Importance of glucose-6-phosphate dehydrogenase activity in cell death

WANG-NI TIAN, LEIGH D. BRAUNSTEIN, KIRA APSE, JIONGDONG PANG, MARK ROSE, XIAONI TIAN, AND ROBERT C. STANTON
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Tian, Wang-Ni, Leigh D. Braunstein, Kira Aps, Jiongdong Pang, Mark Rose, Xiaoni Tian, and Robert C. Stanton. Importance of glucose-6-phosphate dehydrogenase activity in cell death. Am. J. Physiol. 276 (Cell Physiol. 45): C1121–C1131, 1999.—The intracellular redox potential plays an important role in cell survival. The principal intracellular modulator of the redox potential is NADPH, the rate-limiting enzyme, and by 6-phosphogluconate dehydrogenase. Considering the importance of NADPH, we hypothesized that G6PDH plays a critical role in cell death. Our results show that 1) G6PDH inhibitors potentiated H2O2-induced cell death; 2) overexpression of G6PDH increased resistance to H2O2-induced cell death; 3) serum deprivation, a stimulator of cell death, was associated with decreased G6PDH activity and resulted in elevated reactive oxygen species (ROS); 4) additions of substrates for G6PDH to serum-deprived cells almost completely abrogated the serum deprivation-induced rise in ROS; 5) consequences of G6PDH inhibition included a significant increase in apoptosis, loss of protein thiols, and degradation of G6PDH; and 6) G6PDH inhibition caused changes in mitogen-activated protein kinase phosphorylation that were similar to the changes seen with H2O2. We conclude that G6PDH plays a critical role in cell death by affecting the redox potential.

A complex interplay of intracellular signals and metabolic processes is involved in the regulation of cell death (22). Two principal patterns of cell death have been described, necrosis and apoptosis. Necrosis is associated with inflammation (22), whereas apoptosis (programmed cell death) is a regulated process that is usually associated with chromatin condensation and nuclear fragmentation (22). Recent evidence indicates that cell death may be due to a predeterminated genetic program, external triggers, or intracellular stimuli (22). Disturbance of this interplay of extracellular and intracellular factors may trigger cell death.

Although many signals and metabolic events may be important in the regulation of cell death, the intracellular redox level, in particular, has been shown to play a critical role. For example, cell death has been associated with an increase in intracellular levels of reactive oxygen species (ROS) (9, 27). Administration of oxidants such as H2O2 causes cell death. In particular, administration of relatively low concentrations of H2O2 to cells will cause apoptosis, whereas higher concentrations will cause necrosis (3). Antioxidants can prevent cell death (44). For example, exposure of cells to the antioxidant N-acetyl-L-cysteine (NAC) prevents cell death (44). Also, Bcl-2, a critical antiapoptotic gene, has been suggested to work, at least in part, through an antioxidant pathway (11). Thus regulation of the intracellular redox potential is critical for the control of cell death.

The intracellular redox potential is determined by the concentrations of oxidants and reductants. A critical modulator of the redox potential is NADPH, the principal intracellular reductant in all cell types. Glucose-6-phosphate dehydrogenase (G6PDH), the rate-limiting enzyme, and by 6-phosphogluconate dehydrogenase. Considering the importance of G6PDH for cellular antioxidant defenses and the well-documented role of G6PDH in cell death.

In this paper, we show that inhibition of G6PDH potentiated H2O2-mediated cell death. Overexpression of G6PDH rendered the cells more resistant to H2O2-mediated cell death. Serum deprivation, another stimulator of cell death, caused a decrease in G6PDH activity and an increase in ROS. This serum deprivation-induced increase in ROS was almost completely abro-
Glucose → G6PD → 6-phosphogluconolactone → NADPH + H+ → 6-phosphogluconate lactonase → AMP, GMP, UMP, CMP

Citric Acid Cycle

6-Phosphogluconate → Ribulose 5-Phosphate → Ribose 5-Phosphate → Nonoxidative Branch

Gated by providing cells with substrates for G6PDH. By evaluating the pattern of cell death, we show that G6PDH inhibitors greatly enhanced apoptosis. Last, to begin to determine the intracellular signaling proteins that G6PDH activity may affect, we show that H2O2 and G6PDH inhibitors altered mitogen-activated protein kinase (MAP kinase) phosphorylation in a similar manner. We conclude that G6PDH plays a critical role in cell death, likely by affecting the intracellular redox potential.

**EXPERIMENTAL PROCEDURES**

Materials. Cell culture media, growth factors, and sera were obtained from Life Technologies. Rabbit anti-rat G6PDH antibody was generously provided by Dr. Rolf Kletzien (Upjohn). Fluorescent dyes were obtained from Molecular Probes. FLAG antibody was purchased from Eastman Kodak. Extracellular signal-regulated kinase II was from New England Biolabs. All other chemicals were obtained from Sigma.

Cell culture. BALB/c A31 and BALB/c simian virus 40 (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells were grown in RPMI 1640 medium supplemented with 10% inactivated FCS. PC12 cells were grown in DMEM containing 5% calf serum and 5% horse donor serum; 1% penicillin-streptomycin was present in all culture media. In all experiments, separated plates of control cells (without any inhibitor) were simultaneously incubated with cells treated with inhibitor. Bovine aortic endothelial cells (BAEC) were cultured from freshly obtained bovine aorta and maintained in low-glucose DMEM plus 10% calf serum.

Assessment of cell viability. Cell death was measured by trypan blue uptake.

Enzyme activity measurements. Enzyme activities in cell lysates were measured in a spectrophotometer as previously described (37).

Measurement of G6PDH and phosphogluconate dehydrogenase activity in intact cells. The method employed by Van Noorden and colleagues (4, 15) was used. Cells were incubated with medium containing 1/2 volume of 1 mM tetrainingo-blue tetrazolium dye, 0.5 mM glucose 6-phosphate (G6P), and 0.5 mM NADP for 8 h. The increase in blue color is dependent on the production of NADPH, which reduces the tetrazolium dye and produces a blue color. The intensity of the blue color was measured spectrophotometrically. The specificity of this assay for G6PDH has been previously determined (4, 15) and verified by us in control experiments (see Providing substrates for G6PDH to serum-deprived cells abrogates intracellular ROS accumulation).

Measurement of intracellular ROS. Intracellular accumulation of ROS was measured fluorometrically. The nonfluorescent dye 2',7'-dichlorofluorescin diacetate (DCFDA) is freely permeable to cells. DCFDA is hydrolyzed to 2',7'-dichlorofluorescin (DCF) inside the cells, where it converts upon interaction with ROS to a fluorescent DCF (23). DCF fluorescence reading of the samples was conducted using a microplate fluorometer (Cambridge Technology) with the excitation filter set at 485 nm and the emission filter set at 530 nm.

Measurement of protein thios. Thiois were assayed using DTNB (18). Briefly, cell lysate was precipitated and washed twice with TCA. Cellular proteins were suspended in Tris·HCl (pH 7.6). Twenty minutes after the addition of DTNB, the absorbance was measured at 412–520 nm.

Tagged protein and expression. The FLAG-containing G6PDH construct was generated by PCR. The FLAG sequence was tagged to the carboxy terminus of G6PDH. cDNA of G6PDH was a kind gift from Dr. Ye-Shih Ho (Institute of Chemical Toxicology, Detroit, MI). To prepare the FLAG-G6PDH fusion protein, G6PDH-FLAG cDNA was subcloned into the expression vector pcDNA3 (Invitrogen). PC12 cells were transfected by gene pauser (Bio-Rad) and selected in geneticin-containing medium. The expression of epitope-tagged G6PDH was confirmed by Western blotting using monoclonal antibody against FLAG epitope, as well as by enzyme activity (36).

Determination of DNA fragmentation. After incubation with or without H2O2 or G6PDH inhibitors for a specified time, cells were spun down and the pellet was then incubated at 50°C for 1 h in lysis buffer containing 10 mM EDTA, 50 mM Tris·HCl (pH 8.0), 0.5% (wt/vol) Triton X-100, and 0.5 mg/ml proteinase K. Samples were then incubated with additional 0.5 mg/ml RNase A at 50°C for another 3 h. After heating to 70°C, samples were mixed with gel buffer containing 10 mM EDTA (pH 8.0), 1% (wt/vol) low-gelling-temperature agarose, 0.25% (wt/vol) bromphenol blue, and 40% (wt/vol) sucrose before loading into dry wells of 2% (wt/vol) agarose gel containing 0.5 mg/ml ethidium bromide. After electrophoresis, DNA was visualized under ultraviolet light (305 nm).

DNA staining for microscopy. Cells were fixed with 4% paraformaldehyde for 15 min and then stained with 0.4 mg/100 ml 4',6'-diamidine-2'-phenylindole dihydrochloride (DAPI) at 4°C for 2 h. Cells were then washed twice and mounted onto glass slides. An Olympus microscope was used for fluorescence detection. For each treatment, 10 fields (100 cells/field) were counted.

Determination of p42 and p44 MAP kinase phosphorylation. Subconfluent PC12 cells were serum starved for 24 h and then pretreated for 10, 20, or 30 min with one of the following: DMEM alone, the G6PDH inhibitor dehydroepiandrosterone (DHEA; 200 µM), the oxidant menadione (200 µM), or H2O2 (1 and 5 mM). In some experiments, epidermal growth factor (EGF; 10 ng/ml) was added for 5 min after the pretreatment. Cells were then lysed, and MAP kinase p42 and p44 phosphorylation was observed using a phosphotyrosine-specific antibody (New England Biolabs).
RESULTS

Inhibition of G6PDH potentiates H$_2$O$_2$-induced cell death in established cells. DHEA and 6-aminonicotinamide (6-ANAD) are known inhibitors of G6PDH (26, 39). To examine the role of G6PDH in the regulation of cell death, PC12 cells (a neural cell line) and BALB/c cells (a fibroblast cell line) were incubated with either 50 or 100 µM H$_2$O$_2$ in the absence or presence of 100 µM DHEA or 5 mM 6-ANAD. Our previous studies indicated that, when cells were incubated with these concentrations of inhibitors, G6PDH activity was decreased by 40–70% (36). Importantly, we have also previously shown that inhibition of G6PDH by DHEA at concentrations used in this study caused a 30–40% decrease in NADPH levels (36). As shown in Fig. 2A in the absence of serum, 100 µM H$_2$O$_2$ alone decreased cell viability. DHEA and 6-ANAD, two structurally different inhibitors of G6PDH, significantly potentiated the loss of viable cells in the presence of H$_2$O$_2$, suggesting that inhibition of G6PDH enhanced the detrimental effects of H$_2$O$_2$ on cell survival. In the presence of serum but the absence of any G6PDH inhibitor, a modest concentration of H$_2$O$_2$ (100 µM) did not cause cell death (data not shown), suggesting that serum can protect cells from the deleterious effects of modest concentrations of H$_2$O$_2$.

Overexpression of G6PDH increased cell resistance to H$_2$O$_2$-induced cell death. Because the inhibition of G6PDH potentiates H$_2$O$_2$-induced cell death, we tested whether overexpression of G6PDH would provide protection against H$_2$O$_2$-induced cell death. PC12 cells were stably transfected either with vector alone or with a construct of G6PDH tagged with an epitope (FLAG peptide). A clone stably expressing FLAG-G6PDH was isolated. As shown in Fig. 2B, PC12 cells overexpressing G6PDH were more resistant to H$_2$O$_2$-induced cell death than control cells that expressed endogenous levels of G6PDH. This result suggests that increased G6PDH activity enhanced cellular protection against H$_2$O$_2$-induced cell death.

Inhibition of G6PDH potentiates H$_2$O$_2$-induced cell death in nontransformed, freshly isolated cultured cells. Because BALB/c 3T3 cells are a nontumorigenic but established cell line and PC12 cells are transformed cells, freshly isolated cells were studied to determine whether freshly isolated, nontransformed cells also are affected by G6PDH inhibition. Figure 2C shows that DHEA and H$_2$O$_2$ alone as well as the combination of DHEA and H$_2$O$_2$, significantly enhanced cell death, although to a lesser degree than in the transformed cells. The freshly isolated cells were more resistant to the deleterious effects of both DHEA and H$_2$O$_2$. This reduced effect of G6PDH inhibition is likely due to the fact that freshly isolated cells have lower basal activity of the PPP than transformed cells (30, 32, 37, 41) and that freshly isolated cells have increased responsiveness of the PPP to oxidative stress.

Serum deprivation is correlated with decreased G6PDH activity. Our above results indicate that serum protects cells from H$_2$O$_2$-induced death. Previously, researchers have shown that serum withdrawal leads to an increase in ROS (2). Thus deprivation of survival factors might inactivate antioxidant defense systems and trigger a ROS-dependent cell death (3, 19).

Because the function of G6PDH is to provide NADPH for antioxidant defense, we hypothesized that decreased G6PDH activity during serum deprivation may contribute to a decreased antioxidant defense and thus lead to an increase in ROS. We utilized an assay system that measures G6PDH activity in intact cells (see Measurement of G6PDH and phosphogluconate dehydrogenase activity in intact cells). Figure 3A shows that G6PDH activity was reduced in serum-deprived PC12 cells (control, and ***P < 0.001 compared with control).

Serum deprivation caused large increases in intracellular ROS. Because serum deprivation alone decreased G6PDH activity, we suspected that an accumulation of ROS may occur due to a decrease in NADPH availability. Intracellular accumulation of ROS was measured fluorometrically using the nonfluorescent dye DCFDA, to which cells are freely permeable. DCFDA is hydrolyzed to DCF inside the cells, where it converts upon interaction with ROS to a fluorescent DCF. Figure 3B shows that serum deprivation dramatically increased the accumulation of ROS inside the cells. Figure 3C shows that the increase in ROS is time dependent (compare DMEM with serum).

Providing substrates for G6PDH to serum-deprived cells abrogates intracellular ROS accumulation. It has been known that G6P and NADP (the substrates for G6PDH) added to the medium can enter the cells and be converted to 6-phosphogluconate (6PG) and NADPH by G6PDH. A number of researchers have used this method to measure activity of G6PDH in tissues and cells (4, 15). To be sure that the substrates NADP and G6P were taken up by the cells, a number of control experiments were done: 1) there was no change in dye color when either NADP alone or G6P alone was added to the cells; 2) only when both substrates for G6PDH were added was there a change in color; 3) for cells that are almost completely deficient in G6PDH activity (25), addition of either substrate alone or both G6PDH substrates together caused no change in dye color; and 4) using [14C]G6P, we determined that the 14C was converted into CO$_2$ by the PPP using a previously described technique (30, 32, 37). This uptake of G6P was completely blocked by excess cold G6P. These control experiments do not show whether G6P and NADP or their metabolites are taken up. Nevertheless, taken together, these data do suggest that G6P and NADP or their metabolites can be taken up into the cells and utilized by G6PDH.

We therefore tested whether increased activity of G6PDH driven by the addition of G6PDH substrates

Statistics. Student’s t-test was used for statistical analysis. The statistical significance is represented as follows: *P < 0.05 compared with control, **P < 0.01 compared with control, and ***P < 0.001 compared with control.
would ameliorate the rise in ROS due to serum deprivation. Figure 3B shows that the addition of NADP and G6P to the medium abrogated ROS accumulation to a level almost as low as in the presence of serum. Note that only when all substrates for G6PDH were present was the ROS level greatly decreased (Fig. 3B). Providing the substrates (NADP and G6P) for phosphogluconate dehydrogenase (PGD), the next enzyme in the PPP pathway, had only a modest effect on ROS levels. Figure 3C shows the effects of the antioxidants catalase and NAC. Catalase and NAC had only modest effects on reduction of the serum-deprived increase in ROS. Clearly, provision of the substrates for G6PDH (NADP and G6P) was the most effective in reducing ROS levels close to the level seen with serum. These results suggest that G6PDH activity plays an important role in cellular defense against the accumulation of ROS.

DHEA and 6-ANAD enhanced apoptosis. Both necrosis and apoptosis have been described in oxidative stress-induced cell death. To determine whether G6PDH inhibition increased apoptosis, DNA fragmentation and chromatin condensation (42) were evaluated in a variety of cell types.

Figure 4A shows DNA fragmentation in RIN5mAF cells (a pancreatic β-cell line) caused by G6PDH inhibition and by H₂O₂. DHEA and 6-ANAD caused DNA fragmentation, characteristic of apoptosis, in several cell lines tested.

Next we searched for evidence of chromatin condensation, another hallmark of apoptosis. Using immunofluorescence microscopy and DAPI staining of cell nuclei, we observed a significant increase in chromatin condensation in cells treated with G6PDH inhibitors. Figure 4B shows representative photomicrographs depicting the effect of DHEA on chromatin condensation. Both G6PDH inhibitors increased chromatin condensation (6-ANAD photo not shown). Also, the detection of DHEA-enhanced apoptosis was time dependent and varied in different cell lines, being detectable from 18 to 96 h (data not shown). This chromatin condensation enhanced by G6PDH inhibition was seen in all cell lines tested, including PC12, RIN5mAF, COS-7, K-562, and BALB/c 3T3 cells. Figure 4C shows quantitation of the DHEA- and 6-ANAD-induced apoptosis. Figure 4C also shows that the nontransformed BAEC had increased apoptosis after being exposed to DHEA and H₂O₂. The dose dependency of the DHEA effect is seen in Fig. 5 in PC12 cells (EC₅₀ of ~60 µM in the absence of serum). Note that serum rendered the cells more resistant to the apoptotic effects of DHEA.

Although in all cell lines tested apoptosis was universally enhanced by the inhibition of G6PDH, we did find heterogeneity among different cell types. In some cell lines, such as PC12, RIN5mAF, COS-7, and K-562 cells, DHEA induced programmed cell death even without serum deprivation or the addition of an external oxidant. Notably, the induction of apoptosis in these cell
lines was associated with a relatively high basal apoptosis in the presence of serum. In contrast, BALB/c 3T3 cells had a very low basal level of apoptosis in the presence of serum, and serum deprivation was required for DHEA to induce apoptosis in this cell line. Thus it seems possible that DHEA potentiates an existing apoptotic potential in susceptible cells.

Because all tested cell lines displaying higher basal apoptotic level were transformed cells, we suspected that there might be an association between DHEA-triggered apoptosis and cell transformation. To see whether cell transformation per se affected the ability of DHEA to induce apoptosis, chromatin condensation was assessed in SV40-transformed BALB/c cells and in their nontransformed counterpart, BALB/c A31 fibroblasts. The results showed that DHEA caused a similar extent of apoptosis in the transformed BALB/c cells and in the nontransformed cells (data not shown). This result suggests that the degree to which DHEA enhances apoptosis is dependent on cell type rather than cell transformation.

Inhibition of G6PDH caused a decrease in protein thiols. Oxidative stress may cause the decrease of protein thiols, which may consequently impair many enzymes (5). Because the inhibition of G6PDH may decrease cellular reducing equivalents, thus limiting antioxidative defense mechanisms, we tested whether cell death enhanced by the inhibition of G6PDH is associated with loss of protein thiols. Protein-bound sulfhydryl groups were measured using DTNB in control and G6PDH inhibitor-treated PC12 cells. As shown in Fig. 6, upon the inhibition of G6PDH, protein thiols significantly decreased, suggesting that the cells had increased intracellular oxidant levels.

Proteolytic degradation of G6PDH occurs in association with increased cell death. During apoptosis, some important proteins are “executed” by proteases. In cultured cells, apoptosis enhanced by DHEA and 6-ANAD was accompanied by the detection of a 46-kDa fragment of G6PDH (Fig. 7). We have previously shown the specificity of the G6PDH antibody for G6PDH (32, 36, 37). The amount of the 46-kDa fragment of G6PDH correlated closely with apoptotic susceptibility in several cell lines tested. For example, BALB/c 3T3 cells, which have a very low basal apoptotic rate and a moderate increase in cell death after exposure to DHEA, showed little to no evidence of the 46-kDa fragment of G6PDH (data not shown). In contrast, the RIN5mAF cells, which readily undergo apoptosis after exposure to DHEA, had significant cleavage of G6PDH after 48 h of incubation. PC12 cells, which are highly susceptible to DHEA-induced apoptosis, showed degra-

![Graph 1](image1)

**Fig. 3.** A: serum increased G6PDH activity. Enzyme activity was measured from intact BALB/c 3T3 fibroblasts, which were incubated in absence or presence of various concentrations of calf serum. Enzyme activity is expressed as increase in conversion of dye to a dark blue. Data are from a representative experiment that was repeated 4 times with similar results. B: addition of substrates for G6PDH abrogates accumulation of reactive oxygen species (ROS) in serum-deprived cells. Intracellular accumulation of ROS was measured fluorometrically. Conversion of nonfluorescent dye 2’7’-dichlorofluorescin diacetate (DCFDA) to 2’7’-dichlorofluorescin (DCF) is indication of accumulation of ROS. Cells were either incubated with combinations of substrates for G6PDH (100 µM NADP and 200 µM glucose 6-phosphate (G6P)) and PGD (100 µM NADP and 200 µM 6-phosphogluconate (6PG)). Data are means of 4 repeated DCF fluorescence readings, each run in at least triplicate. ***P < 0.001 compared with control. C: addition of substrates for G6PDH is more effective than catalase and N-acetyl-L-cysteine (NAC) in reducing accumulation of ROS in serum-deprived cells. Intracellular accumulation of ROS was measured fluorometrically (see EXPERIMENTAL PROCEDURES). Cells were either incubated with DMEM alone or with one of following: substrates for G6PDH (NADP and G6P), substrates for PGD (NADP and 6PG), catalase (1,000 U/ml), NAC (2 mM), or 1% calf serum. Data are means of 4 repeated DCF fluorescence readings, each run in at least triplicate.
Fig. 4. A: G6PDH inhibitors and H_2O_2 caused DNA fragmentation. Cellular DNA was isolated after cells were treated in absence and presence of 100 µM DHEA, 5 mM 6-ANAD, or 100 µM H_2O_2 for 48 h. B: inhibition of G6PDH enhanced chromatin condensation. Representative micrograph of RIN5mAF cells incubated with one of following for 48 h and then fixed and stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). Top: DMEM alone. Bottom: DMEM + 100 µM DHEA. C: DHEA and 6-ANAD enhanced apoptosis. PC12 and RIN5mAF cells were incubated with H_2O_2 (100 µM), DHEA (100 µM), or 6-ANAD (5 mM) for 48 h. BAEC were maintained in 2% serum and incubated with H_2O_2 (500 µM) and/or DHEA (100 µM) for 3 h. Apoptotic cells were identified by DAPI staining of condensed chromatin. *P < 0.05, **P < 0.01, ***P < 0.001 compared with control.
Fig. 5. DHEA enhanced apoptosis in a dose-dependent manner. PC12 cells were exposed to DHEA for 48 h in presence or absence of serum. Data are means of 3 experiments.

Fig. 6. Inhibition of G6PDH resulted in decreased protein thiols. PC12 cells were treated with or without 100 µM DHEA, 5 mM 6-ANAD, 100 µM menadione, or 100 µM H2O2 for either 4 or 24 h in presence (A) or absence (B) of serum. Cell lysates of control and inhibitor-treated PC12 cells were then assayed for protein-bound thiol groups using DTNB. Data are expressed as means ± SE of 4 separate experiments. *P < 0.05, **P < 0.01, ***P < 0.001 compared with control.

Fig. 7. G6PDH underwent degradation during apoptosis. Western analysis of G6PDH in cell lysate using a specific polyclonal antibody to mammalian G6PDH. PC12 cells were treated for 48 h with or without 100 µM DHEA, 5 mM 6-ANAD, or 100 µM H2O2.

Fig. 8. A: H2O2, menadione, and DHEA stimulated phosphorylation of p42 and p44 MAP kinase in serum-deprived cells. DHEA also had a modest although lower stimulation of p42 and p44 phosphorylation, suggesting that DHEA inhibited phosphorylation of p42 and p44 in a manner similar to known oxidants. B: EGF prevented apoptosis in a variety of cell types and that EGF activates the MAP kinase pathway. We therefore hypothesized that DHEA might decrease EGF-induced phosphorylation of p42 and p44. Figure 8, bottom, shows that H2O2 and menadione stimulated phosphorylation of p42 and p44 in the absence of growth stimuli. DHEA also had a modest although lower stimulation of p42 and p44 phosphorylation, suggesting that DHEA inhibited phosphorylation of p42 and p44 in a manner similar to known oxidants.

PD-98059, the MAP kinase inhibitor, on cell survival. It has been reported that activation of p42 and p44 MAP kinase is part of a cell survival cascade (29, 44). Thus stimulation of p42 and p44 phosphorylation in response to H2O2, menadione, and DHEA likely reflects a cellular protective...
response to increased oxidative stress. PD-98059 alone increased cell death (data not shown). In combination with DHEA, PD-98059 significantly enhanced cell death over DHEA or PD-98059 alone (by Student’s t-test, P < 0.01; data not shown).

**DISCUSSION**

G6PDH plays an important role in cell death by regulating intracellular redox levels. A principal finding of our study was that alterations in G6PDH activity can significantly alter oxidative stress-induced cell death. As noted in the introduction, there is ample evidence showing that an increase in ROS induces cell death (9, 27). This increase in ROS may be due to overproduction of ROS from extracellular or intracellular processes, or there may be a decrease in endogenous antioxidant defense. Much work in recent years has been centered on such intracellular antioxidants as superoxide dismutase, GSH, and catalase. Alterations in the activities of these enzyme systems have been implicated as causes of diseases (e.g., amyotrophic lateral sclerosis) as well as cell death (17). Yet the antioxidant defense mechanisms ultimately rely on the adequate production of NADPH for reducing equivalents during oxidative stress (19). The principal source of NADPH production is the PPP, and many studies have shown that under oxidative stress G6PDH and the PPP are elevated (28, 38). Although there are other metabolic pathways that produce NADPH, research has shown that the PPP is the predominant source of NADPH required to defend against oxidative stress. For example, the work by Pandolfi et al. (24) using G6PDH-deficient cell lines shows that other sources of NADPH do not adequately replace the lack of NADPH production by G6PDH. That is, the G6PDH-deficient cells had decreased growth rates and cloning efficiencies and were highly sensitive to oxidative stress compared with cells expressing endogenous levels of G6PDH. Thus G6PDH is critical for NADPH production and is the principal source of NADPH in a large number of cell types. However, in liver, adipose tissue, pancreatic β-cells, and macrophages, NADP+ -dependent malate dehydrogenase may play a significant role in NADPH production.

The PPP via the nonoxidative branch may also affect cell survival directly and/or by providing substrates for glycolysis. First, Bank et al. (1) showed that levels of transaldolase, an enzyme in the nonoxidative branch of the PPP, can regulate via regulation of the levels of the two dehydrogenases, G6PDH and PGD. Increases in transaldolase led to decreased levels of G6PDH and PGD as well as increased ROS levels and increased apoptosis, whereas decreased transaldolase had the opposite effects. These results, however, also support our hypothesis that G6PDH activity by producing NADPH is important for cell survival. Second, the nonoxidative branch of the PPP also provides substrates for glycolysis. Thus altered flux through the PPP may alter substrate availability for glycolysis, leading to changes in ATP and NADH levels. These metabolic changes could affect cell survival. For example, reduced levels of NAD and NADH have been shown to be associated with decreased growth. Mazurek et al. (20) showed that AMP-induced inhibition of a breast cancer cell line is likely caused by reduced glycolytic carbon flux leading to reductions in NAD and NADH levels. Thus effects of the inhibition of PPP on glycolysis could potentially inhibit cell growth via effects on NAD/NADH levels in addition to changes in NADPH. Also, Street et al. (34) showed that 6-ANAD enhanced irradiation-induced killing of tumor cells. This increased killing was associated with decreased activity of both the PPP and glycolysis. Thus the
nase activity by the thiol-to-disulfide ratio, Gilbert and
Based on their study on modulation of phosphofructoki-
protein folding, conformation, and polymerization.
the thiol-to-disulfide ratio may have significant impact on
intracellular oxidants. It is likely that an altered
ated with decreased protein thiols. A decrease in pro-
We have found that G6PDH inhibition is closely associ-
leading to decreased ROS levels.
out that ectoenzymes convert G6P and NADP into
G6P and NADP is required to decrease ROS levels. It could be argued that these substrates alone rather than G6PDH are able to alter ROS levels. However, Fig. 3B shows that only when both substrates for G6PDH were added was there an effect on ROS levels. Neither substrate alone af-
fected ROS levels, suggesting that the combination of G6P and NADP is required to decrease ROS levels. Although experiments discussed in Providing sub-
strates for G6PDH to serum-deprived cells abrogates intracellular ROS accumulation strongly suggest that
G6P and NADP are taken up by cells, it cannot be ruled out that ectoenzymes convert G6P and NADP into other compounds that are taken up by the cell. However, even if this occurs, it seems likely that the ultimate effect is via G6PDH, as the effect on ROS levels only occurs in the presence of both substrates. Thus we conclude that the substrates are taken up by the cell and utilized by G6PDH to produce NADPH, leading to decreased ROS levels.
Inhibition of G6PDH leads to a loss of protein thiols. We have found that G6PDH inhibition is closely associ-
ated with decreased protein thiols. A decrease in pro-
tein thiol content is a consequence of an increase in intracellular oxidants. It is likely that an altered thiol-to-disulfide ratio may have significant impact on protein folding, conformation, and polymerization. Based on their study on modulation of phosphofructoki-
nase activity by the thiol-to-disulfide ratio, Gilbert and
colleagues (5, 40) further demonstrated the significance of thiol/disulfide in cell biology. They found that cell death was preceded by the loss of protein thiols. Phosphofructokinase activity was greatly decreased following exposure to GSSG, likely due to changes in subunit associations. Glutathione oxidation state is dependent on G6PDH as NADPH is the reductant for GSSG (19). Gilbert and colleagues (5, 40) also showed similar effects on thiols and other enzymes. Thus decreased protein thiol content caused by G6PDH inhibition is consistent with our hypothesis that G6PDH plays a critical role in cell death by regulating intracellular redox levels.
Decreased G6PDH activity leads to enhancement of apoptosis. The effect of oxidants on cell function appears to be related to its intracellular concentration (3, 9, 35). For example, low levels of oxidants (1–5 µM) appear to be stimulatory to cell growth and have been implicated as downstream signals for the growth factors, such as platelet-derived growth factor (35). Midrange concentrations of oxidants (50–100 µM) have been suggested to cause apoptosis, whereas high concen-
trations of oxidants (500–1,000 µM) have been shown to cause necrosis. Because we and others had previ-
ously shown that DHEA can inhibit growth factor-stimulated cell growth (10, 36), we were interested in determining whether G6PDH inhibition led to apopto-
sis and/or necrosis. Our data show that G6PDH inhibi-
tion clearly led to an increase in the numbers of apoptotic cells.
Notably, our data showed that serum was relatively protective against cell death and that cells exposed to serum have a relatively increased antioxidant defense. Importantly, serum deprivation led to a decrease in G6PDH activity (Fig. 3A). Because serum deprivation enhanced DHEA-induced cell death (Fig. 5), we believe that serum deprivation-induced cell death is due, at least in part, to inhibition of G6PDH.
Also of interest, our data showed that there is considerable cell specificity with respect to the suscepti-
bility of a cell to undergo apoptosis. For example, in BALB/c 3T3 fibroblasts, only in the absence of serum did DHEA and 6-ANAD enhance apoptosis, whereas, in PC12, RINm5eAF, COS-7, and K-562 cells, even in the presence of serum, DHEA and 6-ANAD enhanced apopto-
thesis. All of the cells that have increased susceptibility to apoptosis following exposure to G6PDH inhibition are transformed cells. Previous work by a number of researchers showed that cancer cells in vivo and transformed cells in culture have significantly increased activities of G6PDH to levels as high as 20-fold greater than nontransformed cells (41). Thus it is intriguing to speculate that specific inhibition of G6PDH could differentially induce more apoptosis in stressed cells or cells with higher apoptotic tendency and thus may offer therapeutic benefit to patients with cancer. The combi-
nation of G6PDH inhibitor along with other stress-
inducing stimuli (e.g., radiation and/or chemotherapy) might prove to be beneficial.
Increased cell death was associated with degradation of G6PDH. Another interesting observation from this study was the degradation of G6PDH during apoptosis. It has been reported that protease activation may function as the executioner upon apoptotic signal. Despite identification of several proteases, the substrate list for these proteases is far from extensive. The few substrates for proteases identified so far, however, are very critical proteins implicated in the apoptotic cascade. For instance, the cleavage of lamin B1 leads to the collapse of nuclear matrix. (21) The cleavage of poly(ADP-ribose) polymerase inhibits most DNA repair activity (12). Proteolytic cleavage of actin may destroy its ability to inhibit DNase I and its association with fordrin, thus leading to some apoptotic changes in membranes and cytoskeleton (13). The degradation of any enzyme in metabolic pathways has not yet been reported. Given the critical role of G6PDH to provide NADPH, the cleavage of it during apoptosis may be an important event in programmed cell death by inactivating an important antioxidant protein.

Inhibition of G6PDH alters phosphorylation patterns of MAP kinase. Our results also show that G6PDH inhibition is associated with alteration in phosphorylation of p42 and p44 MAP kinase (Fig. 8). The MAP kinase cascade has been implicated in cell growth, cell death, and cell differentiation. Recent work by Stevenson et al. (33) has shown that oxidants such as H₂O₂ can stimulate phosphorylation of p42 and p44 MAP kinase. It is likely that the activation of these kinases is a cellular protective response to oxidative stress (29, 44). In contrast, oxidant-induced activation of p38 kinase, another member of the MAP kinase family, promotes cell death (16). Our data showed that H₂O₂, menadione, and DHEA stimulated phosphorylation of p42 and p44 MAP kinase in cells not exposed to growth factors. Our data indicated that DHEA alone has a modest effect on MAP kinase similar to H₂O₂ (Fig. 8, top). Interestingly, when cells were preincubated with DHEA and then exposed to EGF, the EGF-induced phosphorylation of p42 and p44 MAP kinase was greatly decreased. H₂O₂ displayed similar but lower reduction of EGF-stimulated MAP kinase phosphorylation. Therefore, G6PDH inhibition seemed to cause an effect on phosphorylation similar to that of the oxidants (H₂O₂ and menadione). Last, use of an inhibitor of the MAP kinases, PD-98059, synergistically enhanced DHEA-induced cell death (data not shown). This result suggests that the increase in p42 and p44 MAP kinase phosphorylation seen after DHEA, H₂O₂, and menadione is a protective response to increased oxidative stress. Taken together, these results add further support to the hypothesis that G6PDH is important for cell death regulation by controlling intracellular redox.

In summary, we have found that G6PDH plays an important role in cell death by regulating the intracellular redox status.

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