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

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Tian, Wang-Ni, Leigh D. Braunstein, Kira Apse, Jiongdong Pang, Mark Rose, Xiaoni Tian, and Robert C. Stanton. Importance of glucose-6-phosphate dehydrogenase activity in cell death. Am. J. Physiol. Cell Physiol. 276 (Cell Physiol. 45): C1121–C1131, 1999.—The intracellular redox potential plays an important role in cell survival. The principal intracellular reductant NADPH is mainly produced by the pentose phosphate pathway by glucose-6-phosphate dehydrogenase (G6PDH), 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 thiol, 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.

oxidative stress; pentose phosphate pathway; apoptosis

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 predetermined 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 of the pentose phosphate pathway (PPP; Fig. 1), determines the amount of NADPH by controlling the metabolism of glucose via the PPP (14). It has been traditionally thought that G6PDH was a typical "housekeeping" enzyme that was regulated solely by the ratio of NADPH to NADP (14). But research from our lab as well as others suggests that this enzyme is highly regulated and plays important roles in a variety of cellular processes (14, 32, 37). Work from our lab and others has shown that G6PDH is under close transcriptional, translational, and posttranslational control (14, 32, 37). Specifically, our lab has demonstrated that growth factors can rapidly activate G6PDH and stimulate translocation of G6PDH (32, 37). We have further shown that specific growth factor receptor-associated signaling proteins can affect G6PDH (37). In addition, we recently showed that G6PDH activity plays a critical role in cell growth via its role in intracellular redox regulation (36). In particular, overexpression of G6PDH stimulated cell growth (as determined by increased [3H]thymidine incorporation), whereas inhibition of G6PDH abrogated growth factor stimulation of [3H]thymidine incorporation (36). We also showed that lack of NADPH but not ribose 5-phosphate was responsible for the growth suppression caused by inhibition of G6PDH (36). Others have also found that inhibition of G6PDH abrogated mitogen-stimulated cell proliferation (7, 8). Considering the importance of G6PDH for cellular antioxidant defenses and the well-documented role of oxidant stress in many models of cell death, we studied the 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-
G6PDH Activity Affects Cell Death

Fig. 1. Pentose phosphate pathway. Rate-limiting enzyme is glucose-6-phosphate dehydrogenase (G6PDH). Activation of two dehydrogenase enzymes, G6PDH and phosphogluconate dehydrogenase (PGD), results in production of NADPH, H⁺, and ribose 5-phosphate.

The method employed by Van described (37).

lysates were measured in a spectrophotometer as previously described. Bovine aortic endothelial cells (BAEC) were cultured 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 1 hi nlysis 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) bromophenol 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/ml 4',6'-diamidino-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.

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.

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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 Kletzen (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 (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF cells (SV40) 3T3 fibroblasts, as well as COS-7 cells, were grown in DMEM containing 10% calf serum. K-562 and RIN5mAF c
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.

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 potentiated 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 serum deprivation led to as much as a two- to threefold decrease in G6PDH activity compared with cells cultured in serum-containing medium.

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 DCF DA, which cells are freely permeable. DCF DA 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 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
would ameliorate the rise in ROS due to serum deprivation. Figure 3B shows that the addition of NADP and 6PG 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 6PG) 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 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 induced 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 types was time-dependent.
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-
Fig. 4. A: G6PDH inhibitors and H$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ (100 µM), DHEA (100 µM), or 6-ANAD (5 mM) for 48 h. BAEC were maintained in 2% serum and incubated with H$_2$O$_2$ (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.
dation as early as 12 h (data not shown). This correlation between apoptotic tendency and G6PDH degradation suggests that G6PDH may be a target for proteolysis in apoptosis.

H₂O₂, menadione, and DHEA stimulated phosphorylation of p42 and p44 MAP kinase in serum-deprived cells. DHEA attenuated the EGF-stimulated phosphorylation of p42 and p44 MAP kinase. Many signaling pathways have been implicated in causing apoptosis. We hypothesized that, if G6PDH inhibition is causing cells to be more susceptible to oxidative stress, then signaling proteins associated with apoptosis should be affected by G6PDH inhibitors in a manner similar to the effects of other oxidants. Initially, we evaluated the MAP kinase pathway that has been shown to be implicated in multiple physiological processes including cell death (43). The effects of the oxidants (H₂O₂ and menadione) as well as the G6PDH inhibitor DHEA on the phosphorylation of the critical MAP kinase proteins, p42 and p44, were examined. Figure 8, top, 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.

It has been shown that EGF prevents 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 the stimulation of p42 and p44 phosphorylation by EGF was significantly attenuated by DHEA and to a lesser extent by oxidant exposure (Fig. 8, bottom). These results indicate that both DHEA and H₂O₂ similarly alter phosphorylation patterns of p42 and p44 MAP kinases. Thus the attenuation of EGF-induced phosphorylation by DHEA may be one of the mechanisms underlying DHEA-induced cell death.

Last, we tested the effects of 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 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
nonoxidative branch of the PPP may play a role in cell survival as well as the oxidative branch.

We report here that G6PDH inhibition by DHEA and 6-ANAD enhanced cell death triggered by oxidative stress and serum deprivation. The consequences of enzyme inhibition are the lack of products catalyzed through the enzymatic reaction. The activation of G6PDH provides one molecule of NADPH. Subsequent activation of the next enzyme in PPP, PGD, gives rise to another NADPH. We previously demonstrated that inhibition of G6PDH causes a decrease in intracellular NADPH levels (36). Thus we conclude that lack of NADPH due to G6PDH inhibition made the cells more susceptible to oxidant damage, thus enhancing cell death. Consistent with this conclusion, our results from the stably transfected PC12 cells showed that overexpression of G6PDH made cells more resistant to oxidative stress-induced cell death. A previous study by Costa Rosa et al. (6) appears to be in conflict with our results concerning cell survival and the importance of G6PDH. Although not specifically focused on cell survival, their study reported that epinephrine-induced inhibition of G6PDH did not affect cell survival. A major difference between the two studies is the incubation period. Their experiments were all done within a 1-h period that is likely too short a time to observe significant cell death, whereas exposure to inhibitors in our experiments ranged from 3 to 48 h. Thus we believe there is no conflict between the two studies.

Addition of substrates for G6PDH reduces ROS production in serum-deprived cells. Our interpretation of this data is that the substrates G6P and NADP were taken up by the cells and utilized by G6PDH to produce NADPH and reduce 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 affected ROS levels, suggesting that the combination of G6P and NADP is required to decrease ROS levels. Although experiments discussed in Providing substrates 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 associated with decreased protein thiols. A decrease in protein 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 phosphofructokinase 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 NAPDH is the reductant for GSSG (19). Gilbert and colleagues (5, 40) also showed similar effects on thiolase I, fatty acid synthase, 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 concentrations of oxidants (500–1,000 µM) have been shown to cause necrosis. Because we and others had previously shown that DHEA can inhibit growth-factor-stimulated cell growth (10, 36), we were interested in determining whether G6PDH inhibition led to apoptosis and/or necrosis. Our data show that G6PDH inhibition 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 susceptibility 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, RIN5mAF, COS-7, and K-562 cells, even in the presence of serum, DHEA and 6-ANAD enhanced apoptosis. 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 combination 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. The cleavage of poly(ADP-ribose) polymerase inhibits most DNA repair activity. 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. 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 phosphorylation of p42 and p44 MAP kinase similar to that of the oxidants (H2O2 and menadione). Last, use of an inhibitor of the MAP kinases, PD-98059, displayed similar but lower reduction of EGF-stimulated 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 status.

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

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