This student paper was written as an assignment in the graduate course

Free Radicals in Biology and Medicine

(77:222, Spring 2001)

offered by the

Free Radical and Radiation Biology Program
B-180 Med Labs
The University of Iowa
Iowa City, IA 52242-1181
Spring 2001 Term

Instructors:
GARRY R. BUETTNER, Ph.D.
LARRY W. OBERLEY, Ph.D.

with guest lectures from:
Drs. Freya Q . Schafer, Douglas R. Spitz, and Frederick E. Domann

The Fine Print:

Because this is a paper written by a beginning student as an assignment, there are no guarantees that everything is absolutely correct and accurate.

In view of the possibility of human error or changes in our knowledge due to continued research, neither the author nor The University of Iowa nor any other party who has been involved in the preparation or publication of this work warrants that the information contained herein is in every respect accurate or complete, and they are not responsible for any errors or omissions or for the results obtained from the use of such information. Readers are encouraged to confirm the information contained herein with other sources.

All material contained in this paper is copyright of the author, or the owner of the source that the material was taken from. This work is not intended as a threat to the ownership of said copyrights.

Glucose Metabolism and Cancer Therapy

by

Iman Ahmad

B-180 Medical Laboratories
Free Radical and Radiation Biology Program

The University of Iowa
Iowa City, IA 52242-1181

For 77:222, Spring 2001

3. May 2001

Abbreviations

CAT, catalase
CoQ, coenzyme Q
G-6PDH, glucose-6phosphate dehydrogenase
GR, glutathione reductase
GPx, glutathione peroxidase
GSH, glutathione
MAPK, mitogen activated protein kinase
NAC, N-acetyl cysteine
$NADP^+$, nicotinamide adenine dinucleotide phosphate
$^1O_2$, singlet oxygen
ROS, reactive oxygen species
RNS, reactive nitrogen species
SOD, superoxide dismutase
TCA, tricarboxylic acid

Table of contents

Abstract	2
Introduction	2
Superoxide production in tumor cells	5
Protection mechanism	7
Glucose metabolism	8
Glucose deprivation studies	10
Summary	16
Future study	17
References	21

Abstract

Oxidative stress results when the balance between the production of reactive oxygen species (ROS) overrides the antioxidant capability of the target cell. Cancer cells have low level of main antioxidant enzymes and exhibit a defective respiratory mechanism that leads to increase in steady state levels of hydroperoxide production. In this regard glucose metabolism appears to be an integral component of cellular peroxide detoxification pathways. The current review shows the involvement of ROS in the process of carcinogenesis, and how ROS can be involved in cancer treatment by glucose deprivation. Although the source of the increased prooxidant production is currently unknown, the review presented about glucose deprivation studies on cancer cells led to the proposal that motochondrial hydroperoxide formation causes oxidative stress and cytotoxicity during glucose deprivation.

Introduction

Mammalian cells continuously produce reactive oxygen species (ROS) through different metabolic pathways. ROS are molecules that contain oxygen and have higher reactivity than ground-state molecular oxygen. These species include not only the oxygen radicals (like $O_2^{\bullet-}$, $^{\bullet}OH$, and peroxyl radicals), but also non-radical molecules like

H_2O_2 and $^1O_2$. Superoxide is formed during the reduction of O_2 by the mitochondrial electron transport system [1]. Eukaryotic cells are equipped with an antioxidant system capable of converting ROS to H_2O via different cytosolic enzymes. Oxidative stress results when the balance between the production of ROS overrides the antioxidant capability of the target cell. It is generally thought that low levels of ROS are not harmful to cells and indeed even perform useful functions; high levels of ROS are detrimental through reactions with and modifying cellular protein, lipid, and DNA which results in altered target cell function. The accumulation of oxidative damage has been implicated in both acute and chronic cell injury inducing possible participation in the formation of cancer. Acute oxidative injury may produce selective cell death or sublethal injury, such as mutations, chromosomal aberrations or carcinogenesis [2,3]. In contrast, chronic oxidative injury may lead to a non-lethal modification of normal cellular growth control mechanisms. Cellular oxidative stress may modify intracellular communications, protein kinase activity, membrane structure and function, and gene expression, and result in modulation of cell growth [3]. It has been found that cancer cells demonstrate altered metabolism when compared to normal cells [4-6]. Warburg [4] suggested that cancer was a metabolic disease in which respiration was damaged and anaerobic fermentation was increased resulting in a malignant phenotype. Weber [5-6] suggested that cancer cells exhibit increased rate of pentose phosphate pathway activity that is characterized by an increase in glucose-6-phosphate dehydrogenase. On the other hand, Oberley *et al.* [8] suggested that tumor cells have increased steady state levels of $O_2^{\bullet-}$ and H_2O_2 associated with aberrant respiration, which causes damage and inactivate signaling pathway leading to malignant phenotype. Therefore, it appears that tumor cells have an aberrant respiration, which is compensated by an increase in glycolysis and pentose phosphate

cycle activity as a compensatory mechanism to protect themselves from an increase in the steady state level of H_2O_2 [4-6]. The generation of $O_2^{\bullet-}$ and H_2O_2 within the mitochondria may pose a threat to the cancer cell mitochondria themselves since they are nearly always low in antioxidant enzymes. Glucose metabolism has been shown to be involved with cellular sensitivity to oxidative stress mediated by hydroperoxide, presumably via the formation of pyruvate and NADPH [13]. Since mitochondrial metabolism would be the preferred route of energy production during glucose deprivation, it is reasonable to hypothesize that glucose deprivation would create a metabolic state where $O_2^{\bullet-}$ and H_2O_2 concentrations were increased and peroxide scavenging via pyruvate as well as NADPH dependent reactions was decreased. This would be expected to result in a condition of metabolic oxidative stress characterized by increased levels of pro-oxidant production and increased levels of oxidized glutathione. In this review several areas of literature that point to the involvement of $O_2^{\bullet-}$ and other oxygen species in the process of carcinogenesis and cancer will be reviewed. Data summarizing what is known about the variation in antioxidant enzymatic levels in tumor tissue will be presented. The role of glucose metabolism in cancer protection against increased level of ROS will be discussed. Finally, this study will focus on glucose deprivation studies in different human tumor cells that would be expected to result in increased level of pro-oxidant production as a way of treating cancers, and hypothesizing that mitochondrial H_2O_2 is the prooxidant.

The literature cited would be introduced in order to support the following suppositions upon which this review is based:

1) The decrease in tumor cell antioxidant enzyme activity is an important biochemical difference between tumor and normal cells

2) The loss of naturally occurring antioxidant enzyme activity in tumor cells and reduced rates of respiration due to "damage" to the respiratory mechanism could lead to an increased flux of free-radicals produced from electron transport chain associated phenomena. We hypothesize that tumor cells were thought to compensate for this defect by increasing glycolysis, and pentose phosphate pathway.
3) Glucose deprivation induces cytotoxicity and oxidative stress in a variety of transformed human cell types.
4) Transformed and untransformed human cells are differentially susceptible to glucose deprivation-induced cytotoxicity and oxidative stress.
5) Glucose deprivation-induced cytotoxicity and oxidative stress are dependent upon O_2 metabolism.
6) Mitochondrial H_2O_2 formation causes oxidative stress during glucose deprivation.

Superoxide production in tumor cells

The mitochondrial respiratory chain consists of a series of redox catalysts (pyridine nucleotide, flavoproteins, iron sulfur proteins, ubiquinone and cytochromes) which when assembled constitute the mitochondrial inner membrane. These respiratory carriers are arranged according to their redox potentials. The mitochondria have long been known to be one of the major cellular sources of $O_2^{\bullet-}$. 1-2% of the electrons that traverse the respiratory chain ends up as stray $O_2^{\bullet-}$ ions [1]. Much of the original research into ROS production from mitochondria suggested that the majority of these $O_2^{\bullet-}$ originate from complex III [15-16]. However recent work clearly demonstrates that complex I also plays a major role in the production of $O_2^{\bullet-}$ in mitochondria [17]. In

addition, Ishii *et al.* have suggested based on their work with nematodes that $O_2^{\bullet-}$ may also arise from complex II [18]. Clearly there are a number of potential sources of ROS within the mitochondrial respiratory enzyme chain. Almost, all of these studies have shown that normal mitochondria produces $O_2^{\bullet-}$ by utilizing different electron transport chain inhibitors as, antimycin A, which is known to inhibit the transfer of electrons from cytochrome b to coenzyme Q (CoQ), myxthiazole, which prevents the transfer of electrons from the reduced CoQ to to the FeS (III) (the iron sulfur protein) and then to cytochrome c [19], and rotenone which is specific blocker of NADH-dehydrogenase activity in the mitochondria electron transport chain of site I [21] (Figure 1) [Modified from 21].

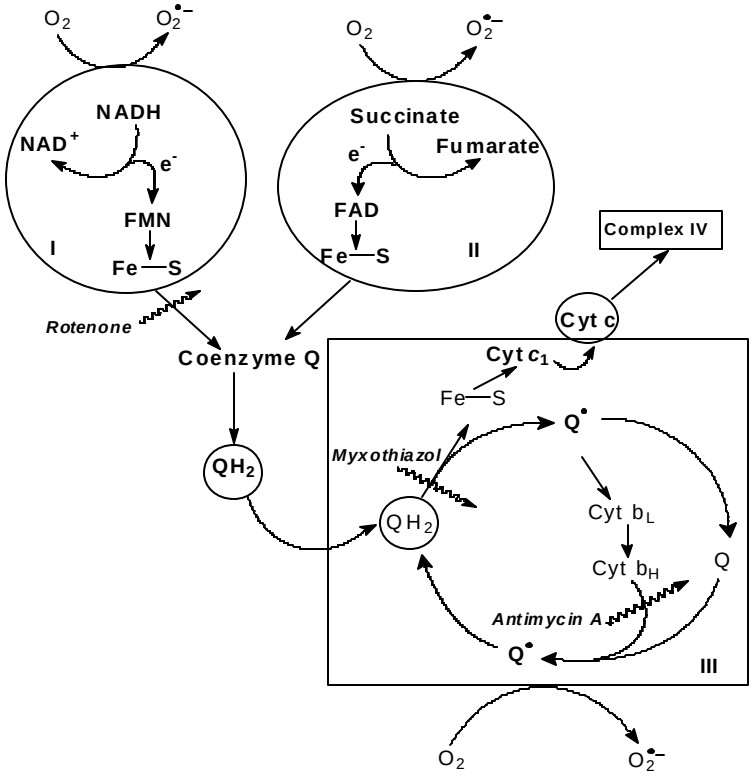


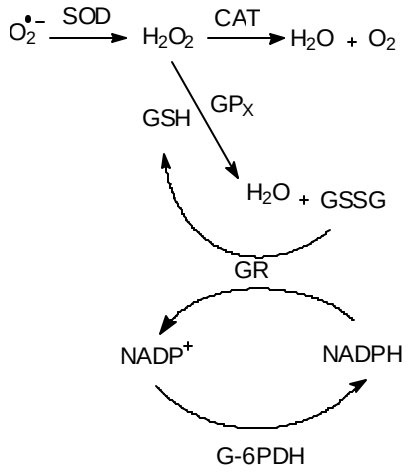
Figure 1. Scheme showing the sites of $O_2^{\bullet-}$ production in the mitochondrial respiratory chain [Modified from 21].

Many studies have been done on the production of $O_2^{\bullet-}$ in tumor cells. Almost all these studies have shown that tumor cells produce $O_2^{\bullet-}$, by utilizing electron transport chain inhibitors or uncouplers in order to increase $O_2^{\bullet-}$ production [22-23]. However, Docampo *et al*. have studied $O_2^{\bullet-}$ production in intact mitochondria from mouse sarcoma 180 cells in the presence of NADH without respiratory chain inhibitors [24]. They found that the tumor did indeed produce $O_2^{\bullet-}$. Dinescu-Romalo and Mihai have performed a study of $O_2^{\bullet-}$ production in several tissues from normal and Guerin T_8 ascites tumor-bearing rats without inhibitors [25]. They found that the tissues from tumor bearing animals produced $O_2^{\bullet-}$ at a much higher rate than the same tissues from non-tumor bearing animals. The results of all of these studies on $O_2^{\bullet-}$ production in tumor cells seems to suggest that tumor cells mitochondria are capable of producing $O_2^{\bullet-}$. The mitochondria of malignant human tumor cells have been shown to exhibit histological pleomorphism when compared to the mitochondria from normal human cells [26]. This pleomorphism is manifested in the abnormal arrangements of the mitochondrial cristae, mitochondrial hypertrophy, and the fragmentation of mitochondria observed with an electron microscope.

Protection mechanism

It is well proven that ROS will be present in high amounts when cells are exposed to any form of stress [33]. These ROS include non-organic molecules, such as $O_2^{\bullet-}$, H_2O_2 and $^{\bullet}OH$, as well as organic molecules such as alkoxyl and peroxyl radicals. ROS are continuously generated during oxidative metabolism. In order to avoid damage caused by ROS, mechanisms exist which remove ROS or prevent the generation of ROS [29-30]. For example, the removal of $O_2^{\bullet-}$ and H_2O_2 prevents the generation of $^{\bullet}OH$, which are formed by the iron-catalyzed Fenton reaction or by Haber-Weiss reaction [31-32], and

are the most reactive species within the ROS family. The mechanism of removal of $O_2^{\bullet-}$ and H_2O_2 is as follow [33]:



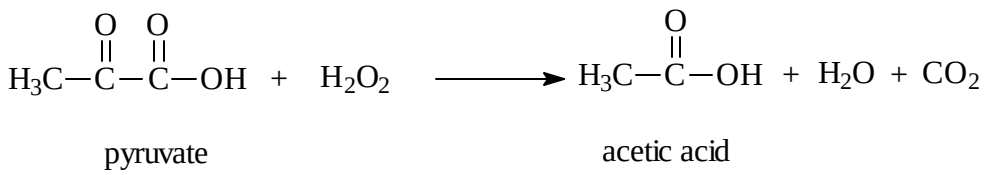
Many studies have shown that cancerous cells have lower MnSOD and catalase activity and that glutathione peroxidase activity is variable in different tumor tissues [11]. How then cancer cells remain viable?

Since oxygen free radicals are produced by mitochondrial membrane bound electron transport chain and mitochondria have been shown to be structurally abnormal in almost all malignant tumor cells and exhibit low level of antioxidant enzymes, it is reasonable to increase their glycolysis and pentose phosphate pathway as a compensatory mechanism to protect themselves via the formation of pyruvate and NADPH. In this regard glucose metabolism appears to be an integral component of cellular peroxide detoxification pathways, which is explained in the following section [13,27-28].

Glucose metabolism

Carbohydrates serve as the primary source of energy in the cell and carbohydrate metabolism is central to all metabolic processes, which can be accomplished either anaerobically or aerobically, resulting in the synthesis of ATP. After the formation of glucose-6-phosphate, the major pathways of glucose metabolism include glycolysis and

pentose phosphate cycle [7,21]. Glycolysis results in the formation of pyruvate, and the pentose phosphate cycle results in the formation of NADPH [7,21]. Pyruvate is an important junction point in glucose metabolism and a substrate for the formation of acetyl-CoA, an entry point to the TCA cycle and mitochondrial oxidative phosphorylation [7,21,34]. As early as 1904, it was shown that H_2O_2 causes a rapid non-enzymatic and stoichiometric decarboxylation of pyruvate to acetic acid, H_2O and CO_2 [35]:



It was recently discovered that mammalian cells secrete pyruvate as an antioxidant defense to O_2 radicals [36], and other hydroperoxides [27]. NADPH, besides being the source of reducing equivalents for the glutathione/ glutathione peroxidase/ glutathione reductase system, has also been shown to participate in the metabolic decomposition of H_2O_2 and organic hydroperoxides [28]. Therefore, in addition to the well known role of glucose metabolism in energy production, it appears to be involved with cellular sensitivity to oxidative stress mediated by hydroperoxides as by products of oxidative metabolism presumably via the formation of pyruvate and NADPH [13]. It has been shown that increasing glucose concentrations in the tissue culture media render CHO cells resistant to H_2O_2-induced cytotoxicity [13]. A schematic diagram showing the role of glucose metabolism as an energy source and detoxifying pathway of H_2O_2 is shown in Figure 2.

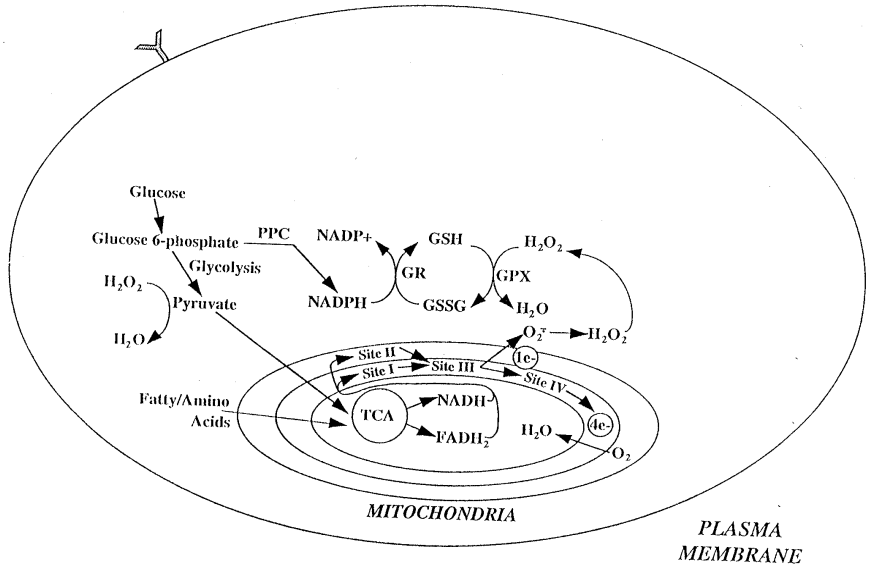


Figure 2. A scheme sowing the role of glucose metabolism as an energy source and detoxifying pathway of H_2O_2. TCA = Tricarboxylic acid cycle; PPC = pentose phosphate cycle; GR = GSSG reductase; GPx = GSH peroxidase [37].

Recently, it has been discovered that simply removing glucose from the cell culture medium (glucose deprivation) induces cytotoxicity and oxidative stress in human tumor cells [37-39]. It also appears that transformed human cell types may be more succeptible to glucose deprivation induced cytotoxicity and oxidative stress, relative to normal human cell types [37,39].

Glucose deprivation studies

Glucose is necessary for survival of cancer cells from pro-oxidants that are produced during respiration. Lee *et al. showed* that glucose deprivation causes cytotoxicity in the MCF-7/ADR human multidrug-resistant breast carcinoma cell line, as shown in Figure 3 [38]

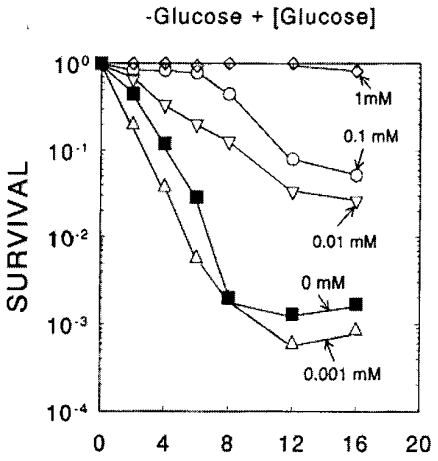


Figure 3. Effect of addition of glucose on glucose deprivation-induced cytotoxicity in MCF-7/ADR cells. Cell monolayers were washed free of glucose, and then placed into media containing the indicated concentrations of glucose. Cell survival after treatment with glucose-free medium (■) or various concentrations (0.001-1 mM) of glucose-supplemented medium for various intervals (up to 16 h) [38].

Also, it was shown that glucose deprivation-induced cytotoxicity was inhibited by adding glucose into the medium, supporting that survival was dependent on the concentration of glucose. Glucose deprivation-induced cytotoxicity in this model system was found to be preceded by activation of MAPK as determined by ERK-2 phosphorylation [38]. Several researchers have shown that ERK-2 is activated *via* phosphorylation of tyrosine and threonine residues [40-41]. Moreover, it has been shown that there is a rapid activation of several other signal transductions pathways such as, Lyn kinase (a src family kinase), c-Jun N-terminal kinase (JNK), MEK, Raf, PKC, and Ras [14,39,45-46]. Furthermore, in glucose-deprived MCF-7/ADR cells, it was shown that there was an increase in the DNA-binding activity of AP-1 transcription factor as well as an increase in the angiogenic factor, basic fibroblast growth factor (bFGF) [14,44]. Finally, the involvement of mitochondrial metabolism in glucose deprivation –induced cytotoxicity might be supported by the protection of these same cell line from glucose deprivation-induced cytotoxicity by the over-expression of the mitochondrial protein, Bcl-2 [44]. These results show that glucose deprivation of these human tumor cell line results in cytotoxicity, activation of signal transduction, and increased gene expression. Metabolic oxidative stress was suggested to

be the mechanism by which glucose deprivation causes cytotoxicity and alterations in signal transduction as evidenced by a significant increase in the steady state level of oxidized glutathione (GSSG) induced by 4 h of glucose deprivation on the same human tumor cell line as shown in Table 1[38].

TABLE I
Total, reduced (GSH), and oxidized (GSSG) glutathione content in 4-h control and glucose-deprived MCF-7/ADR cells

	Total glutathione	GSH	GSSG	Ratio GSH/GSSG
	nmol/mg protein	*nmol/mg protein*	*nmol/mg protein*	
+Glucose	9.1 ± 2.3	6.9 ± 1.9	1.1 ± 0.3	6.3
−Glucose	17.7 ± 3.7[a]	10.9 ± 1.5[a]	3.4 ± 1.8[a]	3.2

[a] Significantly different form +glucose control ($p < 0.05$). Errors represent ±1 S.D. of the mean of three separately collected samples.

Although, there is an increased in GSH synthesis in glucose deprived MCF-7/ADR cells in an attempt to counteract the increased production of intracellular pro-oxidants, but it is clear that the increase in GSH content is not sufficient to counteract the pro-oxidant production as evidenced by the cytotoxicity and accumulation of GSSG. It has been shown previously that ERK-1/ERK-2 were activated by oxidants (such as H_2O_2) and suppressed by reductants [42-43]. This led to the hypothesis that reductants such as NAC could suppress the activation of MAPK induced by glucose deprivation in MCF-7/ADR. This hypothesis was supported by the findings that NAC inhibited the prolonged activation of ERK-2 [38], and the sustained activation of Lyn kinase and JNK [39] (Figure 4).

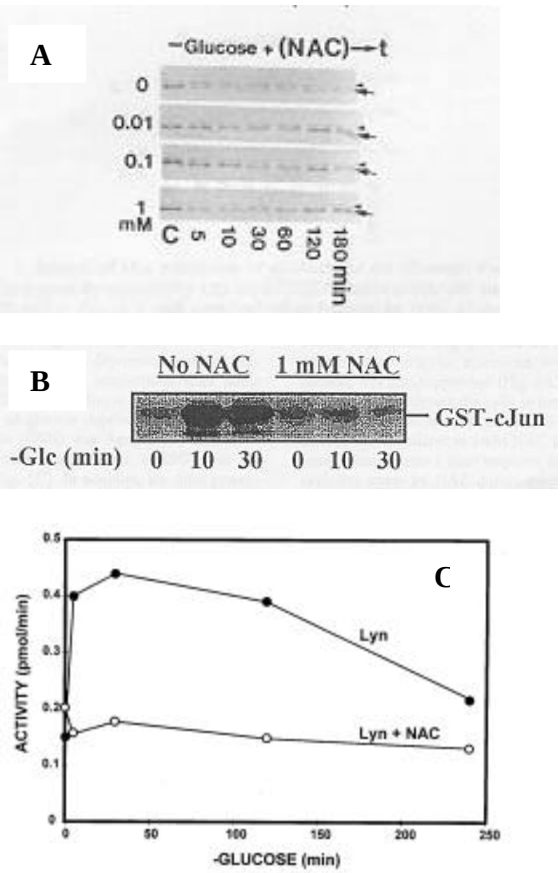


Figure 4. Effect of NAC on ERK-2 (**A**), c-JNK (**B**), and Lyn kinase (**C**) activations in MCF-7/ADR cells. (**A**) ERK-2 activation and deactivation during various concentrations (0.01-1 mM) of NAC-supplemented medium. Control culture (marked C) were untreated. Lysates from equal amounts of protein (30 μg) were separated by SDS-PAGE, transferred onto a nitrocellulose membrane, and processed for immunoblotting with mouse monoclonal antibodies against ERK-2. The activated ERK-2 is marked by the arrowhead and the inactive form is marked by the arrow. (**B**) Cells were incubated in glucose free medium alone or in the presence of 1 mM NAC, cells were lysed at various times, treated with beads coated with GST-c-Jun, and the kinase assay was accomplished. Gel electrophoresis and autoradiography was performed to detect phosphorylation of GST-c-Jun kinases. (**C**) Cells were exposed to glucose free medium alone or in the presence of 1 mM NAC. Lyn was immunoprecipitated from cell lysates, harvested at the various times, and assayed for tyrosine kinase activity using a synthetic peptide as a substrate [38-39].

Moreover, the addition of 1mM of NAC completely protected the cells against glucose deprivation-induced cytotoxicity in MCF-7/ADR cells as shown in Figure 5 [38].

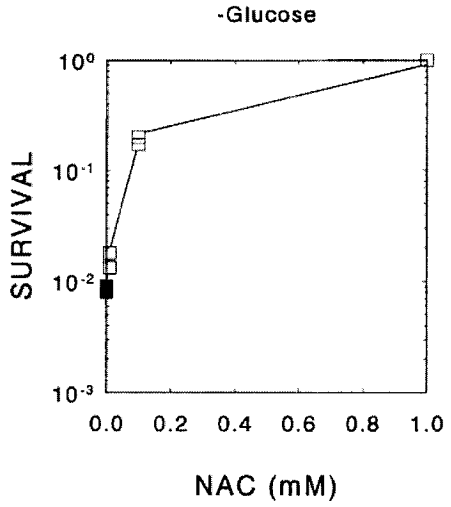


Figure 5. Effect of the addition of NAC on glucose deprivation-induced cytotoxicity in MCF-7/ADR cells. Cell survival after treatment with glucose-free medium (■) or various concentrations (0.01-1 mM) of NAC-supplemented medium for 6 h before and during glucose deprivation [38].

There are several possibilities for how NAC could inhibit oxidative stress during glucose deprivation. One of these possibilities could be by increasing the intracellular cysteine pools, which could directly scavenge radicals as well as provide vital substrates (as cysteine and γ-GC), for increased synthesis of glutathione (Figure 6) [39].

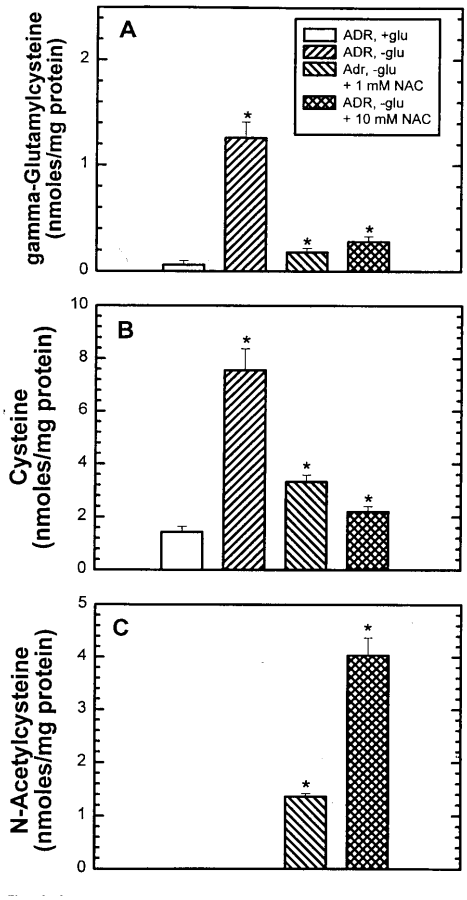


Figure 6. Cysteine, γ-GC, and NAC levels in glucose deprived MCF-7/ADR cells. Lysates from control (+ glu), 4 h glucose deprived (- glu) and glucose deprived cells in the presence of NAC (1 mM and 10 mM) were examined for γ-GC (A), reduced cysteine (B), and NAC (C) content using HPLC. Levels are expressed as nmole/mg protein. Asterisks indicates values are different from control (paired *t*-test, $p < 0.5$). Errors represent ± 1 standard deviation [39].

The major findings of these work is that glucose deprivation induced oxidative stress appears to be account for the cytotoxicity and alterations in signal transduction in MCF-7/ADR cells, and that prooxidant production is the cause for that oxidative stress, which, was measured by using the oxidation-sensitive probe (Table 2) [38]. The increases in mean fluorescence intensity of the probe are interpreted to indicate increases in intracellular pro-oxidant production, and hyroperoxide involvement is inferred by suppressing fluorescence with hyroperoxide scavenging, such as pyruvate [38].

TABLE II
Survival and hydroperoxide production in 2-h glucose-deprived MCF-7/ADR cells supplemented with glutamate and pyruvate

	Survival[a]	Fluorescence intensity[b]
	%	*mean ± S.D.*
+Glucose	100	32 ± 10
−Glucose	20	115 ± 14
−Glucose + 10 mM glutamate	95	39 ± 13
−Glucose + 1 mM pyruvate	80	27 ± 9

[a]Percent survival is normalized to the respective control (+Glu).
[b]Mean F.I. of 25,000 cells analyzed by flow cytometry. Mean F.I. was not different in glucose-deprived and glucose competent cells when labeled with the non-oxidation sensitive dye (C-369) showing that changes in F.I. are indicative of changes in prooxidant production [38].

However, the source of the increased prooxidant production caused by glucose deprivaton is currently unknown, but several observations support intracellular hydroperoxide production by mitochondrial metabolism may be involved [38].

Spitz *et al.* showed that glucose deprivation-induced cytotoxicity and oxidative stress occurs in several human transformed cell lines such as SV40 human transformed fibroblasts (IMR90 SV40, GM00637G), and human colon cancer cells (HT-29), and it is not limited to a single type of transformed human cell type (MCF-7/ADR) (Figure 7) [37].

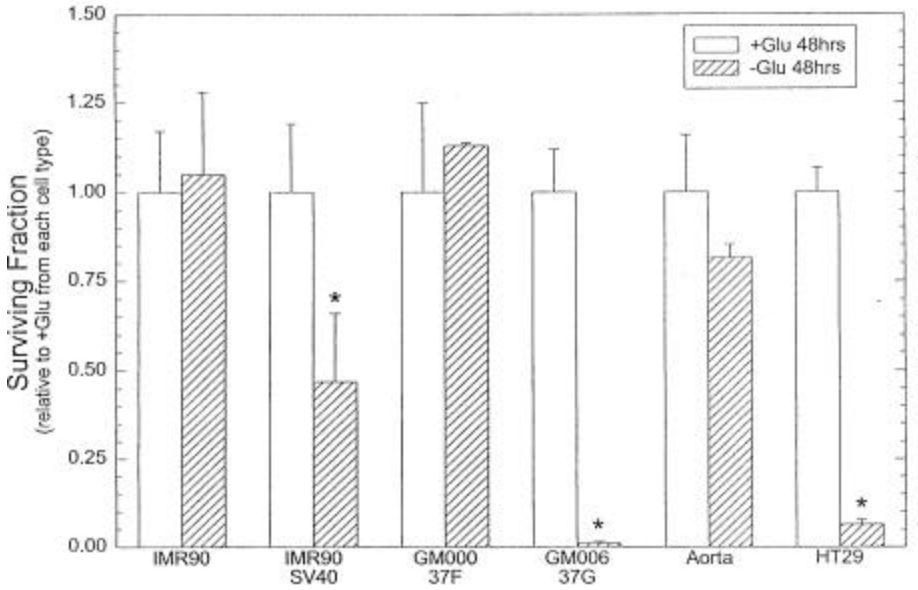


Figure 7. Clonogenic cell survival data showing the differential susceptibility of untransformed (IMR90, GM00037F, Aorta) and transformed (IMR90 SV40, GM00637G, HT29) human cell types to glucose deprivation-induced cytotoxicity. A asterisks indicate the significant differences between +Glu and –Glu from each cell line ($p < 0.05$) [37].

Furthermore, transformed cells appear to be more susceptible to glucose deprivation-induced cytotoxicity and oxidative stress as compared to their normal matched pair cells as shown in Figure 7 [37]. This supports the speculation that cancer cells exhibit a defect in oxidative metabolism, which could be used as a therapeutic advantage when trying to kill cancer cells and spare the normal tissues. Moreover, it was shown that glucose deprivation-induced cytotoxicity in transformed cell line (GM00637G), is dependent upon the metabolism of O_2 [37]. Since most of O_2 metabolism occurs in the mitochondria, this result is consistent with the speculation that a defect in transformed cell's respiratory mechanism may lead to an increase in the (ROS) responsible for glucose deprivation-induced cytotoxicity and oxidative stress.

Summary

All oxygen metabolizing cells are thought to produce a relatively low level of prooxidants, which is thought to be not harmful to cells and indeed even perform useful functions. On the other hand, cancer cells have a defective respiratory mechanism that leads to increase in steady state levels of hydroperoxide production. As a result, metabolism of glucose could be up regulated to produce more pyruvate and NADPH to compensate for this defect. In this regard glucose deprivation can be used as a therapeutic anticancer that will be expected to result in a condition of metabolic oxidative stress characterized by increased levels of prooxidant production. Although, the source of the increased prooxidant production is currently unknown, the review presented above led us to propose that mitochondrial H_2O_2 formation causes oxidative stress during glucose deprivation which is going to be studied by different experiments.

Future studies

During glucose deprivation, steady state levels of intracellular pro-oxidant (presumably H_2O_2) appear to increase immediately in cancer cells [38]. This suggests that H_2O_2 are being produced by ongoing metabolic processes and that the metabolic decomposition of these pro-oxidants is compromised by the removal of glucose, probably *via* a decrease in intracellular NADPH and pyruvate (Figure 2) [37]. It was hypothesized that pro-oxidant production is occurring *via* mitochondrial electron transport chain activity, because in the absence of glucose, mitochondrial metabolism (fatty acids and amino acids) would be the preferred route of energy production [21]. Therefore, it would be expected that glucose deprivation result in a condition of metabolic oxidative stress characterized by increased levels of pro-oxidant production, that leads to increases levels of oxidized glutathione and cytotoxicity [38].

Hypothesis

Mitochondrial H_2O_2 formation causes oxidative stress and cytotoxicity in human tumor cells during glucose deprivation.

To test this hypothesis three main areas will be researched: 1) Mitochondrial involvement in glucose deprivation-induced cytotoxicity and oxidative stress. 2) Pro-oxidant formation causes cytotoxicity and oxidative stress during glucose deprivation. 3) H_2O_2 is the pro-oxidant.

Specific aim #1: Mitochondria is involved in glucose deprivation-induced cytotoxicity and oxidative stress.

Many studies have shown that tumor cells produce $O_2^{\bullet -}$ by utilizing electron transport chain inhibitors in order to increase $O_2^{\bullet -}$ production [22,23]. The results of all of these studies on $O_2^{\bullet -}$ production in tumor cells seem to suggest that tumor cells

mitochondria are capable of producing $O_2^{\bullet -}$. Therefore, using these inhibitors (Antimycin A, Myxthiazole, Rotenone) during glucose deprivation will be expected to accelerate cytotoxicity and oxidative stress in human tumor cells from 48 h to 2 or 4 h, and that antimycin A would be expected to have the greatest effect due to its site of action as mentioned in the background.

To accomplish this goal, the human tumor cell line to be utilized in this study will include HT-29 colon carcinoma. This cell line was chosen based on preliminary results indicating that it demonstrates some degree of sensitivity to glucose deprivation-induced cytotoxicity [37]. This cell line will be deprived of glucose in the presence of the electron transport chain inhibitors for 2 to 4 hs. GSSG content will be used as an index of oxidative stress, and clonogenic cell survival will be used as an indicative of cytotoxicity. We expect more cytotoxicity and more GSSG accumulation with the use of antimycin A during glucose deprivation as compared to other inhibitors. These inhibitors then will show the involvement of mitochondria in glucose deprivation induced-cytotoxicity and oxidative stress.

Specific aim #2: Pro-oxidant production causes cytotoxicity and oxidative stress during glucose deprivation.

Lee *et al*. determined intracellular pro-oxidant production in MCF-7/ADR cells deprived of glucose for 2 h using the oxidative sensitive probe (C-400, 10 μg/ml) and flow cytometry [38]. They showed that mean fluorescent intensity is greater in the glucose deprived cells relative to the glucose competent cells. This probe indicates increases in intracellular pro-oxidant production.

In aim #2, the work done in aim #1 will be repeated using oxidation-sensitive (C-400, 10 μg/ml) and oxidation-insensitive (C-369, 10 μg/ml) fluorescent dyes. The oxidation-

insensitive dye acts as a control for changes in uptake, ester cleavage and efflux, so that any changes in fluorescence seen with the oxidation-sensitive dye will be directly attributed to changes in pro-oxidant production. We expect an increase in the mean fluorescent intensity in the glucose deprived cells relative to the glucose competent cells using oxidative probe and no changes in using insensitive probe; which indicates an increase in the intracellular pro-oxidant production that is responsible for cytotoxicity and oxidative stress seen during glucose deprivation in aim #1; and that this increase will be expected to be higher with the use of antimycin A.

Specific aim #3: H_2O_2 is the pro-oxidant.

The role of H_2O_2 in glucose deprivation-induced cytotoxicity and oxidative stress will be studied. As stated many studies have shown that tumor cells produce $O_2^{\bullet -}$ [22,23], so by using transduction method, $O_2^{\bullet -}$ can be converted into H_2O_2 and also can specifically be removed. Therefore, by using adenovirus-containing cDNA of MnSOD and mitochondrial targeted catalase will indicate the role of H_2O_2 in glucose deprivation-induced cytotoxicity and oxidative stress. The work done in aim #1 will be repeated using only antimycin A and MnSOD alone and in combination with adenovirus catalase with the proper controls. Using AdMnSOD, H_2O_2 formation will increase and expected to result in more oxidative stress and cytotoxicity during glucose deprivation. While catalase cotransfection would be expected to result in a removal of H_2O_2 and enhanced survival. This increase (during catalase cotransfection) and decrease (during MnSOD transfection) in H_2O_2 can be measured using electron paramagnetic resonance in the presence of spin trap DMPO. We expect to see a nice spectrum for DMPO/$^{\bullet}$OH as an indication of H_2O_2 in AdMnSOD transfected cells while a disappearance of that spectrum in catalase cotransfected cells.

However, the inability of AdCAT to rescue cells from killing and oxidative stress during glucose deprivation, suggest the involvement of other pro-oxidant species (RNS) besides the ROS. Nitric oxide is produced during the oxidation of L-arginine to citrulline catalyzed by nitric oxide synthase (NOS). The recent findings of a mitochondrial enzyme (mNOS) in the inner mitochondrial membrane by Giulivi *et al*. [7,20], and by Ghadoufar and Richter [47] supports the idea of a physiological role for $^{\bullet}$NO in mitochondrial respiration. To test the effects of the endogenous $^{\bullet}$NO production on glucose deprivation-induced cytotoxicity and oxidative stress, the HT-29 cells will be supplemented with the inhibitor of NOS N(G)-monomethyl-L-arginine (NMMA) and deprived of glucose in the presence of antimycin A. the experiment then will be analyzed for survival and GSSG content as described before and compare the results with the control group.

References

1. Boveris A, Cadenas E. (1982) Production of superoxide radicals and hydrogen peroxide in mitochondria. In: Oberley LW, ed. *Superoxide Dismutase*. Vol.2. Boca Raton, FL. CRC Press. 15-30.

2. McCord JM, Keele Jr BB, Fridovich I. (1971) An enzyme based theory of oligate anaerobiosis; the physiological function of superoxide dismutase. Proc Natl Acad Sci. USA. **68**:1024-1027.

3. Klaunig JE, Xu Y, Isenberg JS, Bachowski S, Kolaja KL, Jiang J, Stevenson DE, Walborg EF Jr. (1998) The role of oxidative stress in chemical carcinogenesis. **106**:289-295.

4. Warburg, O. (1956) On the origin of cancer cells. *Sci*. **132**:309-314.

5. Weber, G. (1977) Enzymology of cancer cells (first of two parts). *New Eng J Med*. **296**:486-492.

6. Weber, G. (1977) Enzymology of cancer cells (second of two parts). *New Eng J Med*. **296**:541-551.

7. Tatoyan A, Giulivi C. (1998) Purification and characterization of a nitric oxide synthase from rat liver mitochondria. *J Biol Chem.* **273**:11044-11048.

8. Oberley LW, Buettner GR. (1979) Role of superoxide dismutase in cancer: a review. *Cancer Res*. **39**:1141-1149.

9. Oberley LW. (1982) Superoxide dismutase and cancer. In: Oberley LW, ed. *Superoxide Dismutase*. Vol. 2. Boca Raton, FL: CRC Press. 127-165.

10. Oberley LW, Oberley TD. (1986) Free radicals, cancer, and aging. In: Johnson Jr JE, Walford R, Harmon D, Miquel J, eds. *Free radicals, Aging, and Degenerative diseases*. New York: Alan R Liss. 325-371.

11. Oberley TD, Oberley LW. (1993) Oxygen radicals and cancer. In: Yu BP, ed. *Free Radicals in Aging*. Boca Raton, FL: CRC Press. 248-267.

12. Oberley LW, Oberley TD. (1994) Reactive oxygen species in the aetiology of cancer. In: Ionnides C, Lewis DFU, ed. *Drugs, Diet, and Disease.* Vol.1. Hemel Hemp-Stead: Eliis Horwood. 47-63.

13. Averill-Bates, D.A; Przybytkowski, E. (1994) The role of glucose in cellular defenses against cytotoxicity of hydrogen peroxide in Chinese hamster ovary cells. *Arch Biochem Biophys.* **312**: 52-58.

14. Galoforo SS, Berns CM, Erdos G, Corry PM, Lee YJ. (1996) Hypoglycemia-induced AP-1 transcription factor and basic fibroblast growth factor gene expression in multidrug resistant human breast carcinoma MCF-7/ADR cells. *Mol Cell Biochem.* **155**:163-171.

15. Turrens JF, Alexandre A, Lehninger AL. (1985) Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. *Arch Biochem Biophys*. **237**:408-414.

16. Nohl H, Jordan W. (1986) The mitochondrial site of superoxide formation. *Biochem Biophys Res Commun*. **138**:533-539.

17. Robinson BH. (1998) Human complex I defeciency: clinical spectrum and involvement of oxygen free radicals in the pathogenecity of the defect. *Biophys Acta*. **1364**:271-286.

18. Ischii N, Fujii M, Hartman PS, Tsuda M, Yasuda K, Senoo-Matsuda N, Yanase S, Ayusawa D, Suzuki K. (1998) A mutation in succinate dehydrogenase cytochrome b causes oxidative stress and aging in nematods. *Nature*. **394**:694-697.

19. Matsuno-Yagi A, Hatefi Y. (1996) Ubiquinol-cytochrome c oxidoreductase. The redox reactions of the bis-heme cytochrome b in ubiquinone-sufficient and ubiquinone-deficient systems. *J Biol Chem*. **271**:6164-6171.

20. Giulivi C, Poderoso JJ, Boveris A.(1998) Production of nitric oxide by mitochondria. *J Biol Chem*. **273**:11038-11043.

21. Campbell MK. (1999) *Biochemistry*. 3rd ed. Saunders college publishing. pp: 454-481.

22. Bartoli GM, Galeotti T, Azzi A. (1977) Production of superoxide anion and hydrogen peroxide in Ehrlich ascites tumor cell nuclei. *Biochem Biophys Acta*. **497**:622.

23. Peskin AV, Zbarsky IB, Konstantinov AA. (1980) A novel type of superoxide generating system in nuclear membranes from hepatoma 22a ascites cells. *FEBS Lett*. **117**:44.

24. Docampo R, Cruz FS, Bovaris A, Muniz RPA, Esquivel DMS. (1979) Beta-lapachone enhancement of lipid peroxidation and superoxide anion and hydrogen peroxide formation by sarcoma 180 ascites tumor cells. *Biochem Pharm*. **28**:723.

25. Dinescu-Romalo G, Mihai C. (1979) Superoxide anion production and superoxide dismutase activity in several tissues from normal and Guerin T_8 ascites tumor-bearing rats. *Cell Mol Biol*. **25**:101-106.

26. Springer EL.(1980) Comparative study of the cytoplasmic organelles of epithelial cell lines derived from human carcinomas and non-malignant tissues. *Cancer Res*. **40**:803-817.

27. Nath KA, NGO EO, Hebbel RP, Croatt AJ, Zhau B, Nutter LM. (1995) α-ketoacids scavenge H_2O_2 in vitro and in vivo and reduce menadione-induced DNA injury and cytotoxicity. *Am J Physiol*. **268**:C227-C236.

28. Tuttle, S.W., Varnes, M.E., Mitchell, J.B., & Biaglow, J.E. (1992) Sensitivity to chemical oxidants and radiation in CHO cell lines deficient in oxidative pentose cycle activity. *Int J Radiat Onc Biol Phys*. **22**:671-675.

29. Sies H. (1991) *Oxidative Stress: oxidants and antioxidants*. Academic Press, New York.

30. Halliwell B, Gutteridge JMC. (1999) *Free Radicals in Biology and Medicine*. Oxford University Press, New York.

31. Winterbourn CC. (1995) Toxicity of iron and hydrogen peroxide: The Fenton reaction. *Toxicol Lett*. **82/83**:969-974.

32. Wardman P, Candeias LP. (1996) Fenton chemistry: an introduction. *Radiat Res*. **145**:523-531.

33. Oberley LW, Oberley TD. (1997) Role of antioxidant enzyme in the cancer phenotype. In: Clerch LB, Massaro DJ, eds. *Oxygen, Gene Expression, and Cellular Function*. New York: Marcel Dekker, Inc. 279-287.

34. Campbell MK. (1999) *Biochemistry*. 3rd ed. Saunders college publishing. pp: 454-481.

35. Holleman MAF. (1904) Note on the action of oxygenated water on α-ketoacids and 1,2, dicetones. *Recl Trav Chi Pays-Bas Belg*. **23**:169-172.

36. O'Donnell-Torney J, Nathan CF, Lanks K, DeBoer CJ, Harpe JD. (1987) Secretion of pyruvate: an antioxidant defense of mammalian cells. *J Exp Med.* **165**:500-514.

37. Spitz DR, Sim JE, Ridnour LA, Galoforo SS, Lee YJ. (2000) Glucose deprivation-induced oxidative stress in human tumor cells. *Ann NY Acad Sci.* **899**:349-362.

38. Lee YJ, Galoforo SS, Berns CM, Chen JC, Davis BH, Sim JE, Corry PM, Spitz DR. (1998) Glucose deprivation-induced cytotoxicity and alterations in mitogen-activated protein kinase activation are mediated by oxidative stress in multidrug-resistant human breast carcinoma cells. *J Boil Chem.* **273**:5294-5299.

39. Blackburn, RV, Spitz DR, Liu X, Galoforo SS, Sim JE, Ridnour LA, Chen JC, Davis BH, Corry PM, Lee YJ. (1999) Metabolic oxidative stress activates sognal transduction and gene expression during glucose deprivation in human tumor cells. *Free Rad Biol. Med.* **26**:419-430.

40. Gomez N, Cohen P. (1991) Dissection of the protein kinase cascade by which nerve growth factor activates MAP kinases. *Nature.* 353(6340):170-173.

41. Meloche S, Pages G, Pouyssegur J. (1992) Functional expression and growth factor activation of an epitope-tagged p44 mitogen-activated protein kinase, p44mapk. *Mol Biol Cell.* 3(1):63-71.

42. Stevenson MA, Pollock SS, Coleman CN, Calderwood SK. (1994) X-irradiation, phorbol esters and hydrogen peroxide stimulate mitogen-activated protein kinase activity in NIH-3T3 cells through the formation of reactive oxygen intermediates. *Cancer Res.* **54**:12-15.

43. Guyton KZ, Liu Y, Gorospe M, Xu Q, Holbrook NJ. (1996) Activation of mitogen-activated protein kinase by hydrogen peroxide. *J Biol Chem.* **271**:4138-4142.

44. Lee YJ, Galoforo SS, Berns CM, Tong WP, Kim HR, Corry PM. (1997) Glucose deprivation-induced cytotoxicity in drug resistant human breast carcinoma MCF-7/ADR cells: role of C-Myc and bcl-2 in apoptotic cell death. *J Cell Sci.* **111**:681-686.

45. Gupta AK, Lee YJ, Galoforo SS, Berns CM, Martine AA, Corry PM, Guan Kl. (1997) Differential effect of glucose deprivation on MAPK activation in drug sensitive human breast carcinoma MCF-7 and multidrug resistant MCF-7/ADR cells. *Mol Cell Biochem.* **170**:23-30.

46. Liu X, Gupta AK, Corry PM, Lee YJ. (1997) Hypoglycemia induced c-Jun phosphorylation is mediated by c-Jun N-terminal kinase 1 and Lyn kinase in drug-resistant human breast carcinoma MCF-7/ADR cells. *J Biol Chem.* **272**:11690-11693.

47. Ghafourifar P, Richter C. (1997) Nitric oxide synthase activity in mitochondria. *FEBS Lett*. **419**:291-296.