination of intracellular stability of ATP, ADP and G6P, cell extracts were prepared after two- and four-hour incubations at 37°C in 25K or 3.5K buffer. At the end of the incubation period, the buffer was removed from the chamber. The cells were quickly washed twice with 1mL of cold phosphate-buffered saline supplemented with glucose (154mM NaCl, 2.7mM KCl, 8.2mM Na_2HPO_4, 1.8mM KH_2PO_4, 10mM glucose, pH 7.35). Ice-cold 1M $HClO_4$ (350uL) was added to each well to lyse the cells, and then incubated for ten minutes at 4°C with gentle agitation. It was found that if the cells were scraped from the bottom of the well, there would be a stronger signal. The extract was removed from each chamber and transferred to a pre-cooled microfuge tube. For most experiments (5 of 8), the extracts were homogenized using a motor driven plastic pestle. The homogenates were then centrifuged for 10 minutes at 18,000xg, 4°to remove precipitated proteins and cell debris. The supernatant was partially neutralized by addition of 145uL of 4M KOH for fifteen minutes on ice. The tubes were centrifuged for two minutes at 18,000xg, 4°C to pellet insoluble $KClO_4$. The supernatants were then further neutralized with 7% 0.5M Tris base solution. The volume of the supernatant was measured, then 0.5M Tris base was added (7% of measured volume) to make up the final volume of the extract. The cell extracts were then stored at -80°C.

D. Glucose assay

In order to determine the rate of glucose consumption, the following enzymatic assay was performed on each buffer sample in triplicate. Figure 3 shows the principle of the assay, which is based on phosphorylation of glucose, followed by oxidation of

glucose-6-phosphate, with spectrophoton metric detection of NADPH by absorbance at 340nm. A 485uL volume of 1x reaction buffer (contents listed in Table 2) was added to each cuvette and an initial reading was taken. A 15uL volume of the glucose standard or cell extract was added to the first cuvette and the timer set for fourteen minutes. Each subsequent sample was added at every 30-second mark. At 7 minutes, the final reading of the first cuvette was taken, with the remaining readings taken at one-minute intervals. The difference between the two readings was used to calculate the concentration of glucose consumed by the cells during the experiment. The readings for the glucose standards were used to generate a standard curve for calculating the concentrations in the unknown samples (Figure 4).

Figure 3 Reaction scheme used to measure glucose concentration. The enzymes hexokinase and glucose-6-phosphate dehydrogenase (G6PDH) were coupled with ATP and NADP to generate the products NADPH and 6-phosphoglucono-δ-lactone. Glucose is phosphorylated through the activity of hexokinase, which removes a phosphate from ATP thus yielding ADP and G6P. In the presence of G6PDH and NADP, G6P will be drawn into the PPP and will produce the reduced coenzyme NADPH. NADPH absorbs at λ=340nm and can be detected by a spectrophotometer. The amount of NADPH produced is equivalent to the amount of glucose present in a solution since the amount of added glucose is the limiting component in this reaction scheme.

Table 2: Buffer Solution for the Glucose Assay

0.1M TEA (pH 7.5)
7mM $MgCl_2$
2mM ATP
2mM NADP
1u/mL hexokinase
1u/mL G6PDH

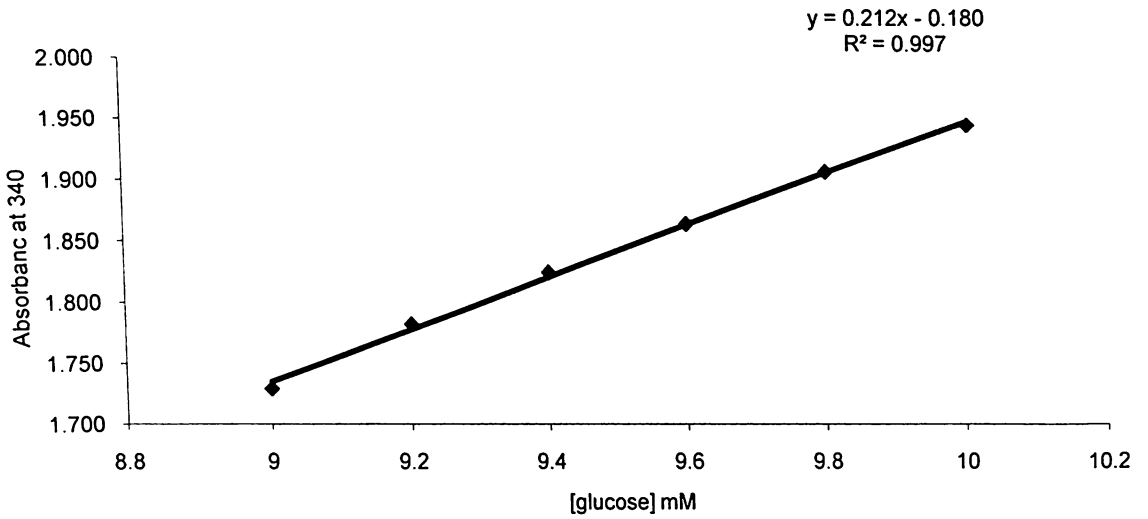

Figure 4 Glucose present in samples from 19Feb09. The amount of glucose present in the buffer samples was determined using the absorbances obained from a set of standards prepared in the high K^+ buffer. The relationship between NADPH production (as measured by A_{340}) and glucose concentration is illustrated from a representative assay. The equation from the graph of the known concentration of glucose of the standards vs. the absorbance at λ340 was used to calculate the concentration of glucose in the unknown samples by substituting the absorbance for y. These concentrations were multiplied by the calculated volume of each sample to determine the nmol of glucose in each sample, which was plotted against the time at which the sample was taken.

E. Lactate Assay

In order to determine the rate of lactate production by the cells, the following assay was performed on each buffer sample in triplicate. Figure 5 shows the reaction scheme for this assay, which is based on the oxidation of lactate and concomitant production of NADH, which fluoresces at 460nm upon excitation at 340nm. A 480uL volume of 1x reaction buffer (contents listed in Table 3) was added to each cuvette. A 20uL volume of standard or unknown was added to the reaction buffer at one-minute intervals. After sixty minutes of incubation, the fluorescence of each sample was measured at one-minute intervals. The values of the standards were used to determine the equation for calculating the amount of lactate present in each sample. This equation was determined using a standard curve, as in Figure 6. The values of the unknowns were compared to these and used to determine the amount of lactate produced by the cells.

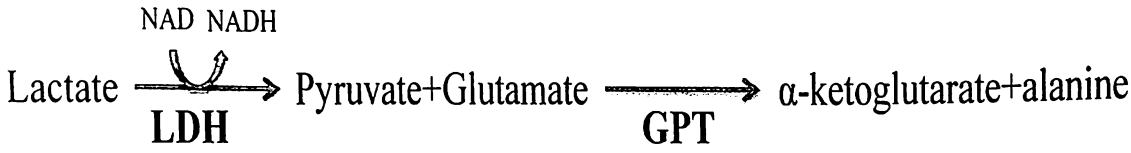

Figure 5 Reaction scheme to measure lactate concentration. The enzymes lactate dehydrogenase (LDH) and glutamate pyruvate transaminase (GPT) and the coenzyme NAD were used to oxidize lactate to pyruvate with concomitant production of NADH. To facilitate NADH production, pyruvate was removed by transaminase-catalyzed reaction with glutamate. NADH emits light at λ_{em}=460nm. Since lactate is forced to produce NADH, a fluorometer can be used to measure the fluorescence of the NADH produced from these reactions. The fluorescence of the samples is proportional to the amount of lactate present.

Table 3: Buffer solution for Lactate Assay

1x assay buffer (100mM glycylglycine, 100mM glutamate, pH 8.8)
1U/mL lactate dehydrogenase
1U/mL GPT
1.5mM NAD

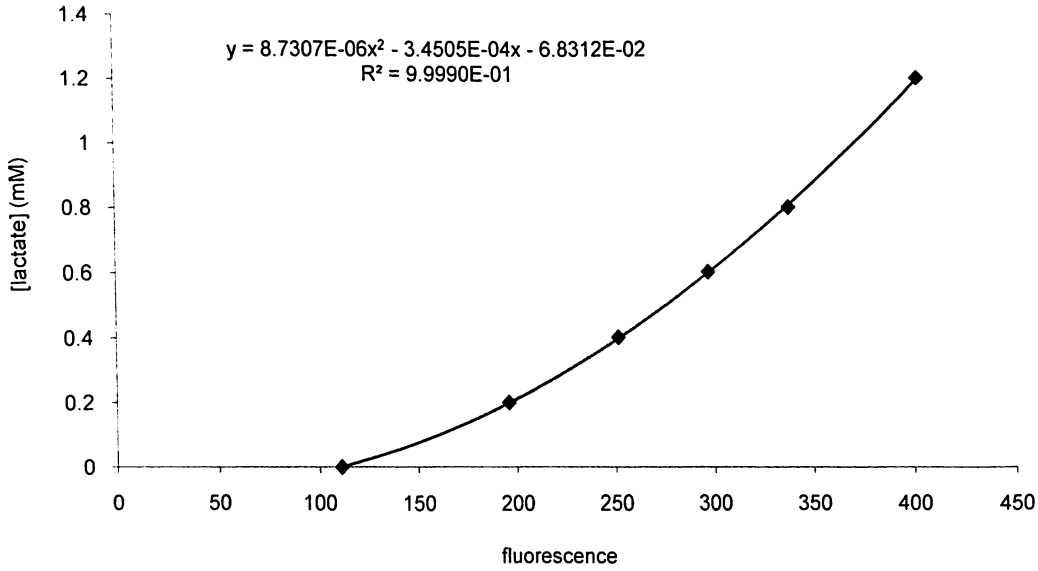

Figure 6 Lactate present in samples from 19Feb09. The amount of lactate present in the buffer samples of supernatant was determined using the fluorescences obtained from a set of standards prepared in high or low K^+ buffer. The relationship between NADH production (as measured by emission intensity at 460nm) and lactate concentration is illustrated from a representative assay. The equation from the graph of the known concentration of lactate of the standards vs. the fluorescence, a), was used to calculate the concentration of lactate in the unknown samples by substituting the fluorescence of the uknowns for x. These concentrations were multiplied by the calculated volume of each sample (refer to Figure 2) to determine the nmol of lactate in each sample, which was plotted against the time at which the sample was taken.

F. ATP and ADP assay

To determine the ATP/ADP ratio, the cell extracts and ATP/ADP standards were treated with a 1.11x buffer solution (contents listed in Table 4). The reaction pathways for both the ATP and ADP assays are shown in Figure 7 and Figure 8. The unknowns and standards were assayed in triplicate according to the following procedure. For the ATP and ADP standards, 2uL of ATP standard was added to 3uL of the corresponding ADP standard. 45uL of the reaction buffer was added to this mixture. For the unknowns, 45uL of the reaction buffer was added to 5uL of the cell extract. Immediately after adding the reaction buffer, the luminescence of each sample was measured in a luminometer at 60-second intervals with five readings per interval. A standard curve created from the relationship between luminescence and concentration of the standards was created, and the equation was used to determine [ATP] in the samples (see Figure 9). After the luminescence of each sample was measured, 1uL 70mM PEP was added and an initial luminescence reading was acquired. The samples were then allowed to incubate at room temperature for approximately 30 minutes before acquiring a final luminescence reading. The difference between these two readings from the standards was used in a standard curve to determine an equation for calculating [ADP] in the unknown samples, as shown in Figure 10. The average reading from the 60-second interval and the difference between the two readings after the PEP was added were used to determine the ATP/ADP ratio in the cell extracts*.

* For the experiment on 30June09, a separate buffer was made for the third set of the triplicate in the ATP/ADP assay.

Table 4: Buffer Solution for ATP/ADP assay

-70mM Tris-acetate pH 7.4
-1mM EDTA
-50mM KCl
-60uM DTT
-10mM Mg Acetate
-1:100 luciferin/luciferase reagent (Sigma)
-0.1% BSA
-1U/mL pyruvate kinase

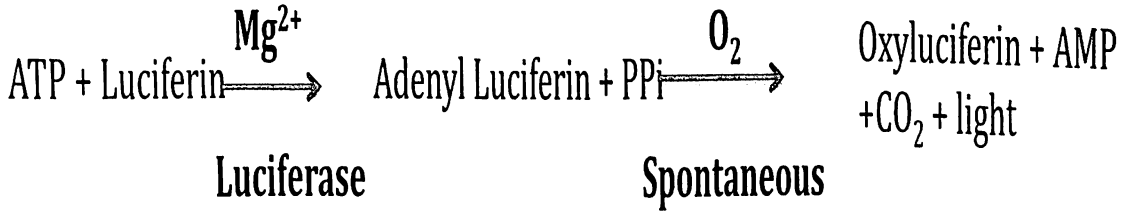

Figure 7 Reaction scheme used to measure ATP concentration. Luciferin is added to the sample along with Mg^{2+} and luciferase. If ATP is present, it will by hydrolyzed by luciferase to produce Adenyl luciferin and pyrophosphate (PPi). In the presence of O_2, oxyluciferin, AMP, CO_2, and light will spontaneously be produced. This light can be detected by a luminometer, and the luminescence is directly proportional to the amount of ATP in the sample[3].

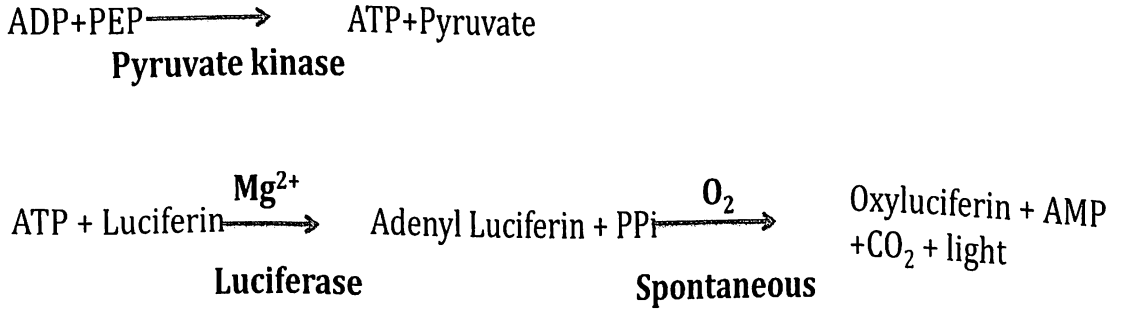

Figure 8 Reactions used to measure ADP concentration. In the presence of phosphoenolpyruvate (PEP) and pyruvate kinase, ADP will be phosphorylated, producing ATP and pyruvate. This ATP then reacts with the remaining luciferin from the ATP assay to produce light detectable by a luminometer. This luminescence is proportional with the amount of ADP present since the ATP formed from ADP in the sample produces it[3].

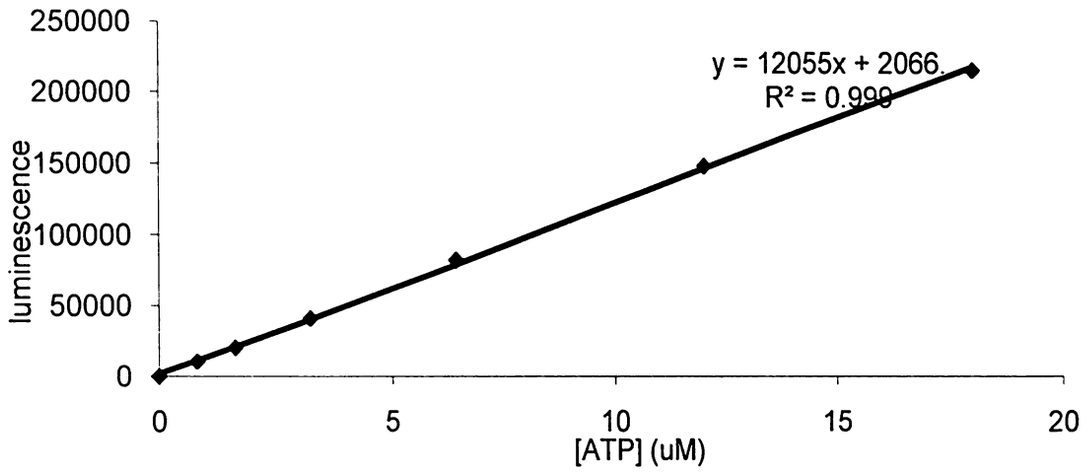

Figure 9 ATP present in cell extracts from 11Aug09. The amount of ATP present in the neutralized $HClO_4$ cell extracts was obtained from a set of ATP standards prepared in neutralized $HClO_4$. The relationship between luminescence and ATP concentration is illustrated from a representative assay.

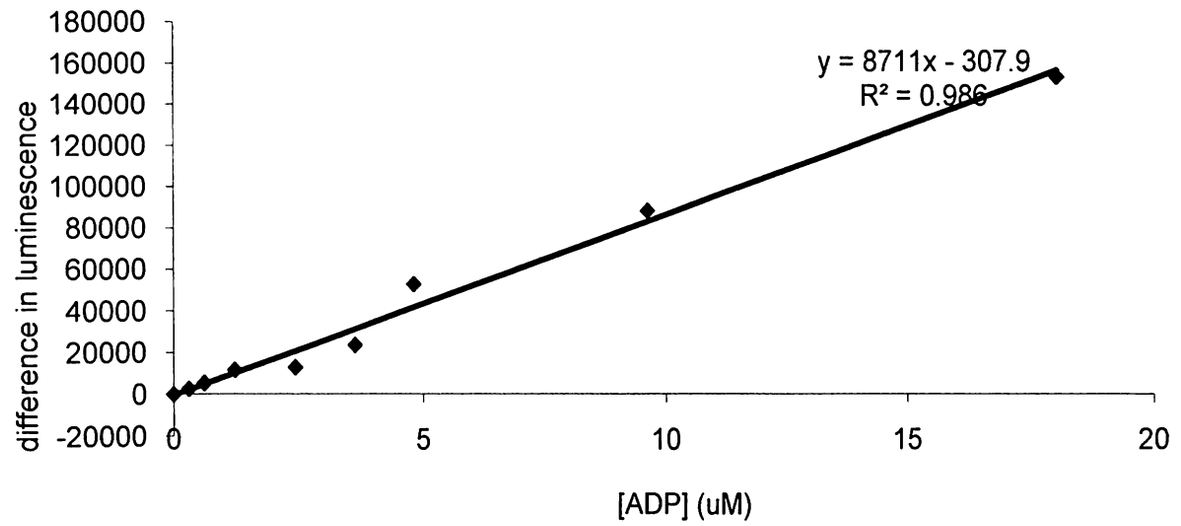

Figure 10 ADP present in cell extracts from 11Aug09. The relationship between the difference in initial and final luminescence of the ADP standards and concentration of ADP is illustrated from a representative assay. This equation, the equation from the ATP assay, and the measured luminescence of each of the unknown samples could be used to determine the concentration of ATP and ADP and therefore the ATP/ADP ratio in the cell extract.

G6P Assay

In order to determine the total pmol of G6P in the cell extracts, the samples and standards were treated with a 1.5x buffer solution (contents listed in Table 5), and were assayed according to the following procedure. The reaction scheme for the assay is shown in Figure 11. Before preparing the buffer solution, fresh FMN was prepared in a dark tube by adding 2.8mg to 585uL of 10mM TEA. The 1.5x buffer was then prepared in a dark tube and put on ice for 30-45 minutes. During this time, 32uL of each of the G6P standards and unknowns was added to a separate tube. After the buffer solution had finished incubating, 68uL of it was added to separate tubes and a background luminescence was taken in the luminometer over an 80 second interval with 6 readings. The 32uL of standard/sample was then added and another reading was taken over an 80 second interval. The concentration of G6P in the extract was calculated using the net luminescence of each extract and the equation generated by the standard curve from the net luminescence of each G6P standard, as seen in Figure 12. Using this calculated [G6P] and the extract volume measured during the two- and four-hour experiments, the total amount of G6P per cell extract could be calculated. Once this value was calculated, the in situ [G6P] could be calculated using the cytoplasmic volume (uL) of 7 million CGNs (from CGN Z stack data) for both high and low K^+ conditions

Table 5: Buffer Solution for G6P assay

100mM TEA
1mM EDTA
60uM DTT
25uM FMN[†]
2mM NAD
1mM decanal
200ug/mL NFOR/luciferase
2U/mL G6PDH[‡]
930uL H_2O

Figure 11 Reaction scheme used to measure G6P concentration. Glucose-6-phosphate dehydrogenase (G6PDH) oxidizes glucose-6-phosphate (G6P), forming 6-P-gluconolactone and NADH. In the presence of flavin-mononucloetide (FMN) and NADH-FMN oxidoreductase (NFOR), NADH is oxidized to NAD and $FMNH_2$ is produced. In the presence of decanal and luciferase from the photobacterium *V. fischeri*, a fatty acid (FA) is produced along with FMN and light. This light is detectable by a luminometer and the luminescence is proportional with the amount of G6P present in the sample[3].

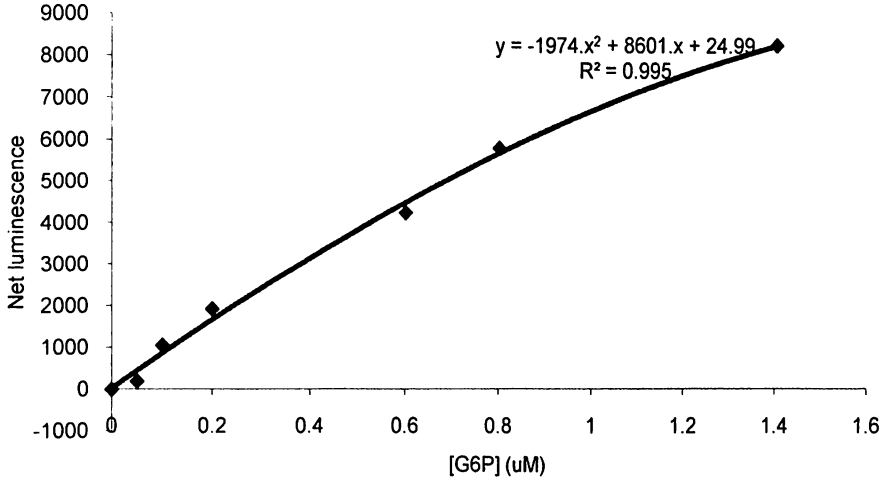

Figure 12 Relationship between net luminescence and concentration of G6P. An equation for calculating [G6P] was found using the relationship between the luminescence of the G6P standards and their known concentrations. The measured luminescence of the samples could then be plugged into the equation to calculate [G6P] in the cell extracts.

[†] For experiments prior to 22July09, 50uM FMN

[‡] For experiments prior to 22July09, 200u/mL stock solution. After 22July09, 300u/mL stock solution

Results

Glucose consumption rate in cells does not change over time in both normal and apoptotic cells

The concentration of glucose in the extracellular media of both 25K and 3.5K buffers showed a steady decrease each hour over the five-hour measurement (Figures 13 and 14). The overall glucose consumption rate was lower in the apoptotic (3.5K) cells. Table 6 shows the average concentrations of glucose in each sample at each time point to demonstrate the lower glucose consumption rate in 3.5K cells.

Lactate production rate does not change over time in both normal and apoptotic cells

By using the raw measurements of fluorescence of the samples, the concentration of lactate produced by the cells at each time interval could be measured. The data collected showed a steady increase in lactate in the extracellular supernatant over the given time period in both the high K^+ and low K^+ conditions. In should be noted that the high K^+ cells showed a significant higher concentration of lactate in the final measurement, thus showing a higher lactate production rate than that of the low K^+ cells. These data are illustrated in Figure 15, Figure 16, and Table 7.

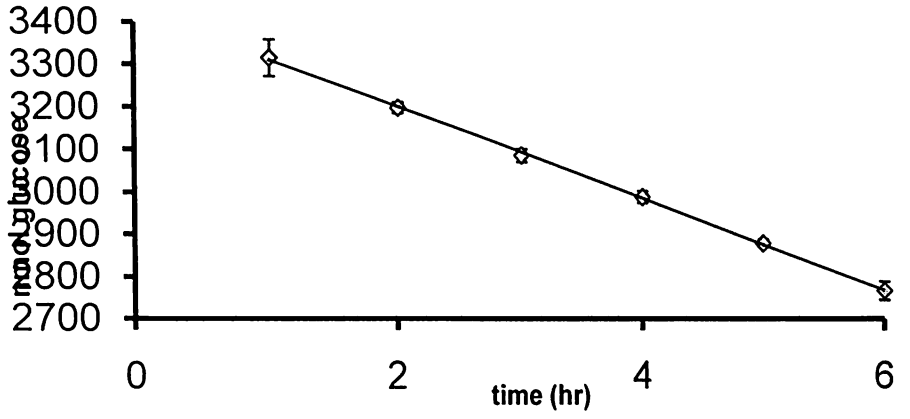

Figure 13 Glucose consumption remained steady in healthy cells. Cells were incubated in high K^+ buffer which was removed and replaced every hour for five hours. The amount of glucose in each sample was measured using a spectrophotometer. The concentration of glucose at each hour was determined using these values, thus showing a steady decrease in the amount of glucose present in media over five hours. Data are mean ± S.E.M. of 5 experiments. Results are presented as if the buffer had not been replaced each hour simply to illustrate the linear nature of consumption with time.

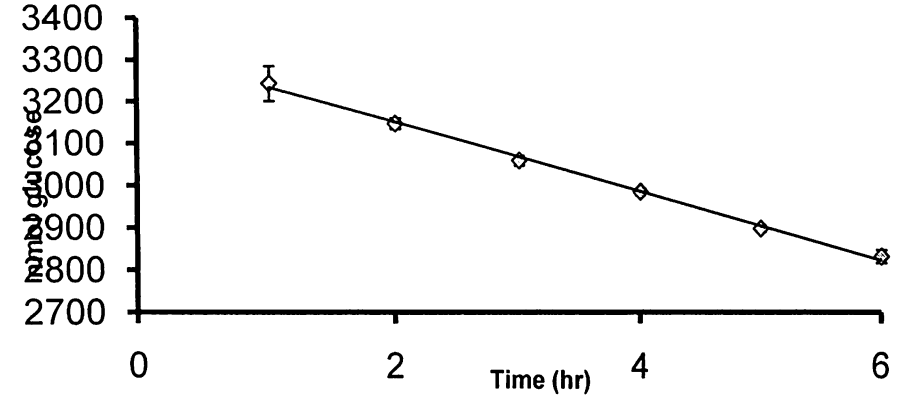

Figure 14 Glucose consumption rate remained steady in apoptotic cells. Cells were incubated in low K^+ buffer which was removed and replaced every hour for five hours. The amount of glucose in each sample was measured using a spectrophotometer. The concentration of glucose at each hour was determined using these values, thus showing a steady decrease in the amount of glucose present in media over five hours. Data are mean ± S.E.M. of 5 experiments. Results are presented as if the buffer had not been replaced each hour simply to illustrate the linear nature of consumption with time.

Table 6 Glucose consumption rate every hour in both healthy and apoptotic CGNs

Time (min)	25K	3.5K
60-120	116.8±12.6	96.3±13.3
120-180	113.7±15.2	87.0±11.6
180-240	97.9±14.0	74.3±7.9
240-300	107.8±8.8	87.4±7.7
300-360	112.8±22.1	66.4±15.5

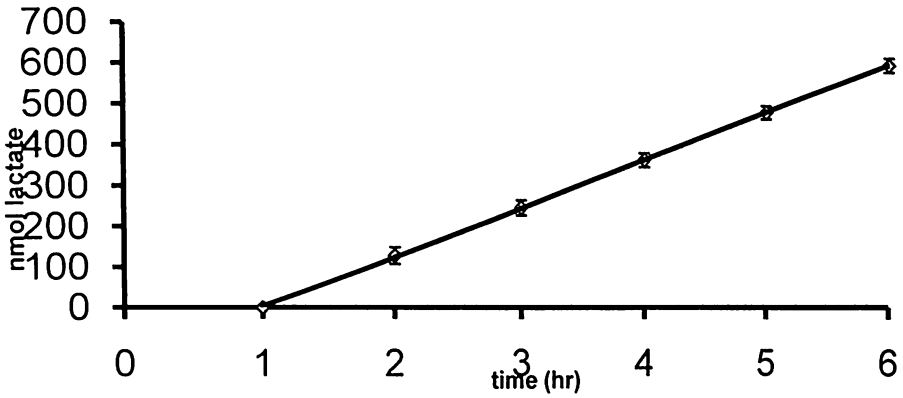

Figure 15 Lactate production rate remained steady in healthy cells. Cells were exposed to high K^+ buffer, which was removed, stored, and replaced every hour for five hours. Assay buffer was added to each sample and the fluorescence was measured in a fluorometer. The concentration of lactate was determined from these raw measurements, thus showing a steady increase in lactate in the extracellular supernatant collected from the chamber containing healthy cells in 25K FBS. Data are mean ± S.E.M. of 5 experiments. Results are presented as if the buffer had not been replaced each hour simply to illustrate the linear nature of consumption with time.

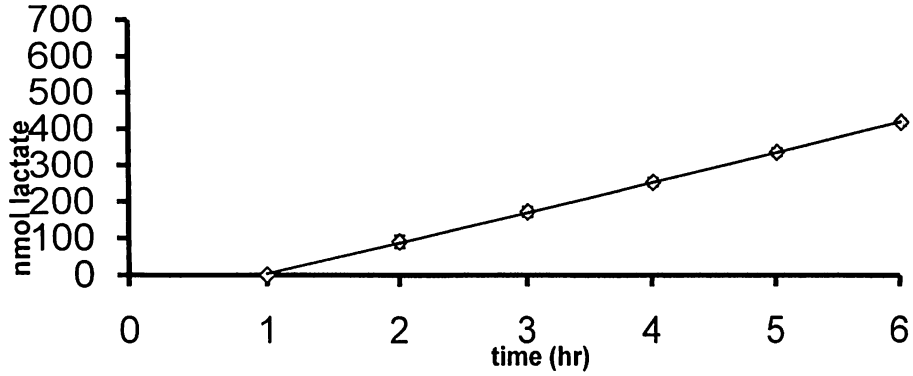

Figure 16 Lactate production rate remained steady in apoptotic cells. Cells were exposed to low K^+ buffer, which was removed, stored, and replaced every hour for five hours. Assay buffer was added to each sample and the fluorescence was measured in a fluorometer. The concentration of lactate was determined from these raw measurements, thus showing a steady increase in lactate in the extracellular supernatant collected from the chamber containing healthy cells in 3.5K BSA. Data are mean ± S.E.M. of 5 experiments. Results are presented as if the buffer had not been replaced each hour simply to illustrate the linear nature of consumption with time.

Table 7 Lactate production rate every hour in healthy and apoptotic CGNs

Time (min)	25K	3.5K
60-120	128.4±20.2	91.6±17.0
120-180	116.8±18.2	82.9±13.5
180-240	116.9±17.1	80.7±11.6
240-300	119.0±16.0	84.4±10.1
300-360	120.3±17.7	81.3±9.1

ATP/ADP ratio remained steady over four hours for healthy and apoptotic neurons

The ATP/ADP ratio in the cell extracts showed little change between the two- and four-hour time points. The luminescence of the standards was used to determine the equation for the relationship between luminescence and concentration of ATP and ADP. The luminescence of the cell extract samples was then used to determine the concentration of ATP and ADP in the high K^+ and low K^+ cell extracts from two and four hours of incubation. These measurements were then used to determine the ATP/ADP ratio in the cell extracts. The average and standard deviation of each data set is summarized in Figure 17.

G6P concentration remained steady over four hours in both healthy and apoptotic cells

The concentration of G6P showed little change between the two- and four-hour cell extracts in both the healthy and apoptotic neurons. The luminescence of the standards was used to determine the equation for the relationship between luminescence and concentration of G6P. The luminescence of the cell extract samples was then used to determine the concentration of G6P in the high K^+ and low K^+ cell extracts from two and four hours of incubation. The average was taken of each set of data and is summarized in Figure 18. There is no significant difference in [G6P] between the two time periods in both healthy and apoptotic neurons.

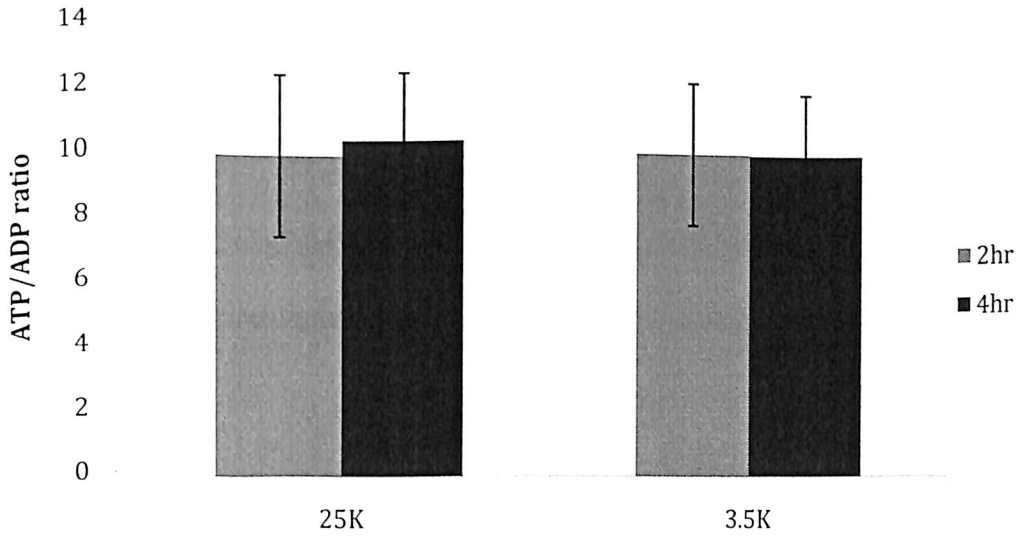

Figure 17 ATP/ADP ratio remained steady in both healthy and apoptotic neurons. The average ATP/ADP ratio of the cell extracts remained steady in neurons exposed for two or four hours to high (25K) or low (3.5K) K^+ buffer. The cell extracts were assayed along with standards in a luminometer. After each assay, the luminescence of the standards was used to determine an equation for calculating the concentration of ATP and ADP in the cell extracts and the ATP/ADP ratio was determined. Data are mean ± S.E.M. of 5 experiments.

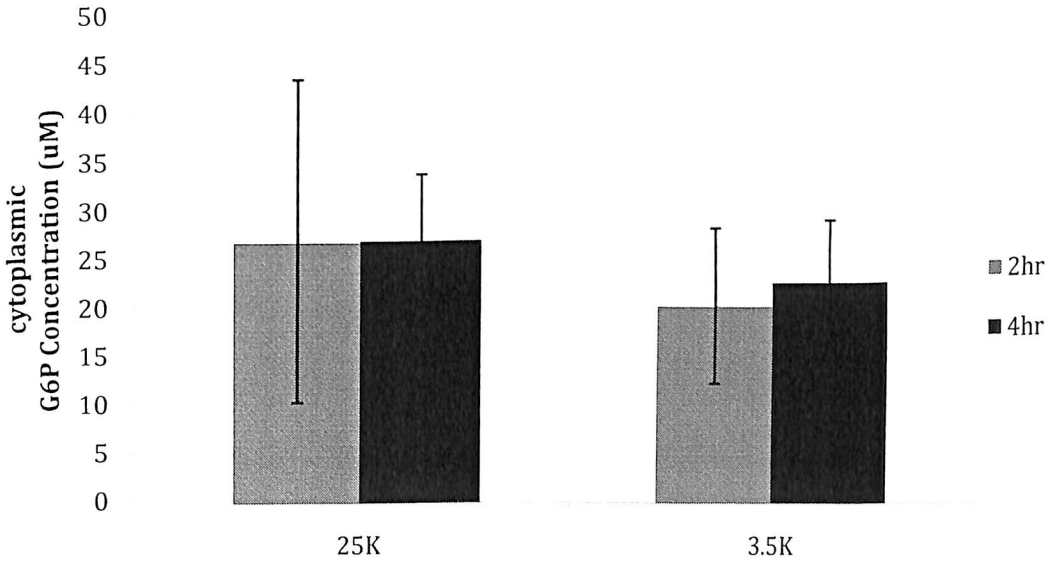

Figure 18 G6P remained steady in both healthy and apoptotic neurons. The average cytoplasmic G6P concentration in the cell extracts remained steady over four hours in both the healthy and apoptotic neurons. Cells were treated with either high K^+ or low K^+ buffer and incubated for either two or four hours. The cell extracts were assayed along with standards in a luminometer. After each assay, the luminescence of the standards was used to determine an equation for calculating the concentration of G6P in the cell extracts. Data are mean ± S.E.M. of 5 experiments. The cytoplasmic concentrations were calculated by dividing the total G6P obtained in each extract by the average cytoplasmic volume of $7x10^6$ neurons, which was previously determined to be 2.90uL and 2.97uL for neurons in 25K and 3.5K buffers, respectively.

Discussion

The goal of this project was to determine if both healthy and apoptotic neurons maintain steady-state metabolism so that carbon flux through all reaction blocks shown in Figure 1 can be calculated. Assays were conducted to determine the rate of consumption of glucose, lactate production rate, ATP/ADP ratio, and concentration of glucose-6-phosphate within the cell. If all of these factors remained steady, it could be concluded that both healthy and apoptotic neurons maintained a metabolic steady state. The results of this project confirm the hypothesis that the neurons do maintain this steady state of metabolism.

CGNs incubated in either high K^+ or low K^+ for five hours with samples taken at each hour were used to determine glucose consumption rate and lactate production rate. The high K^+ cells represented the healthy cells and low K^+ cell represented the apoptotic cells. Samples from each time point and each condition along with parallel control samples from wells containing no cells were assayed for extracellular glucose concentration using a spectrophotometer. The results show a steady decrease in the amount of extracellular glucose for both high K^+ and low K^+ neurons. This indicates that both the healthy and apoptotic neurons maintained a constant rate of glucose consumption for at least 5h. These data support the hypothesis since both healthy and apoptotic neurons exhibit a steady rate of glucose consumption.

Despite the fact that glucose consumption remains constant, it is possible that the fate of glucose within the cell is changing with time. This could occur if the proportion of glucose-6-phosphate consumed by glycolysis and the pentose phosphate pathway changes, while the overall demand for glucose-6-phosphate remains unchanged.

Therefore, the same samples used for the glucose assays were also used to determine lactate production in healthy and apoptotic neurons. Lactate production was measured by measuring the concentration of lactate present in the extracellular media in each sample through the use of fluorometer. Results indicate a steady increase in extracellular lactate in both the high K^+ and low K^+ neurons, indicating that the neurons were able to produce lactate at a steady rate. These date also support the hypothesis that both healthy and apoptotic neurons maintain a steady state metabolism.

CGNs incubated in either high K^+ or low K^+ growth media were incubated for either two to four hours and then were further treated with perchloric acid and neutralized with Tris Base to prepare the cells for the ATP, ADP, and G6P assays. The concentrations of each of these metabolites was measured using a luminometer. If the rates of consumption and/or production of ATP change with time due to changes in fluxes through glycolysis, oxidative phosphorylation, and/or ATP consumer blocks, then the ATP/ADP ratio would be expected to change during the 5h period. Results show that the ATP/ADP ratio does not change over the 5h period in either healthy or apoptotic neurons, which is consistent with the fluxes through these blocks remaining constant. By similar reasoning, if the rates of consumption and/or production of glucose-6-phosphate changes due to changes in fluxes through glucose uptake and phosphorylation, glycolysis, and/or the pentose phosphate pathway, then the concentration of glucose-6-phosphate would be expected to change. The results indicate that the concentration of glucose-6-phosphate is constant in both healthy and apoptotic neurons, which indicates fluxes through these blocks is constant. The constancy of glucose-6-phosphate is also consistent with the measured flux data showing that glycolysis (as measured by lactate production)

and glucose uptake and phosphorylation fluxes (as measured by glucose uptake) are constant. These data indicate that levels of each intermediate in cell metabolism did not change over a four-hour time period since there was little difference between the time points. The steady state of these metabolite levels also supports the hypothesis of this project since both healthy and apoptotic neurons exhibit no change between the two time points.

It should be noted that metabolism in the low K^+ CGNs is lower than that of the high K^+ CGNs. Therefore, this apoptotic stimuli does have an effect on cell metabolism within one hour. This data is consistent with previous results showing decreased mitochondrial respiration in low K^+ cells, indicating that at least one reaction block is directly affected by the apoptotic stimulus. Further research would be needed to determine which specific reactions in the metabolic pathway are directly targeted by apoptotic stimuli, and if this is important in signaling the neuron to undergo apoptosis.

Each set of data from these assays exhibits metabolic steady-state behavior since they show little change over time. These results indicate that both healthy and apoptotic neurons maintain a steady state in cell metabolism. Using this conclusion, further studies can be conducted to determine carbon flux in healthy and apoptotic neurons since metabolic steady state has been established for both conditions. The glucose consumption rate, lactate production rate, and the cell respiration rate can all be measured experimentally to measure carbon flux throughout the cell metabolic pathway. By comparing the measurements of both healthy and apoptotic neurons, it would be possible to detect impairments on cell metabolism in apoptotic neurons.

References

1. Green, D. R. (2007) "Apoptosis." Cells. Eds. Benjamin Lewin, Lynne Cassimeris, Vishwanath R. Lingappa, George Plopper. Jones and Bartlett Publishers, Sudbury, MA. Pgs. 534-557.
2. Jekabsons, M. B. (2009) "A Systems Biology Approach to Investigating Apoptotic Stimuli as Effectors of Cell Metabolism: Practical Application of Top-Down Control Analysis to Attached Neurons." *International Journal of Molecular Sciences*. Vol. 10. Pgs. 702-722.
3. Chatterjee, Jahnavi. (2008). "Development of metabolite assays in order to determine the quantitative contributions of different reactions to the control of energy metabolism flux in neurons." Summer Research Institute for Undergraduates 2008.
4. Jekabsons, M. B., Nicholls, D. G. (2006) "Bioenergetic analysis of cerebellar granule neurons undergoing apoptotsis by potassium/serum deprivation." *Cell Death and Differentiation*. Pgs. 1-16.
5. Chipuk, J.E., Bouchier-Hayes, L., Green, D.R. (2006) "Mitochondrial outer membrane permeabilization during apoptosis: the innocent bystander scenario." *Cell Death and Differentiation*. Pgs. 1396-1402.
6. Thompson, J.E., Thompson, C.B. (2004) "Putting the Rap on Akt." *Journal of Clinical Oncology: Biology of Neoplasia*. Vol. 22. Pgs. 4217-4226.
7. Vila, M., Przedborski, S. (2003) "Targeting Programmed Cell Death in Neurodegenerative Diseases." *Nature Review: Neuroscience*. Vol. 4. Pgs. 1-11.
8. Hammerman, P.S., Fox, C.J., Thompson, C.B. (2004) "Beginnings of a signal-transduction pathway for bioenergetic control of cell survival." Trends in Biochemical Science. Vol. 29. Pgs. 586-592.