Glucose Deprivation-induced Cytotoxicity and Alterations in Mitogen-activated Protein Kinase Activation Are Mediated by Oxidative Stress in Multidrug-resistant Human Breast Carcinoma Cells*

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We previously observed that glucose deprivation induces cell death in multidrug-resistant human breast carcinoma cells (MCF-7/ADR). As a follow up we wished to test the hypothesis that metabolic oxidative stress was the causative process or at least the link between causative processes behind the cytotoxicity. In the studies described here, we demonstrate that mitogen-activated protein kinase (MAPK) was activated within 3 min of being in glucose-free medium and remained activated for 3 h. Glucose deprivation for 2–4 h also caused oxidative stress as evidenced by a 3-fold greater steady state concentration of oxidized glutathione and a 3-fold increase in pro-oxidant production. Glucose and glutamate treatment rapidly suppressed MAPK activation and rescued cells from cytotoxicity. Glutamate and the peroxide scavenger, pyruvate, rescued the cells from cell killing as well as suppressed pro-oxidant production. In addition the thiol antioxidant, N-acetyl-l-cysteine, rescued cells from glucose deprivation-induced cytotoxicity and suppressed MAPK activation. These results suggest that glucose deprivation-induced cytotoxicity and alterations in MAPK signal transduction are mediated by oxidative stress in MCF-7/ADR. These results also support the speculation that a common mechanism of glucose deprivation-induced cytotoxicity in mammalian cells may involve metabolic oxidative stress.

We have previously observed that glucose deprivation activates mitogen-activated protein kinase (MAPK) and induces cell death in multidrug-resistant human breast carcinoma MCF-7/ADR cells (1, 2). Since activation of MAPK has been suggested to result from oxidative stress (3–6), the hypothesis that glucose deprivation-induced cytotoxicity and alterations in MAPK signal transduction could result from metabolic oxidative stress was tested. Metabolic oxidative stress is defined as oxidative stress generated by mitochondrial biochemical pathways.

MAPK, also known as extracellular regulated protein kinases (ERKs), are serine/threonine kinases (7). Three MAPK homologs have been identified in mammalian cells as follows: ERK1, ERK2, and ERK3 (7). ERK1 and ERK2 are expressed ubiquitously, and their molecular masses are 44 and 42 kDa, respectively. The MAPK pathway is activated by various growth factors (8, 9), alterations in intracellular redox state (3, 6), and polyunsaturated fatty acids (10, 11). MAPK activation appears to involve extracellular signals including oxidants stimulating the formation of active Ras, GTP (12, 13). The GTP-bound Ras protein binds to Raf kinase and initiates a protein kinase cascade that leads to MAPK activation (14, 15). Activated MAPK is believed to translocate into the nucleus (16) and phosphorylate nuclear substrates including redox regulated transcription factors believed to be involved with cellular responses to oxidative stress (e.g. ternary complex factor Elk-1, c-Myc, and c-Fos) (9, 17–21). Therefore, the MAPK pathway appears to be activated in response to oxidative stress and could be involved in cellular responses to oxidative stress.

Glucose metabolism has been shown to be involved with cellular sensitivity to oxidative stress mediated by hydroperoxides (22) presumably via the formation of pyruvate and NADPH. Furthermore, superoxide and hydroperoxides (i.e. H$_2$O$_2$) have been shown to be produced as by-products of mitochondrial respiration (23). 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 superoxide and hydroperoxide 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 report, we demonstrate that glucose deprivation-induced cell death in MCF-7/ADR is accompanied by activation of the MAPK pathway as well as oxidative stress as evidenced by significant increases in intracellular oxidized glutathione content and pro-oxidant production. Glucose or glutamate treatment shortened the duration of MAPK activation and rescued cells from cytotoxicity indicating that cellular metabolism was integrally related to both processes. In addition pyruvate (a known peroxide scavenger) as well as glutamate rescued MCF-7/ADR from glucose deprivation-induced cytotoxicity.

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Glucose Deprivation-induced Oxidative Stress in MCF-7/ADR Cells

Effect of the Addition of Glucose on Glucose Deprivation-induced Cytotoxicity (A) and ERK-2 Activation (B) in MCF-7/ADR Cells. Cell monolayers were washed free of glucose (which took 10 min) and then placed into media containing the indicated concentrations of glucose. Panel A, cell survival after treatment with glucose-free medium (C) or various concentrations (0.001–1 mM) of glucose-supplemented medium for various intervals (up to 16 h). Panel B, ERK-2 activation and deactivation during various treatment times (5–180 min) with various concentrations (0.01–10 mM) of glucose-supplemented medium. Control cultures (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 monoclonal antibodies against ERK-2. The activated ERK-2 is marked by the arrowhead and the inactive form is marked by the arrow.

Immunoassays were performed with Winlist software (Verity Software House, Topsham, ME).

RESULTS

Effect of the Addition of Glucose on Glucose Deprivation-induced Cytotoxicity and ERK-2 Activation—Fig. 1A shows glucose deprivation-induced cytotoxicity in MCF-7/ADR cells. The survival of the cells decreased exponentially to 1.5 × 10⁻³ by 8 h of incubation in glucose-free medium. Fig. 1A also shows that the glucose deprivation-induced cytotoxicity was inhibited by adding glucose into the medium at time 0. Survival was dependent on the concentration of glucose (0.001–1 mM) added. Fig. 1B shows glucose-induced MAPK activation as determined by ERK-2 phosphorylation in MCF-7/ADR cells. Several researchers, including our own group, have shown that ERK-2 is activated via phosphorylation of tyrosine and threonine residues (28, 29), and activated ERK-2 changes its mobility on SDS-PAGE (1, 30). Furthermore, we have shown in the MCF-7/ADR model system that changes in mobility on the SDS-
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**TABLE I**
Total, reduced (GSH), and oxidized (GSSG) glutathione content in 4-h control and glucose-deprived MCF-7/ADR cells

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

* Significantly different from + glucose control (p < 0.05). Errors represent ±1 S.D. of the mean of three separately collected samples.

PAG gel are accompanied by increased activation of MAPK as determined directly by an in-gel kinase assay (1). ERK-2 was activated within 5 min in MCF-7/ADR, and its activation was maintained for 3 h during treatment with glucose-free medium (Fig. 1B). Similar results were observed in ERK-1 by treatment with glucose-free medium (data not shown). Fig. 1B also shows the effect of the addition of glucose on the time course of activation and deactivation of ERK-2. The duration of sustained ERK-2 activation was shortened in glucose-supplemented medium in a dose-response fashion similar to that seen for glucose deprivation-induced cytotoxicity. For example, the activated (phosphorylated) ERK-2 became inactivated (presumably by dephosphorylation) within 30 min by adding 1 mM glucose and by 60 min with 0.1 mM glucose.

Glucose Deprivation of MCF-7/ADR Results in Oxidative Stress as Determined by Oxidized Glutathione Content and Increased Hydroperoxide Production—Data from Table I indicate that oxidative stress is induced by glucose deprivation as evidenced by a disruption in glutathione metabolism. This was determined by measuring the intracellular concentrations of oxidized, reduced, and total glutathione as an index of oxidative stress during glucose deprivation. Table I shows that total and reduced glutathione concentrations were increased after 4 h of glucose deprivation. This suggests that glutathione synthesis was induced by glucose deprivation during the same time frame as cytotoxicity was occurring. Table I also shows a significant increase in the steady state levels of oxidized glutathione (GSSG) induced by 4 h of glucose deprivation. These results are consistent with the hypothesis that glucose deprivation induced intracellular oxidative stress during the same time frame as cytotoxicity and alterations in signal transduction through the MAPK pathway were occurring.

Fig. 2 and Table I show intracellular pro-oxidant production determined in the cells deprived of glucose for 2 h using the oxidation-sensitive probe and flow cytometry. Fig. 2, panels B-D, shows that mean fluorescence intensity is greater in the glucose-deprived cells relative to the glucose-competent cells (compare panels C and D). This increase is roughly equivalent to the increase in fluorescence intensity caused by treatment with 5 μM genuine H₂O₂ (panel B). This probe is not specific and has been shown to be sensitive to H₂O₂ as well as organic hydroperoxides. Therefore, increases in mean fluorescence intensity are interpreted to indicate increases in intracellular pro-oxidant production, and hydroperoxide involvement is inferred by suppressing fluorescence with hydroperoxide scavengers such as pyruvate (31). Table II shows that the increase in fluorescence intensity caused by glucose deprivation is suppressed by pyruvate as is glucose deprivation-induced cytotoxicity; this strongly suggests the involvement of intracellular hydroperoxide production. Taken together the results of the glutathione analysis as well as the hydroperoxide measurements support the hypothesis that glucose deprivation induces an increase in intracellular hydroperoxide production which leads to an increase in oxidized glutathione causing oxidative stress and cytotoxicity during the same time frame that activation of MAPK signal transduction is occurring.

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

| Survival | Fluorescence intensitya |
|----------|-------------------------|
| + Glucose| 100 ± 3.2               |
| − Glucose| 20 ± 1.5                |
| + Glucose + 10 mM glutamate | 95 ± 2.3 |
| + Glucose + 1 mM pyruvate | 80 ± 2.7 |

a Percent survival is normalized to the respective control (+ glucose). Mean F.I. was not different in glucose-deprived and glucose-competent cells when labeled with the non-oxidation sensitive dye (C369) showing that changes in F.I. are indicative of changes in pro-oxidant production. 1—significantly different from + glucose control (p < 0.05).

**Effect of the Addition of N-Acetyl-l-cysteine on Glucose Deprivation-induced Cytotoxicity and ERK-2 Activation—** Previous studies have shown that glucose deprivation promotes the production of free radicals in neuronal cells (32). Free radicals as well as H₂O₂ have been suggested to activate MAPK (3, 4, 6). The hypothesis that oxidative stress induced by glucose deprivation could be responsible for cytotoxicity and alterations in signal transduction in MCF-7/ADR was further tested by adding the free radical scavenging thiol antioxidant N-acetyl-l-cysteine (NAC) to MCF-7/ADR cells during glucose deprivation. Data from Fig. 3A show that NAC protected the cells from glucose deprivation-induced cytotoxicity in a dose-response fashion, and addition of 1 mM NAC completely protected against glucose deprivation-induced cytotoxicity in MCF-7/ADR cells. Fig. 3B shows that prolonged activation of ERK-2 was also inhibited by treatment with NAC. These results indicate that treatment with a thiol antioxidant was capable of inhibiting both the cytotoxicity and the alterations in MAPK signal transduction induced by glucose deprivation in the MCF-7/ADR cells. These results provide strong support for the...
hypothesis that glucose deprivation-induced oxidative stress (seen in Fig. 2 and Tables I and II) was responsible for both the cytotoxicity and alterations in signal transduction seen in the MCF-7/ADR cell line.

The Effect of Glutamine or Glutamate on Glucose Deprivation-induced Cytotoxicity and ERK-2 Activation—To examine if glucose deprivation-induced cytotoxicity could be inhibited by adding glutamine, which is known in some cell types to be interchangeable with glucose in generating ATP, MCF-7/ADR cells were incubated in glucose-free medium for 8 h in the presence of an excess amount of glutamine (0.15–150 mM). Data from Fig. 4A show that there was no significant protection from glucose deprivation-induced killing by adding glutamine. Also, addition of glutamine did not alter the duration of ERK-2 activation (Fig. 4B). In striking contrast, glucose deprivation-induced cytotoxicity was significantly reduced by adding glutamate (Fig. 5A), and 10.5 mM glutamate was capable of completely inhibiting the cytotoxicity associated with exposure to glucose-free medium. In addition, the duration of sustained ERK-2 activation was shortened by addition of glutamate (Fig. 5B). Finally, adding glutamate was also capable of inhibiting glucose deprivation-induced increases in pro-oxidant production (Table II). These results suggest that glutamate, by virtue of being able to enter the tricarboxylic acid cycle through the glutamate dehydrogenase conversion to α-ketoglutarate, can protect against glucose deprivation-induced cytotoxicity and alterations in signal transduction caused by oxidative stress. Although glutamine can be converted into glutamate by the enzyme glutaminase, the contrasting results obtained in Figs. 4 and 5 suggest that MCF-7/ADR cells are deficient in this enzyme activity.

**DISCUSSION**

The major finding of this work is that glucose deprivation-induced oxidative stress appears to account for the cytotoxicity and alterations in MAPK signal transduction which were observed in MCF-7/ADR cells. The source of the increased pro-oxidant production caused by glucose deprivation is currently unknown, but several observations support the hypothesis that intracellular hydroperoxide production (i.e., hydrogen peroxide or organic hydroperoxides) by mitochondrial metabolism may be involved. First, glucose deprivation-induced oxidative stress clearly involves a disruption in the steady state levels of total and reduced glutathione levels in the MCF-7/ADR cells (Table II). This suggests that cells exposed to glucose deprivation are increasing glutathione synthesis in an attempt to counteract the increased production of intracellular pro-oxidant(s). In addition, it is clear that the increase in glutathione content is not sufficient to counteract the pro-oxidant production. This is evident by significant increases in oxidized glutathione and hydroperoxide production during the first 4 h of deprivation and significant cytotoxicity during the same time. Second, the most direct pathway to obtain elevated levels of oxidized glutathione is through the action of glutathione peroxidases. These enzymes metabolize H₂O₂ and organic hydroperoxides ROOH at the expense of reduced glutathione, with two molecules of GSH being consumed and one molecule of GSSG formed for each molecule of hydroperoxide which is decom-
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**Fig. 5.** Effect of the addition of glutamate on glucose deprivation-induced cytotoxicity (A) and ERK-2 activation (B) in MCF-7/ADR cells. Panel A, cell survival after treatment with glucose-free medium (■) or various concentrations (0.15–10.5 mM) of glutamate-supplemented medium (□) for 8 h. Panel B, ERK-2 activation and deactivation during treatment with various concentrations (0.15–15 mM) of glutamate-supplemented medium. Western blot analysis was done as described in Fig. 1. The activated ERK-2 is marked by the arrowhead, and the inactive form is marked by the arrow.

Another major finding of this work is that oxidative stress during glucose deprivation was suppressed by addition of an α-ketoacid (pyruvate) which has previously been known to be an excellent scavenger of hydroperoxides. Glutathione reductase then uses the reducing equivalents in NADPH to reduce GSSG back to GSH. In most mammalian cells exhibiting steady state metabolism of O2, the major source of superoxide and H2O2 is thought to be derived from NADPH to reduce GSSG back to GSH. In most mammalian cells exhibiting steady state metabolism of O2, the major source of superoxide and H2O2 is thought to be derived from NADPH to reduce GSSG back to GSH. Glutathione reductase then uses the reducing equivalents in NADPH to maintain glutathione in the reduced form, and therefore, oxidized glutathione accumulates. This idea was tested by measuring pro-oxidant production with an oxidation-sensitive dye, and glucose deprivation was shown to lead to an increase in pro-oxidant production (Table II). Furthermore, the increase in pro-oxidant production and cytotoxicity during glucose deprivation was suppressed by addition of an α-ketoacid (pyruvate) which has previously been shown to be an excellent scavenger of hydroperoxides.

During any condition of oxidative stress the combination of glutathione peroxidase, glutathione, NADPH, and glutathione reductase represents a major pathway available to cells for reducing hydroperoxide concentration. NADPH production is an integral part of the pentose phosphate pathway and is an integral part of the pentose phosphate dehydrogenase pathway. Since MCF-7/ADR have been shown to have a 3-fold elevation in glycolysis when compared with parental MCF-7 (33, 34) and would be expected to shift to mitochondrial energy production during glucose deprivation, they would be expected to generate substantial amounts of hydroperoxides as a by-product of respiratory activity and use up glucose and NADPH stores rapidly. When the combination of reduced glutathione and NADPH was no longer capable of effectively decomposing hydroperoxides, oxidative stress and cytotoxicity would result from the build-up of intracellular hydroperoxides and oxidized glutathione would accumulate. Our data are therefore completely consistent with the hypothesis that glucose deprivation results in oxidative stress via increased steady state concentrations of hydroperoxides from mitochondrial metabolism. In support of this hypothesis other workers (22) have previously provided convincing evidence demonstrating that glucose deprivation results in dramatic cellular sensitization to the cytotoxic effects of exogenous H2O2 exposure.

The ability of the thiol antioxidant, N-acetylcysteine (NAC), to completely block both the cytotoxicity and alterations in signal transduction induced by glucose deprivation also supports the assumption that glucose deprivation-induced oxidative stress and disruption of thiol metabolism are causally related to the biological effects which were observed. Several possibilities exist for how NAC could be blocking oxidative stress during glucose deprivation. NAC can function directly as an antioxidant to scavenge radicals generated by hydroperoxides undergoing Fenton chemistry. NAC could also increase intracellular cysteine pools which could directly scavenge radicals as well as provide a vital substrate for increased synthesis of glutathione. Finally, by increasing intracellular reduced thiol pools NAC could block oxidative stress-induced changes in signal transduction by maintaining redox-sensitive proteins in their reduced form (35).

The results of this work showing that glutamate was capable of reversing the glucose deprivation-induced oxidative stress and alterations in signal transduction in the MCF-7/ADR cells can also be interpreted in light of the aforementioned hypothesis. It is well known that the energy metabolism of cancer cells is quite different from that of normal cells. Whereas normal cells depend primarily on oxidative phosphorylation to synthesize ATP, in normal steady state conditions, cancer cells demonstrate relatively high rates of glycolysis in the presence of oxygen as well as demonstrating slightly reduced levels of oxidative phosphorylation (36). Moreover, since MCF-7/ADR cells are known to have high rates of glycolysis (33, 34), glucose deprivation would be expected to rapidly impact upon the process of ATP generation as well as NADPH production in these cells due to their rapid utilization of glucose. During glucose deprivation energy generation would be expected to become increasingly dependent upon the tricarboxylic acid cycle, the common central pathway for the degradation of the two-carbon acetyl residues derived not only from carbohydrates but also from fatty acids and amino acids, as well as ATP production by oxidative phosphorylation. As previously stated, if metabolism in the glucose-deprived condition was shifted to primarily respiration, the steady state levels of superoxide and hydroperoxides would be expected to increase. Glutamate could therefore rescue the cells from glucose deprivation by directly entering the tricarboxylic acid cycle leading to increased NADH production and increased ATP levels. In addition excess glutamate would be expected to increase the formation of α-ketoacids such as pyruvate which have been shown to be excellent scavengers of hydroperoxides (31). In fact, our data show that α-ketoacids are capable of suppressing pro-oxidant production as well as cytotoxicity during glucose deprivation (Table II). Glutamate is also required for the first committed step in the synthesis of glutathione catalyzed by γ-glutamylcysteine synthetase. By using these different pathways glutamate could rapidly contribute to increased glutathione synthesis, increased peroxide scavenging, as well as ATP and NADPH production.

Another major finding of this work is that oxidative stress
associated with glucose deprivation appears to be capable of causing alterations in signal transduction through the ERK1/ERK2 MAPK pathways that are also reversed by the thiol antioxidant NAC and glutamate. This supports the speculation that alterations in signal transduction caused by hydroperoxide-induced metabolic oxidative stress could represent one possible mechanism by which signal transduction and gene expression are mechanistically linked to metabolism resulting in a diversity of biological effects, including induction of protective responses as well as possible triggers for the cell death signal. In support of this assumption, oxidative stress has been shown by other investigators to trigger signal transduction pathways (3–6), and recent studies also suggest that reactive oxygen species such as superoxide anion can play a role in the Ras signaling pathway (13, 37). Collectively, our studies show that glucose deprivation induces activation of ERK1/ERK2 (this report), Ras, Lyn kinase, and c-Jun N-terminal kinase (JNK1) cascades (38), all of which could be involved with alterations in gene expression seen during glucose deprivation. The activation of MAPK is clearly associated with oxidative stress during glucose deprivation, and MAPK activation has previously been associated with regulation of glucose transport to supply energy for cell growth and division (14, 39). Our observations therefore suggest that the prolonged activation of MAPK (and the resulting changes in gene expression) caused by metabolic oxidative stress could represent an attempt by the cells to induce protective mechanisms to try and alleviate the cytotoxic effects of glucose deprivation. Likewise, activation of other kinases such as JNK1 could contribute to the induction of cytotoxic responses during glucose deprivation.

Since tumor cells in general have been suggested to have a disruption in intracellular oxidation/reduction reactions as well as glycolytic metabolism (40–42), the current results linking glucose metabolism to MAPK signal transduction as well as cytotoxicity via oxidation/reduction reactions may have far reaching implications to understanding the contribution of metabolism to the phenotypic changes associated with cancer as well as suggesting metabolic targets for the design of novel therapeutic interventions.

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