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Differential effect of glucose deprivation on MAPK activation in drug sensitive human breast carcinoma MCF-7 and multidrug resistant MCF-7/ADR cells

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Abstract

We have investigated the effect of glucose deprivation treatment on the activation of mitogen activated protein kinases (MAPKs) in the drug-sensitive human breast carcinoma cells (MCF-7) and its drug resistant variant (MCF-7/ADR) cells. Western blots and in-gel kinase assays showed that glucose free medium was a strong stimulus for the activation of MAPK in MCF-7/ADR cells. No activation was seen in MCF-7 cells. MAPK was activated within 3 min of being in glucose free medium and it remained activated for over 1 h in MCF-7/ADR cells. After being returned to complete medium, 1 h was required for the MAPK to become deactivated. To investigate whether alternative sources of ATP could inhibit glucose deprivation induced MAPK activation, we added glutamine and glutamate to glucose deprived medium. The addition of glutamine did not reverse glucose deprivation induced MAPK activation in MCF-7/ADR cells. The addition of glutamate, however, decreased the MAPK activation and the length of time of activation. We observed an increase greater than three fold in MEK, Raf, Ras, and PKC activity with glucose deprivation in MCF-7/ADR cells. This suggests that glucose deprivation-induced MAPK activation is mediated through this signal transduction pathway. (Mol Cell Biochem **170:** 23–30, 1997)

Key words: glucose deprivation, MAPK, MCF-7, MCF-7/ADR, signal transduction

Introduction

Angiogenic factor-related genes such as basic fibroblast growth factor (bFGF) contain AP-1 *cis*-acting regulatory elements (TPA response elements; TRE) in their promoter region [1]. These regulatory elements are recognized by AP-1 transcription factors (Jun and Fos family proteins) [2]. The induction of *jun* and *fos* gene transcripts and post-translational modification of their products regulates the activity of AP-1 transcription factor [3]. Environmental stresses such as heat shock exposure [4], UV-irradiation [5], ionizing radiation exposure [6, 7], and treatment with chemical agents [4, 5] have been shown to induce *c-jun* and *c-fos* gene expression in mammalian cells. Recent work from this laboratory has shown that stress induced *bFGF* gene expression is mediated through the activation of protein kinase C (PKC) and AP-1 transcription factors (Jun and Fos) [8, 9].

A fundamental question that remains unanswered is how

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the stresses stimulate the synthesis and/or post-translational modification of Jun and Fos. It is known that phosphorylation of *c-jun* at two serine residues (ser-63 and 73) results in the activation of *c-jun* [10]. Mitogen activated protein kinases (MAPKs) also known as extracellular protein kinases (ERKs) have been proposed to play a role in this phosphorylation [11]. MAPK is also involved in the transcriptional regulation of *c-fos*. The transcription of *c-fos* is mediated by two major *cis*-elements, the serum response element (SRE) and the *c-sis* conditioned medium induction element (SIE). The SIE interacts with activated Stat3, a member of the STAT (signal transducer and activator of transcription) family of transcription factors [12]. MAPK has been shown to be involved in the activation of STAT [13]. The SRE is recognized and stably found by a homodimer of serum response factor (SRF) [14]. The binary SRE-SRF complex interacts with another factor, the ternary complex factor (TCF) [15]. The activity of TCF is rapidly increased in response to cell stimulation with agents such as growth factors which led to MAPK activation. MAPK appears to be responsible for the phosphorylation of TCF [16] and subsequently its transcriptional activity [17]. Thus, in an effort to elucidate how environment stress induces angiogenesis, we propose that environmental stress activates MAPK.

We have previously shown that glucose deprivation activates the expression of *c-jun* and *c-fos* genes which results in an increase in AP-1 activity and subsequent bFGF gene expression in the drug-resistance human breast carcinoma MCF-7/ADR cells [9]. We hypothesized that the induction of these gene expressions by glucose deprivation was mediated through the activation of MAPK. To test our hypothesis, we used MCF-7/ADR cells and compared them to the drug sensitive human breast carcinoma MCF-7 cells. We observed differences in cellular response between these two cell lines with glucose deprivation. Whereas glucose deprivation in MCF-7/ADR cells resulted in cytotoxicity, little/no effect was observed in MCF-7 cells (unpublished data). Furthermore, we have previously shown that radiation induced expression of *c-jun*, *c-fos*, and *bFGF* genes is more prominent in MCF-7/ADR cells than in MCF-7 cells [8]. In this study, we observed MAPK activation with glucose deprivation in MCF-7/ADR cells but not in MCF-7 cells. We also observed an increase in MEK, Raf, Ras, and PKC activity in MCF-7/ADR cells with glucose deprivation. It is hypothesized that the activation of this signal transduction pathway is responsible for the cytotoxicity in MCF-7/ADR cells with glucose deprivation. At a time when intracellular ATP levels are depleted, the cell is signalling for increased protein synthesis leading to an incompatible signal transduction and cell death. Certainly, further work is needed to elucidate the exact mechanism by which this is mediated; in this paper, however, we present the involvement of MAPK. This model will also provide a framework for future studies.

Materials and methods

Cell culture

Human breast carcinoma cells (MCF-7) and its multidrug-resistant subline (MCF-7/ADR) were elucidated in McCoy's 5a medium (Cellgro) with 10% iron-supplemented bovine calf serum (HyClone Laboratories, Logan, UT) and 26 mM sodium bicarbonate for monolayer cell culture. Two or three days prior to the experiment, cells were plated into 35 mm petri dishes, T-150 flasks, or T-75 flasks. The petri dishes/flasks containing cells were kept in a 37°C humidified incubator with a mixture of 95% air and 5% CO_2.

Glucose deprivation treatment

Cells were rinsed three times with Hanks' balanced salt solution (HBSS). Cells were treated with glucose-free McCoy's 5A medium (Gibco BRL, Gaithersburg, MD) for the specified time intervals.

Drug treatment

Glutamine and glutamate were obtained from Sigma Chemical Co (St. Louis, MO). Drug treatment was accomplished by aspirating the medium and replacing it with medium containing the drug. The drug treatment was terminated by aspiration of the medium and rinsing the cells 3 times with HBSS.

Polyacrylamide gel electrophoresis

Samples were mixed with 2 × Laemmli buffer (1 × buffer: 2.4 M glycerol; 0.14 M Tris, pH 6.8; 0.21 M sodium dodecyl sulfate (SDS); 0.3 mM bromophenol blue), and boiled for 5–15 min. Protein content was measured with BCA protein assay reagent (Pierce, Rockford, IL). The samples were diluted with 1 × lysis buffer containing 1.28 M β-mercaptoethanol and an equal amount of protein (30 μg) was applied to a one dimensional PAGE. Electrophoresis was carried out on 10–18% linear gradient SDS-polyacrylamide gels [18].

Western blot

Proteins that were separated by SDS-PAGE were transferred onto a nitrocellulose membrane by electroblotting. The transfer was performed at a current of 0.12 A at 30 V overnight. The membrane was incubated in blocking solution (3% bovine albumin; 20 mM Tris, pH 7.5; 400 mM NaCl; 0.05% Tween-20) for 1 h at 37°C, washed, and then incubated with the MAPK monoclonal antibody (Gibco BRL, 1:500 dilution). The primary antibodies were diluted with diluting so-

lution (1% bovine albumin; 20 mM Tris, pH 7.5; 400 mM NaCl; 0.05% Tween-20). After incubation with the primary antibody, the membrane was washed 3 times for 5 min each in TBST (20 mM Tris, pH 7.5; 400 mM NaCl; 0.05% Tween-20), and incubated with alkaline phosphatase-conjugated rabbit anti-mouse IgG in the diluting solution at 1:3000 for 1–2 h. The membrane was then stained using nitro blue tetrazolium (NBT) and 5′-bromo-4-chloro-3-indolylphosphate (BCIP) solution.

In-gel kinase assay

A 10% acrylamide SDS-PAGE gel was made with 0.1 mg/ml myelin basic protein embedded within. The samples were prepared as for the polyacrylamide gel electrophoresis detailed above, loaded and electrophoresed [19]. The gel was washed with 50 mM Hepes, pH 7.4; 5 mM 2-mercaptoethanol; and 20% isopropanol for 1 h at room temperature 2 times. The next wash was with 50 mM Hepes, pH 7.4 and 5 mM 2-mercaptoethanol for 1 h at room temperature. This was followed by a wash in 50 mM Hepes, pH 7.4; 5 mM 2-mercaptoethanol; and 6 M guanidine chloride for 1 h at room temperature repeated once. The gel was then washed with 50 mM Hepes, pH 7.4; 5 mM 2-mercaptoethanol; and 6 M guanidine chloride for 1 h at room temperature repeated once. The gel was then washed with 50 mM Hepes, pH 7.4; 5 mM 2-mercaptoethanol; and 0.04% tween-20 for 16 h at 4°C with two changes of solution. The final wash was carried out in 25 mM Hepes, pH 7.4; 5 mM 2-mercaptoethanol; 10 mM $MgCl_2$; and 90 μM sodium vanadate for 30 min at 30°C. Phosphorylation of the gel was carried out in 25 mM Hepes, pH 7.4; 5 mM 2-mercaptoethanol; 10 mM $MgCl_2$; 90 μM sodium vanadate; 50 μM ATP; and 150–250 μCi ^{32}P-ATP for 30 min at 30°C. The gel was washed with 5% TCA and 10 mM sodium pyrophosphate until the background was low. The gel was dried and autoradiographed.

MEK activity assay

Cells were washed twice with ice cold PBS and harvested in IP buffer (25 mM Hepes, pH 7.5; 0.2 mM PMSF; 0.05% 2-mercaptoethanol; 1% triton; 2 mM sodium vanadate; 50 mM NaF; proteinase inhibitors of leupeptin and chymostatin; and the trypsin inhibitor aprotinin). The cells were sonicated and the lysate centrifuged for 10 min at 10,000 rpm in a microfuge. The supernatant (1 mg protein) was incubated with 2 μl of anti-MEK antibody (K.L. Guan, University of Michigan) for 2 h at 4°C. 40 μl of protein A agarose (Pierce) was added in the immunoprecipitation reaction and incubated at 4°C for an additional 1 h. The samples were centrifuged for 2 min at 4,000 rpm and washed with IP buffer (minus proteinase inhibitors) four times. The immunoprecipitates were washed once with kinase buffer (18 mM Hepes, pH 7.5; 10 mm magnesium acetate). The immunoprecipitated MEK was incubated in 20 μl of kinase buffer with 50 mM ATP and 0.3 μg of purified ERK1 for 30 min at 30°C with gentle shaking. The myelin basic protein (MBP) kinase assay of the activated ERK1 was performed in 40 μl kinase buffer containing 1.5 μCi 32P-γ-ATP and 20 μg of MBP for 20–30 min at 30°C. 20 μl of the sample was spotted onto a p81 phosphocellulose paper and washed 4 times with 80 mM phosphate solution followed by one wash with 95% ethanol. The amount of P^{32} incorporated in MBP was determined by liquid scintillation counting.

Raf activity assay

The samples were immunoprecipitated as for MEK assay except that 2 μg of anti-Raf antibody (Santa Cruz) was used. The immunoprecipitated Raf was incubated in 20 μl of kinase buffer with 50 mM ATP and 0.3 μg of purified GST-MEK1 (K.L. Guan, University of Michigan) for 30 min at 30°C with gentle shaking. The activated GST-MEK1 was used to activate 0.3 μg of purified ERK1 in 20 μl of kinase buffer with 50 mM ATP for 10 min at 30°C with gentle shaking. The MBP kinase assay of the activated ERK1 was performed as in the MEK assay.

Ras activity assay

Cells were grown in 60 mm petri dishes and washed with phosphate buffered saline (PBS). These cells were then incubated in phosphate free McCoy's containing dialyzed serum for 45 min. The medium was replaced by phosphate free McCoy's containing 0.25 mCi ^{32}P-orthophosphoric acid per 1 ml and incubated for an additonal 3 h. The cells were then washed twice with warm Hank's solution, incubated in glucose free medium for 5 min, and washed twice with cold PBS. Lysis of the cells was performed in 500 μl of lysis buffer (50 mM Tris buffer, pH 7.4; 20 mM $MgCl_2$; 150 mM NaCl; 1% NP-40; 2 mM PMSF; 10 μg/ml leupeptin; 10 μg/ml aprotinin; 1 μg/ml pepstatin A; 2 mM vanadate) containing 20 μg of monoclonal rat-anti-Ras antibody (Oncogene Science, Cambridge, MA). Nuclei were removed by centrifugation. The supernatant was incubated at 4°C for 2 h with mixing on rotating wheel. 50 μl of Protein G Agarose (Oncogene Science, Cambridge, MA) linked to goat anti rat-IgG (Oncogene Science, Cambridge, MA) was added and the mixture further incubated for 1 h. The beads were washed four times with lysis buffer and once with PBS. The pellet was resuspended in 15 μl of 0.75 M KH_2PO_4, pH 3.4 and boiled for 5 min. Separation of the GTP and GDP Ras was on PEI-cellulose

TLC plates in 0.75 M KH_2PO_4, pH 3.4. Autoradiography was then performed.

PKC activity assay

Amersham's PKC enzyme assay system was used to determine the PKC activity in each sample. Cells were mixed with extraction buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 10 mM EGTA, 0.3% w/v β-mercaptoethanol, 50 μg/ml PMSF, 1 μg/ml leupeptin), sonicated, and then centrifuged. Supernatant was saved and mixed with glycerol for storage. Protein content was determined by the Bradford method [20]. The reaction buffer (calcium buffer, lipid, peptide buffer, and DTT buffer) was added to the sample (50–100 μg of protein). The reaction was started by adding γ^{32}P-ATP and continued for 15 min at 25°C. The reaction was terminated by adding the stop reagent. One hundred μl of the reaction mixture was blotted onto cellulose filter paper. The filter paper was rinsed with acetic acid three times and then air dried. The filter paper was counted in a scintillation counter.

Results

Glucose deprivation-induced MAPK activation

Mitogen activated protein kinases (MAPKs) also known as extracellular regulated protein kinases (ERKs) are modulated via phosphorylation and dephosphorylation at tyrosine and threonine residues [21]. Activation of MAPK, thus, changes the mobility of the protein on SDS-PAGE and on Western blot this can be visualized with the activated form having a MW of 44 kDa and the inactive form having a MW of 42 kDa [22]. Two distinct MAPKs (ERK1 and ERK2) have been isolated and amino acid sequencing has shown that they are 90% homologous [23]. The response to stimuli for both is similar and in this paper we report results specifically for ERK2. The Western blot in Fig. 1 depicts MAPK activation with different medium conditions in MCF-7 and MCF-7/ADR cells. We observed little or no activation of MAPK in MCF-7 cells (Fig. 1A). In MCF-7/ADR cells, however, a low level of activation can be seen with dialyzed serum and full serum (Fig. 1B, lanes 3 and 4). Glucose free medium alone caused a strong activation of MAPK (Fig. 1B, lane 5). Full medium by itself resulted in little or no activation (Fig. 1B, lane 6). Both glucose free medium containing dialyzed serum (Fig. 1B, lane 7) and full medium containing full serum (Fig. 1B, lane 8) caused an activation of MAPK. Figure 2 shows an in-gel MAPK assay with various medium conditions in MCF-7 and MCF-7/ADR cells. MCF-7 cells again show little or no activation of MAPK (Fig. 2A). The MCF-7/ADR cells show strong activation with glucose free medium (Fig. 2B, lane 2) and glucose free medium containing dialyzed serum (Fig. 2B, lane 4). Full medium alone (Fig. 2B, lane 3) did not cause activation and full medium containing full serum (Fig. 2B, lane 5) caused small activation. There is somewhat of a discrepancy in the data with full medium containing full serum between the Western blot (Fig. 1B, lane 8) and the in-gel MAPK assay (Fig. 2B, lane 5) to the level of MAPK activation. Further work is needed to clarify this issue.

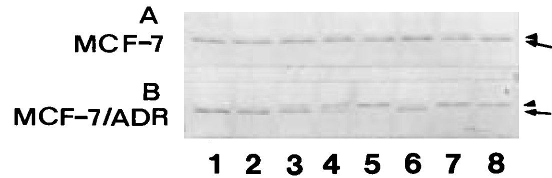

Fig. 1. MAPK activation in MCF-7 and MCF-7/ADR cells with various medium conditions. Lysates from an equal amount of protein (30 μg) were separated by SDS-PAGE, transferred onto nitrocellulose membrane and processed for immunoblotting with an anti-ERK2 antibody. Lane 1 is control and contains extracts from cells that were washed with HBSS three times. All of the other lanes contain extracts from cells that were washed 3 times with HBSS and then incubated for 10 min in HBSS (lane 2), HBSS containing dialyzed serum (lane 3), HBSS containing full serum (lane 4), glucose free medium only (lane 5), full medium only (lane 6), glucose free medium containing dialyzed serum (lane 7), and full medium containing full serum (lane 8). The activated MAPK is marked by the arrow head and the inactive one is marked by the arrow.

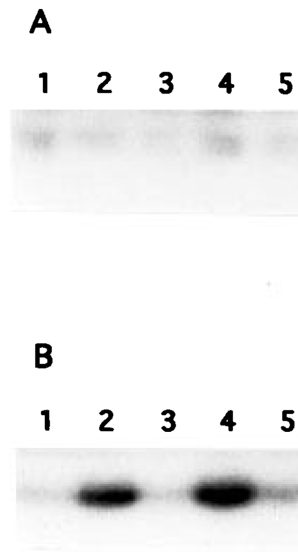

Fig. 2. In-gel MAP Kinase assay of MCF-7 and MCF-7/ADR cells with various medium conditions. The in-gel kinase assay was carried out as described in materials and methods of MCF-7 (panel A) and MCF-7/ADR (panel B) cells after having been washed with HBSS 3X and incubated for 10 min in HBSS containing full serum (lane 1), glucose free medium only (lane 2), full medium (lane 3), glucose free medium containing dialyzed serum (lane 4), and full medium containing full serum (lane 5).

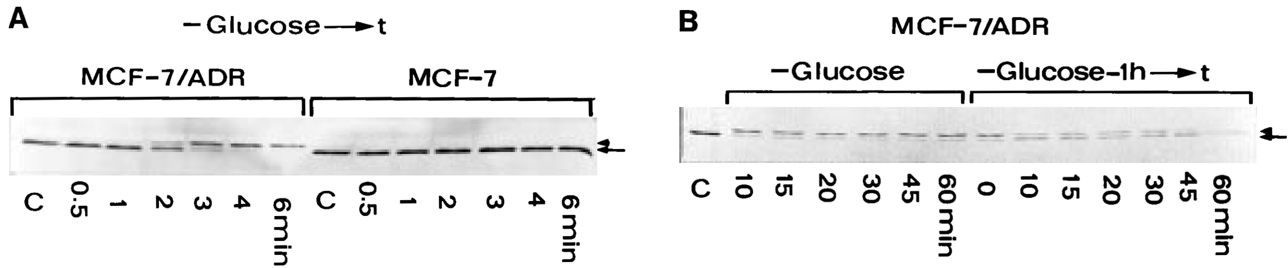

Fig. 3. Time course of glucose deprivation-induced MAPK activation and deactivation. Panel A: MCF-7 and MCF-7/ADR cells were washed with HBSS three times and incubated in glucose free medium for the intervals (0.5, 1, 2, 3, 4, and 6 min) indicated at the bottom of each lane; Panel B: -Glucose: MCF-7/ADR cells were washed 3 × with HBSS and incubated in glucose free medium for the intervals (10, 15, 20, 30, 45, and 60 min) indicated at the bottom of each lane. Glucose 1 h –>t: MCF-7/ADR cells were washed 3× with HBSS and incubated in glucose free medium for 1 h. The medium was then replaced with full medium for the intervals (10, 15, 20, 30, 45, and 60 min) indicated at the bottom of each lane. Western blot analysis was done as described in Fig. 1.

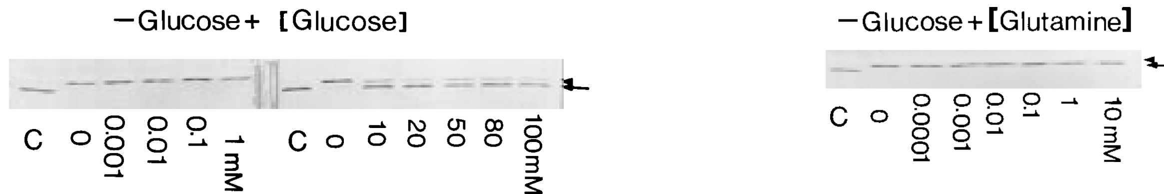

Fig. 4. The effect of the concentration of glucose on MAPK activation. Glucose free medium was supplemented with glucose to make the concentrations (0.001, 0.01, 0.1, 1, 10, 20, 50, 80, and 100 mM) indicated at the bottom of each lane. MCF-7/ADR cells were washed with HBSS 3 × and incubated in their respective medium for 10 min each. Western blot analysis was done as described in Fig. 1.

Fig. 5. The effect of the addition of Glutamine on glucose deprivation-induced MAPK activation. Glucose free medium was supplemented with glutamine to make the concentrations (0.0001, 0.001, 0.01, 0.1, 1, and 10 mM) indicated at the bottom of each lane. MCF-7/ADR cells were washed with HBSS 3× and incubated in their respective medium for 10 min. Western blot analysis was done as described in Fig. 1.

Time course for activation and deactivation

The time course for activation is shown in Fig. 3A. MCF-7/ ADR cells showed MAPK activation in 3 min after being in glucose free medium. This activation was maintained for more than 1 h if still incubated in glucose free medium (Fig. 3B). Once removed from glucose free medium and replaced with full medium, deactivation of MAPK started to occur immediately but took upto 1 h to completely return to the normal deactivated state (Fig. 3B).

Effect of glucose, glutamine and glutamate on MAPK activation

The concentration of glucose in full medium is 17.7 mM. We investigated the effect of various concentrations of glucose on MAPK activation. In Fig. 4, it can be seen that glucose concentrations less than 10 mM resulted in strong MAPK activation. Since glutamine is known to be a replacement for glucose as a source of ATP, we added it to glucose deprived medium to see if we could reverse glucose deprivation induced MAPK activation. As seen in Fig. 5, no effect of glutamine was noted at concentrations upto 10 mM. We similarly looked at glutamate except we also did a time

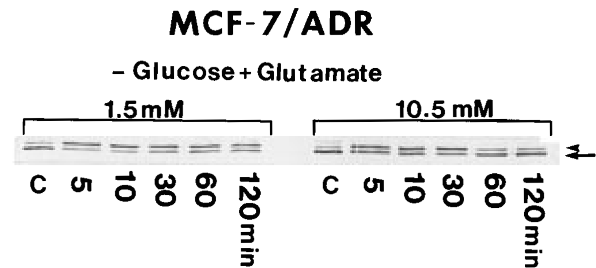

Fig. 6. The effect of the addition of Glutamate on glucose deprivation-induced MAPK activation. Glucose free medium was supplemented with glutamate to make the concentrations 1.5 and 10.5 mM as indicated at the top of the sections. MCF-7/ADR cells were washed with HBSS 3 × and incubated in this medium for 5, 10, 30, 60, or 120 min as indicated at the bottom of each lane. Western blot analysis was done as described in Fig. 1.

course experiment (Fig. 6). At concentrations of 10.5 mM, there is still activation of MAPK but there is a large amount that is inactive and it appears to become inactive at a shorter time interval.

Glucose deprivation-induced MEK, Raf, Ras, and PKC activity

We wanted to further study the pathway by which glucose deprivation causes MAPK activation. We, therefore, investigated some of the upstream elements. Figure 7 shows the

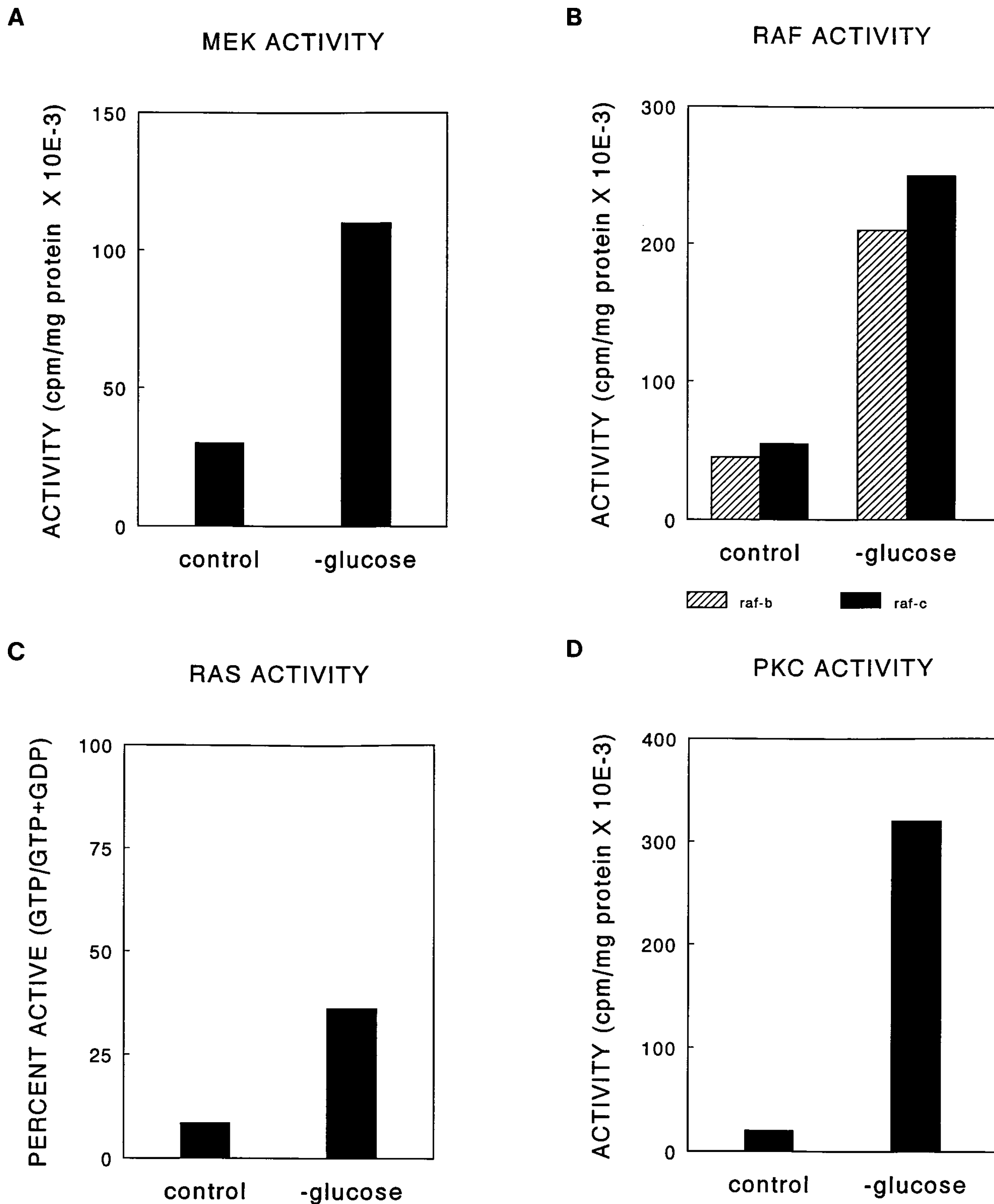

Fig. 7. MEK, Raf, Ras and PKC activity in MCF-7/ADR cells. MEK (panel A), Raf (panel B), Ras (panel C), and PKC (panel D) activity were measured as described under materials and methods in MCF-7/ADR cells under normal conditions (control) and after being in glucose free medium for 4–5 min (-glucose) as labelled at the bottom of each graph.

changes that occur in MEK, Raf, Ras and PKC activity after being in glucose free medium for 4–5 min. MEK activity increased by 3 fold (Fig. 7A). Both Raf-b and Raf-c were looked at and their activity increased by 4 fold (Fig. 7B). The Ras activity was obtained from densitometric analysis of the autoradiograph of the TLC separated samples (data not shown). The percent active Ras increased 4.5 times (Fig. 7C). Similarly, a 14 fold increase in PKC activity was also seen (Fig. 7D).

Discussion

In this study, we have observed that mitogen activated protein kinases (MAPKs) are activated in response to glucose deprivation in drug resistant human breast carcinoma (MCF-7/ADR) cells. This activation occurred in 3 min after being in glucose free medium (Fig. 3A) and was maintained for more than 1 h if still incubated in glucose free medium (Fig. 3B). MAPKs also known as extracellular regulated protein kinases (ERKs) are protein kinases having molecular masses of 42–45 kDa that are stimulated in response to a wide range of stimuli [24]. The best studied of these kinases are $p42^{MAPK}$ (ERK2) and $p44^{MAPK}$ (ERK1) [25]. We have presented our data specifically for ERK2, however, experiments done using ERK1 showed similar results (data not shown).

It is well known that stimuli which promote growth and differentiation in cells activate MAPK. These include growth factors like epidermal growth factor (EGF) [22, 26] and platelet derived growth factor (PDGF) [26] or hormones like insulin [27] and progesterone [28]. It is also known that nonmitogenic stimuli can activate MAPK. These include thrombin [29], bradykinin [30], osmotic shock [31], ionizing radiation [32], and oxidant treatment [33]. We have here shown that glucose deprivation in MCF-7/ADR cells can also activate MAPK.

Glucose as an energy source is crucial for cell growth. It has been shown that glucose deprivation in an interleukin 3 dependent cell line resulted in programmed cell death or apoptosis [34]. Glutamine as an alternative energy source could prevent this. In our data, the addition of glutamine did not reverse glucose deprivation induced cytotoxicity (unpublished data) nor MAPK activation in MCF-7/ADR cells (Fig. 5). The addition of glutamate, however, rescued cells from the cytotoxicity (unpublished data), decreased the MAPK activation, and the length of time of activation (Fig. 6). Although glutamine can be converted into glutamate by the enzyme glutamate synthase, it may be that MCF-7/ADR cells are deficient or have a defect in this enzyme. Glutamate can be converted by the cell into α-ketoglutarate which can enter the tricarboxylic acid cycle and generate ATP. We also found that MAPK activation is dependent on the concentration of glucose (Fig. 4). This suggests a role for the energy supply within a cell regulating the activation of MAPK, AP-1 transcription factors, and bFGF. In addition, the effects of glucose deprivation are seen in other pathways like those involving JNK which is also activated (data not shown).

It is known that MAPK is activated by phosphorylation at tyrosine and threonine [21]. The direct upstream activator of MAPK is MAP kinase or MEK [35]. MEK is activated by phosphorylation on serine and possibly threonine residues [36]. Some evidence has indicated the Raf proto-oncogene product, which is a serine/threonine kinase activated by both receptor and nonreceptor tyrosine kinases via activation of p21Ras, activates MEK [37, 38]. Raf has also been shown to be activated by protein kinase C (PKC) [39]. The data that we have presented (Fig. 7) shows that glucose deprivation results in a greater than three fold increase in MEK, RAF, RAS, and PKC activity.

At this time only speculation can be made concerning the mechanism of MAPK activation with glucose deprivation. In neuronal cells, glucose deprivation has been shown to promote the production of free radicals [40], and free radicals in turn can activate MAPK [33]. We have measured the levels of catalase in MCF-7 and MCF-7/ADR cells and found that MCF-7 cells have four times as much catalase as MCF-7/ADR cells (unpublished data). We have observed that the addition of the free radical scavenger N-acetylcysteine at concentrations of 1 mM can prevent glucose deprivation induced MAPK activation in MCF-7/ADR cells (unpublished data). Obviously, further studies at the biochemical level are necessary to understand the mechanism of MAPK activation.

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