

Enhanced Glutathione Levels and Oxidoresistance Mediated by Increased Glucose-6-phosphate Dehydrogenase Expression*

(Received for publication, June 23, 1998, and in revised form, October 30, 1998)

Francesca Salvemini‡, Annamaria Franzé‡, Angela Iervolino‡, Stefania Filosa‡, Salvatore Salzano§, and Matilde Valeria Ursini‡¶

From the ‡International Institute of Genetics and Biophysics, Consiglio Nazionale delle Ricerche, Via Guglielmo Marconi 12, 80125 Naples and the §Center of Endocrinology and Experimental Oncology, Consiglio Nazionale delle Ricerche, Via Pansini 10, 80131 Naples, Italy

Glucose-6-phosphate dehydrogenase (G6PD) is the key enzyme of the pentose phosphate pathway that is responsible for the generation of NADPH, which is required in many detoxifying reactions. We have recently demonstrated that G6PD expression is induced by a variety of chemical agents acting at different steps in the biochemical pathway controlling the intracellular redox status. Although we obtained evidence that the oxidative stress-mediated enhancement of G6PD expression is a general phenomenon, the functional significance of such G6PD induction after oxidant insult is still poorly understood. In this report, we used a GSH-depleting drug that determines a marked decrease in the intracellular pool of reduced glutathione and a gradual but notable increase in G6PD expression. Both effects are seen soon after drug addition. Once G6PD activity has reached the maximum, the GSH pool is restored. We suggest and also provide the first direct evidence that G6PD induction serves to maintain and regenerate the intracellular GSH pool. We used HeLa cell clones stably transfected with the human G6PD gene that display higher G6PD activity than the parent HeLa cells. Although the activities of glutathione peroxidase, glutathione reductase, and catalase were comparable in all strains, the concentrations of GSH were significantly higher in G6PD-overexpressing clones. A direct consequence of GSH increase in these cells is a decreased reactive oxygen species production, which makes these cells less sensitive to the oxidative burst produced by external stimuli. Indeed, all clones that constitutively overexpress G6PD exhibited strong protection against oxidants-mediated cell killing. We also observe that NF- κ B activation, in response to tumor necrosis factor- α treatment, is strongly reduced in human HeLa cells overexpressing G6PD.

Reactive oxygen species (ROS)¹ are produced inside the cell during oxidative metabolism, and they are involved in human

diseases such as arteriosclerosis, amyotrophic lateral sclerosis, Down's syndrome, re-perfusion shock syndrome, and cancer (1–3). Abnormal production of ROSs can damage macromolecules such as nucleic acids, lipids, and proteins and therefore participate in necrotic cell death and apoptosis (4). Inside the cell, ROSs are scavenged by both enzymatic and non-enzymatic antioxidant pathways. Reduced glutathione (GSH), a cysteine-containing tripeptide, is required to maintain the normal reduced state of the cells and to counteract all the deleterious effects of oxidative stress. GSH is synthesized inside the cells through a complex biochemical pathway composed of several well known enzymes (5). During the reaction of H₂O₂ scavenging, GSH is oxidized to GSSG by the enzyme GSH peroxidase (6). The reduction of GSSG to GSH is catalyzed by GSSG reductase, which uses NADPH as reducing potential. NADPH is also required for the formation of active catalase tetramers. This latter enzyme catalyzes the reduction of H₂O₂ in H₂O and O₂ (7, 8). Catalase is mainly peroxisomal, whereas the GSSG reductase/GSH peroxidase cycle is active in the cytoplasm. The NADPH required for the production of both GSH and catalase is produced by the pentose phosphate pathway (9, 10).

Glucose-6-phosphate dehydrogenase (G6PD), the first and rate-limiting enzyme of the pentose phosphate pathway, has long been regarded as important in the biosynthesis of the sugar moiety of nucleic acids (11). Until recently, the role of this housekeeping enzyme in the cell response to oxidative stress was limited to human erythrocytes that lack any other NADPH-producing route (12, 13). However, recent results have demonstrated that this enzyme also plays a protective role against ROSs in nucleated eucaryotic cells that possess alternative routes for the production of NADPH. Indeed, in the lower eucaryote *Saccharomyces cerevisiae*, mutants in the G6PD gene are sensitive to oxidants that specifically deplete the intracellular pool of GSH (14). Furthermore, mouse ES cells containing G6PD null mutation are uniquely sensitive to oxidants (15).

In every cell line so far tested, as well as in lymphocyte primary cultures, we demonstrated that G6PD expression is enhanced by oxidative stress induced by agents that either increase the intracellular concentration of O₂⁻ or decrease the GSH pool. The mechanism regulating G6PD expression appears to affect the rate of transcription initiation (16).

Here, we confirm that G6PD expression is induced by drugs that decrease the intracellular GSH pool; indeed, this increase is blocked by treatment with antioxidants that specifically replenish the intracellular GSH. We also observed that, rapidly after drug treatment, GSH decreases and G6PD is enhanced. When G6PD reaches maximal induction, GSH pool is restored, suggesting that GSH equilibrium could be dependent from G6PD expression.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: International Inst. of Genetics and Biophysics, Consiglio Nazionale delle Ricerche, Via Guglielmo Marconi 12, 80125 Naples, Italy. Tel.: 39-81-7257-248; Fax: 39-81-5936-123; E-mail: ursini@iigbna.iigb.na.cnr.it.

¹ The abbreviations used are: ROS, reactive oxygen species; G6PD, glucose-6-phosphate dehydrogenase; BSO, L-buthionine-(S,R)-sulfoximine; 6PGD, 6-phosphogluconate dehydrogenase; PDTC, pyrrolidine dithiocarbamate; NAC, N-acetylcysteine; DOC, sodium deoxycholate; DCFH-DA, dichlorofluorescein diacetate; DCF, dichlorofluorescein; TNF- α , tumor necrosis factor- α .

We have isolated and extensively characterized HeLa clones that overexpress G6PD and thus have increased G6PD activity. We observe that the activity of other enzymes in these cells, such as GSSG reductase, GSH peroxidase, catalase, or 6PGD (the second enzyme of pentose phosphate pathway) remain unchanged. Furthermore, these cells feature increased intracellular levels of GSH and consequently, lower ROS levels with respect to control cells. The G6PD-overexpressing cells exhibit a markedly decreased NF- κ B DNA binding activity, produced by the TNF- α -dependent ROS burst, and a marked increase in cellular resistance to apoptosis induced by hydrogen peroxide and TNF- α .

In conclusion, the results reported here suggest that G6PD is part of an inducible mechanism of cell response to oxidative stress; furthermore, they support the hypothesis that G6PD plays a key role in the control of intracellular reductive potential by increasing the intracellular content of glutathione, which, in turn, decreases ROS levels. This overall intracellular reduced environment may facilitate cellular protection against oxidant injuries.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—The human hepatoma Hep3B cell line and HeLa cell line were grown in Dulbecco's modified minimal essential medium. Media were supplemented with 10% fetal calf serum and 1% penicillin/streptomycin (Life Technologies, Inc.). The cells were cultured and stably transfected as described elsewhere (17). The plasmid pGD15neo has been described (18); it also contains a Neo gene driven by the SV40 early promoter. Control HeLa cells were transfected with the same plasmid devoid of all the human G6PD coding sequences. Cells were harvested 48 h after transfection, and clones were selected and cultured in 400 μ g/ml G418. No significant change in G6PD levels (mRNA, protein, and activity) was detectable between wild type and vector control-transfected clones (HeLa-neo).

Cell Treatment and Enzyme Assay—All the experiments described here were performed on actively growing cells. To summarize briefly, the medium was removed and cells were incubated in phosphate-buffered saline containing the indicated concentrations of H₂O₂ (Merck), NAC, PDTC (Sigma), and diamide (Calbiochem); the concentration of each oxidant used was empirically determined. After a 30-min incubation at 37 °C, cells were rinsed with phosphate-buffered saline and fresh medium was added.

G6PD activity was determined, as already described (19), by measuring the rate of production of NADPH. Since 6PGD, the second enzyme of the pentose phosphate pathway, also produces NADPH, both 6PGD and total dehydrogenase activity (G6PD+6PGD) were measured separately, as described elsewhere (20), in order to obtain accurate enzyme activity. G6PD activity was calculated by subtracting the activity of 6PGD from total enzyme activity.

Catalase, glutathione reductase, glutathione peroxidase activities, and total protein concentration were determined according to Aebi (21), Carlberg (22), Mannervick (23), and Bradford (24), respectively.

G6PD Electrophoresis—Cellulose acetate gel electrophoresis and staining for G6PD was carried out according to published methods (25), with the following modifications. (a) Gel running buffer was 85 mM Trizma base, pH 9.2, 35 mM glycine, 58 mM sucrose, 0.2 mM EDTA, 36.6 mM NADP. (b) Length of run was 90 min at 100 V at room temperature. (c) Gel staining solution was 0.1 mM Tris-Cl, pH 8.0, 0.2 mg/ml glucose 6-phosphate, 0.1 mg/ml NADP, 0.15 mg/ml dimethylthiazol-2-diphenyltetrazolium bromide, 0.1 mg/ml phenazine methosulfate, 20 mM MgCl₂.

Immunoblot—15 μ g of protein lysates were separated by SDS-polyacrylamide gel electrophoresis and electroblotted to nitrocellulose. Nitrocellulose filter was incubated with rabbit anti-human G6PD antibody supplied by U. Benatti (26) in 3% bovine serum albumin. For detection of rabbit antibodies, the filter was incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (Bio-Rad) and developed with a substrate of 0.5 mg/ml 4-chloronaphthol and 0.33% hydrogen peroxide in 0.1 M Tris-HCl. Immunoblots using an antibody raised against human β -actin (Amersham Corp.) were performed according to the manufacturer's instructions. They were revealed with the ECL kit from Amersham, and autoradiographs were recorded onto X-Omat AR films (Eastman Kodak Co.).

Electrophoretic Mobility Shift Assays—Gel shift assays were per-

formed using standard methods; a double-stranded κ B oligonucleotide, whose sequence has already been described (27), was used as probe. The oligonucleotide was labeled using Klenow enzyme. The radiolabeled probe was incubated with HeLa nuclear extract for 30 min at room temperature. For oligonucleotide competition analysis, a 100-fold molar excess of cold competitor was also added to the mixture. In order to carry out DNA binding assays in which antibodies were included, antibodies were added to the binding assay mixtures and incubated for 2 h on ice before the addition of radiolabeled probe. The p65 antibody was purchased from Santa Cruz Biotechnology. HeLa cell nuclear extracts were made using Lee's method (28). DOC treatment was performed by incubating the cytosolic fraction from unstimulated cells for 15 min with 0.8% DOC and 1% Nonidet P-40 before electrophoretic mobility shift assay (29). A Molecular Dynamics PhosphorImager™ system was used to analyze the gels.

Intracellular Glutathione Measurement—Intracellular reduced glutathione content was estimated using the Biotex GSH-400 enzymatic method (OXIS) in accordance with the manufacturer's instructions. Briefly, total cellular protein material (from 6×10^6 cells) was precipitated in 5% metaphosphoric acid and the resulting supernatant was used for the test. The level of GSH present in each cell type was calculated according to standard curves of increasing GSH concentrations.

Fluorescent Measurement of Intracellular ROS—Formation of ROS was measured using DCFH-DA according to Royall and Ischiropoulos (30). Cells (6×10^6) were pre-loaded with 5 mM DCFH (Molecular Probes) in the culture medium for 30 min. Measurement were carried out in duplicates using a FACScan (Becton Dickinson) flow cytometer. Dead cells and debris were excluded by forward/side scatter gating.

Induction of Apoptosis—Cells were plated 24 h before the assay at a density of 1×10^6 cells/10-cm plate. Apoptotic cell death was induced by H₂O₂ and TNF- α used at the indicated concentration. 24 h after induction of apoptosis, cells were washed in phosphate-buffered saline and then resuspended in the same buffer and fixed with ice cold 70% ethanol. Fixed cells were stained with 50 μ g/ml propidium iodide and DNA content was analyzed using a FACScan (Becton Dickinson) flow cytometer by red fluorescence. Apoptotic cells appear as a broad hypodiploid DNA peak preceding the peak of diploid DNA from viable cells. Cells containing a lower amount of DNA and a side scatter higher than that of G₀/G₁ cells were considered to be apoptotic (31, 32).

RESULTS

G6PD Expression Is Enhanced by GSH-depleting Drugs: This Effect Is Specifically Counteracted by NAC—We have already reported that several oxidative drugs, whose final effect is to decrease the intracellular GSH pool, enhanced the expression of G6PD and that this regulation is achieved at the levels of transcription of this gene (16). In the first experiment, human Hep3B cells were treated with diamide, a powerful sulfhydryl group-oxidizing agent that specifically causes the depletion of glutathione without any other observable effect (33). During the same experiment, we tested the antioxidant ability of both NAC and PDTC. NAC is a GSH precursor (34), whereas PDTC is a metal-chelating agent, which complexes Fe³⁺ and other metal ions; these, by means of the Fenton reaction, participate in the production of hydroxyl radicals from H₂O₂ (35). Hep3B cells were exposed to 500 μ M diamide for 30 min in phosphate-buffered saline; the buffer was then replaced with fresh medium. In the cases in which NAC (30 mM) or PDTC (500 μ M) were used, each antioxidant was added to the cells 1 h before diamide treatment. Two hour after cell treatments, G6PD activity was analyzed by both spectrophotometric assay and cellulose acetate gel electrophoresis. This last method allows a specific determination of G6PD activity without the interference of other NADPH-producing enzymes (25). During the same experiments, we also measured the levels of G6PD protein by immunoblot analysis of total cellular proteins probed with anti-G6PD antiserum (26). In the same immunoblot, β -actin protein levels was also measured as reference (Fig. 1). As reported in Table I, we found that G6PD activity was 2.1-fold increased in diamide-treated cells; this increase is abolished by NAC pretreatment (see also Fig. 1B for cellulose acetate gel electrophoresis analysis of G6PD activity). A similar result was

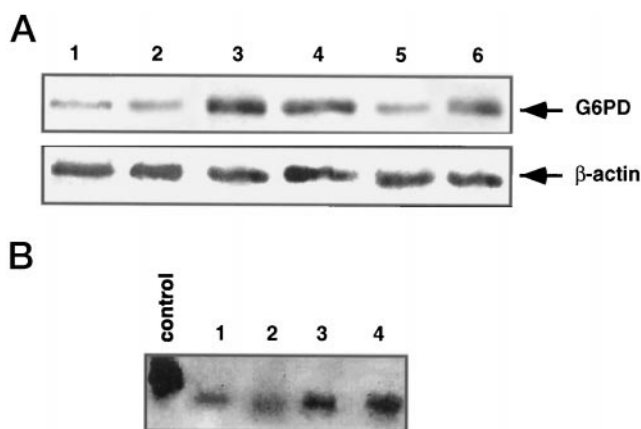


FIG. 1. Regulation of G6PD expression by oxidants and antioxidants. *A*, Western blot analysis of G6PD protein levels made with extracts of Hep3B cells treated with oxidants and/or antioxidants, as described under "Results". Protein extracts from Hep3B cells left untreated (lane 1) or treated with 30 mM NAC (lane 2), 500 μ M PDTC (lane 3), or 500 μ M diamide (lane 4), NAC + diamide (lane 5), and PDTC + diamide (lane 6) were analyzed. G6PD protein was detected with rabbit anti-human G6PD polyclonal antibodies. The membrane was then stripped, and ECL-Western blot analysis was performed with monoclonal antibody against β -actin to verify the loading. A representative experiment is presented. *B*, G6PD enzyme activity was estimated using cellulose acetate gel electrophoresis of cell lysates. 2 μ g of protein extracts from human blood cells (control lane) and Hep3B cells left untreated (lane 1) or treated with 30 mM NAC (lane 2), 500 μ M PDTC (lane 3), or 500 μ M diamide (lane 4) were analyzed using a staining method allowing the specific determination of G6PD activity. A representative experiment is presented.

TABLE I

Effects of oxidants and antioxidants on G6PD activity in Hep3B cells

Hep3B cells were treated for 30 min in phosphate-buffered saline with or without (No addition) 500 μ M diamide, 500 μ M BSO, or 500 μ M PDTC (listed in the first column) preceded or not (No addition) by pretreatment with either 30 mM NAC or 500 μ M PDTC for 1 h in growth medium (listed in the first row). The buffer was then replaced with fresh medium and cells were collected after 2 h. At the end of incubation period G6PD activity was determined and defined as nanomoles of reduced NADP/min/mg of protein as described under "Experimental Procedures." Results are given as mean \pm S.D. of three independent experiments. ND, not determined.

	No addition	NAC	PDTC
No addition	45.6 \pm 2.9	35.8 \pm 3.7	96.5 \pm 7.7
Diamide	94.6 \pm 4.3	37.7 \pm 5.6	95.6 \pm 8.0
BSO	93.9 \pm 2.6	39.9 \pm 2.9	90.8 \pm 7.5
PDTC	96.5 \pm 7.7	37.3 \pm 2.6	ND

observed when another GSH precursor, glutathione diethyl ester, was used instead of NAC (data not shown). PDTC does not counteract diamide effect but, rather, determines an enhancement of G6PD protein levels and of G6PD activity comparable to that observed with diamide (2.1-fold). This is not surprising since this compound can exert either antioxidant or pro-oxidant effects in different situations (36). Indeed, it was demonstrated that PDTC and other dithiocarbamates may cause oxidation of GSH by means of a non-radical mechanism (35). We further demonstrated that the PDTC-mediated increase of G6PD is also counteracted by NAC (Table I).

As shown in Fig. 1A, the results obtained by activity determination, perfectly parallel to what was observed using protein analysis. Hence, the GSH-mediated regulation of G6PD activity results from regulated levels of this protein. Identical results were obtained when BSO, an effective inhibitor of γ -glutamylcysteine synthetase (the enzyme that catalyzes the first step in biosynthesis of GSH) (6), was used in place of diamide to reduce the intracellular GSH content (Table I).

Results comparable to those reported in Table I were ob-

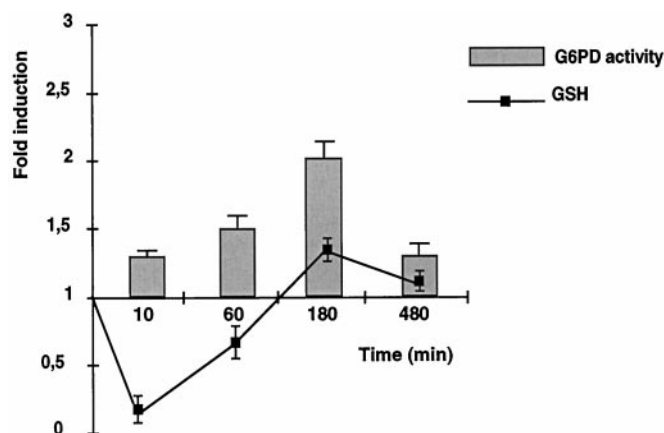


FIG. 2. Kinetic analysis of G6PD activity and GSH levels. Hep3B cells were treated with 500 μ M diamide as reported in Fig. 1 and collected at the indicated time points. G6PD activity and GSH levels were determined as described under "Experimental Procedures." The time point values reported in the graph represent the fold induction normalized to time 0, for each cellular type. Results are given as mean \pm S.D. of three independent experiments. Note the GSH replenishment soon after G6PD induction.

tained in HeLa cells after diamide or BSO treatments (data not shown).

Rapid G6PD Induction after Diamide Treatment Precedes GSH Replenishment—We then compared the kinetics of increase of G6PD activity and the levels of intracellular GSH after diamide treatment. Hep3B cells were treated with diamide as reported in Fig. 1; cells were collected at the indicated time points. As reported in Fig. 2, we observed a slight but reproducible enhancement of G6PD activity (1.3-fold) as soon as 10 min after drug treatment; simultaneously, GSH was dramatically reduced (80% reduction). As G6PD activity gradually increases, the levels of intracellular GSH had restored and, when G6PD reaches the maximum levels, GSH levels are 30% higher than in untreated control cells. 8 h after treatment, both G6PD activity and GSH levels returned to normal level.

These observations indicate that G6PD expression is inversely correlated to GSH intracellular levels and may also suggest that GSH replenishment may depend on G6PD expression.

Regulation of GSH Levels by G6PD Overexpression—In order to assess how G6PD expression may influence GSH levels, cell lines producing increased levels of G6PD were generated. Human HeLa cells were stably transfected with an eucaryotic expression vector containing the entire human G6PD gene (18). Western blot analysis revealed several cell clones carrying pGd15neo that had increased levels of G6PD expression. As reported in Fig. 3, two of these clones were analyzed in more detail. Clone HeLa-Gd3 and clone HeLa-Gd4 show 3-fold increase in G6PD protein and G6PD activity compared with vector-control transfected clones (HeLa-neo). The increased levels of G6PD protein were also confirmed by the increase in G6PD mRNA as determined by Northern blot analysis (data not shown).

Subsequently, we evaluated the effect of G6PD expression on the activities of the enzyme of GSSG recycling (GSSG reductase and GSH peroxidase), on the activity of H_2O_2 -detoxifying enzyme catalase and on 6PGD, the second enzyme of the pentose phosphate pathway. We also analyzed the levels of intracellular GSH. Overexpression and increased activity of G6PD have no significant effect on GSSG reductase, GSH peroxidase, catalase, and 6PGD activities; on the other hand, a 2.3-fold increase of GSH levels were detectable in both HeLa-Gd3 and HeLa-Gd4 clones with respect to control HeLa-neo cells (Table II).

Overexpression of G6PD Decreases the Intracellular Levels of ROSs—We then investigated whether the increased GSH levels associated with G6PD overexpression might have resulted from decreased levels of ROSs production in those cells. Therefore, we analyzed the ROS levels in control HeLa-neo, Gd3 (Fig. 3), and Gd4 (not shown) clones with DCFH-DA, which is converted to DCF by ROS-mediated oxidation (30). Cells stained with DCF were analyzed by fluorescent-activated cell sorting. The results reported in Fig. 4 clearly show a significant decrease in the intensity of DCF fluorescence resulting from the overexpression of G6PD (44% of the control cells).

The question remained as to whether the G6PD overexpression-mediated lower ROS intracellular levels resulted from a higher detoxification of ROSs due to elevated concentrations of reduced glutathione; alternatively, G6PD may have directly reduced the basic ROS formation and, as a consequence, increased the cellular concentration of glutathione. The production of ROSs was therefore analyzed in glutathione-depleted control HeLa-neo cells as well as Gd3 and Gd4 clones by using the *in vivo* conversion of DCFH-DA to fluorescent DCF. Gluta-

thione depletion was induced by 24-h incubation of the cells with 1 mM BSO, a glutathione synthesis inhibitor (6). This treatment depleted glutathione by >99% in all types of cells (data not shown) and increased the rate of ROS formation (Fig. 4). It is of interest that the G6PD-mediated lower rate of ROS formation, which was clearly detectable in non-BSO-treated cells, was almost completely abolished by the glutathione depletion. This supports the hypothesis that G6PD acts at the level of glutathione metabolism rather than the level of ROS formation.

TNF- α -mediated Activation of NF- κ B Is Attenuated by G6PD Overexpression—The ability of G6PD expression to decrease ROS levels was investigated using targets that are particularly sensitive to oxidative burst. We therefore analyzed the TNF- α -mediated activation of the transcription factor NF- κ B in HeLa cells together with Gd3 and Gd7 clones. Most inducers of NF- κ B seem to rely on the production of ROSs, as evidenced by the inhibitory effect of several antioxidants, including NAC (33). A causal link between ROS production and TNF- α -mediated NF- κ B induction was clearly demonstrated (27, 37). The activation of NF- κ B by TNF- α was analyzed by *in vitro* DNA binding and electrophoretic mobility shift assays. Nuclear extracts were prepared from control HeLa cells as well as Gd3 and Gd4 clones that were either left untreated or exposed to TNF- α . DNA binding assays were performed using a DNA probe encompassing the κ B motif (see “Experimental Procedures”). As seen in Fig. 4, 2-h treatment with 25–200 units/ml TNF- α induced, in control HeLa cells, the binding of a protein factor to κ B oligonucleotide. Competition experiments revealed that the binding to the radioactive κ B DNA was no more detectable when increasing concentration of nonradioactive κ B DNA were added to the binding mixture. A supershift band was also observed when the reaction mixture was incubated with an antibody that recognizes the p65/RelA subunit of NF- κ B. Hence, TNF- α induced the binding of NF- κ B to the κ B oligonucleotide in HeLa cells. The binding was greatly reduced in Gd3 cells (Fig. 5) and Gd4 cells (not shown), which overexpress G6PD with respect to control HeLa-neo cells. In order to determine the amount of inducible NF- κ B present in the cytoplasm of HeLa-neo and HeLa-Gd3 cells, we treated cytoplasmic fraction obtained from both cells with 0.8% DOC and 1% Nonidet P-40. This treatment allows the dissociation of I κ B- α from NF- κ B and thus restores the DNA binding ability of this factor. As shown in Fig. 5 (lanes 13 and 15), DOC treatment promotes similar levels of NF- κ B binding to κ B DNA in HeLa-neo and HeLa-Gd3. This indicates that the increase in G6PD activity did not alter the intrinsic ability of NF- κ B to bind DNA, but rather at least partially impairs the process that leads to the activation of this factor due to oxidative stress.

Sensitivity to Apoptotic Signals Is Influenced by Levels of G6PD Expression—Since G6PD overexpression raises the intracellular glutathione content and, as consequence, decrease the intracellular ROS production, we investigated whether this phenomenon can influence the survival of these cells exposed to oxidants burst. Apoptosis was produced in adherent cells treated with both hydrogen peroxide and TNF- α at the indi-

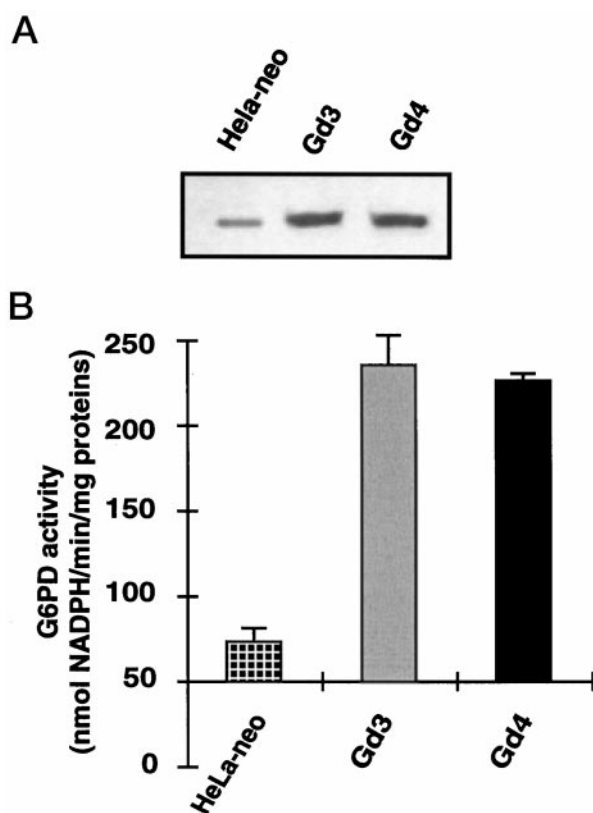


FIG. 3. Characterization of G6PD-overexpressing clones. A, Western blot analysis of G6PD protein levels in two HeLa cell clones overexpressing G6PD (Gd3 and Gd4) in comparison to control HeLa-neo cells. G6PD protein was detected with rabbit anti-human G6PD serum. B, G6PD activity measured in HeLa-neo control cells and HeLa-Gd3 and HeLa-Gd4 clones. Note the 3-fold increase of both G6PD activity and protein respect to HeLa-neo cells.

TABLE II

Activity of antioxidant enzymes in HeLa cells overexpressing or not overexpressing human G6PD

The enzyme activities are defined as following: G6PD and 6PGD, nanomoles of reduced NADPH/min/mg of protein; catalase, micromoles of H₂O₂/min/mg of protein assayed at 30 mM H₂O₂; GSHPx, nanomoles of NADPH/min/mg of protein assayed at 2 mM GSH; GR, nanomoles of NADPH/min/mg of protein; GSH, nmol/10⁶ cells. Numbers are means \pm S.D. of three independent experiments. GR, glutathione reductase; GSHPx, glutathione peroxidase.

	GSHPx	GR	Catalase	6PGD	GSH	G6PD
HeLa-neo	102.5 \pm 1.5	18.6 \pm 1.5	7.3 \pm 0.1	42.5 \pm 2.5	10.9 \pm 1.7	73.5 \pm 12.5
Gd3	90.5 \pm 16.5	19.6 \pm 4.1	6.6 \pm 0.7	38.8 \pm 0.7	22.4 \pm 2.6	235.5 \pm 37.0
Gd4	106.5 \pm 6.5	17.9 \pm 3.9	6.6 \pm 0.2	40.0 \pm 2.0	25.4 \pm 2.9	226.0 \pm 18.0

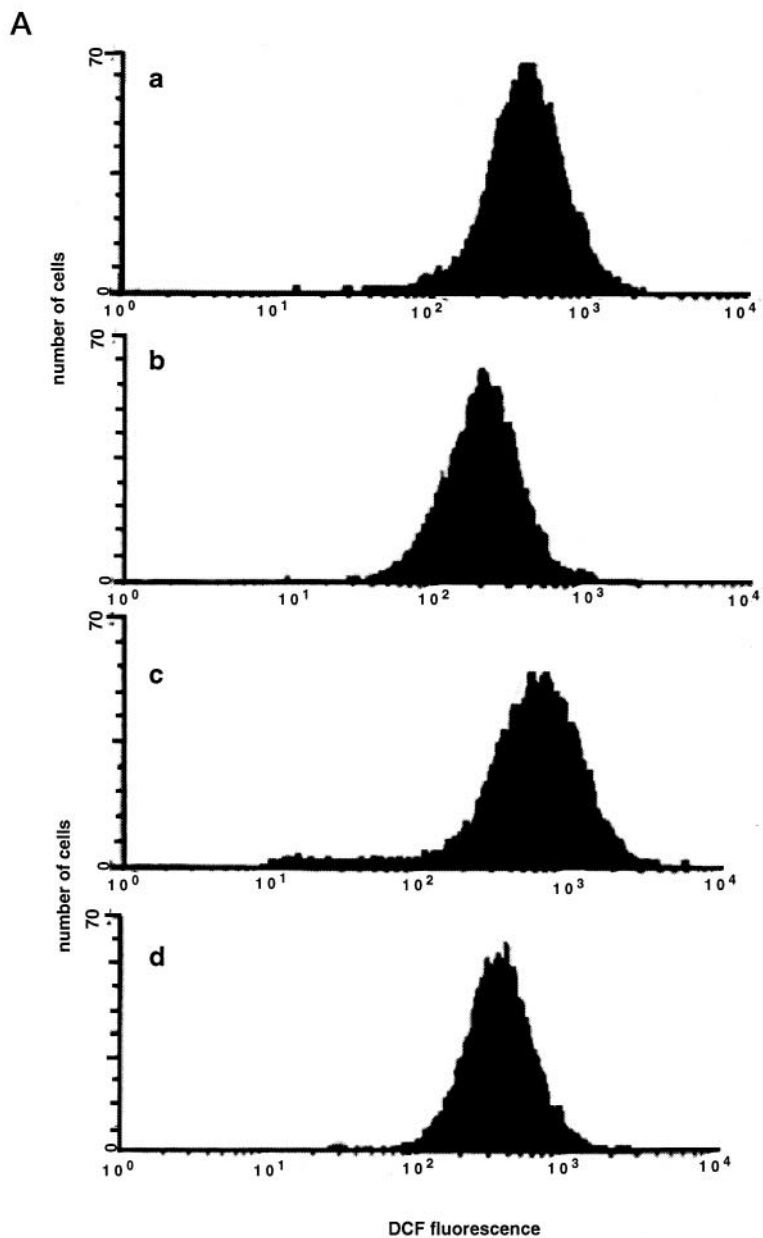
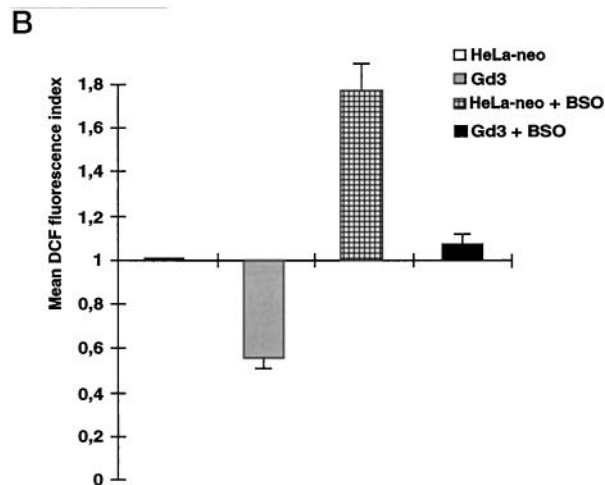


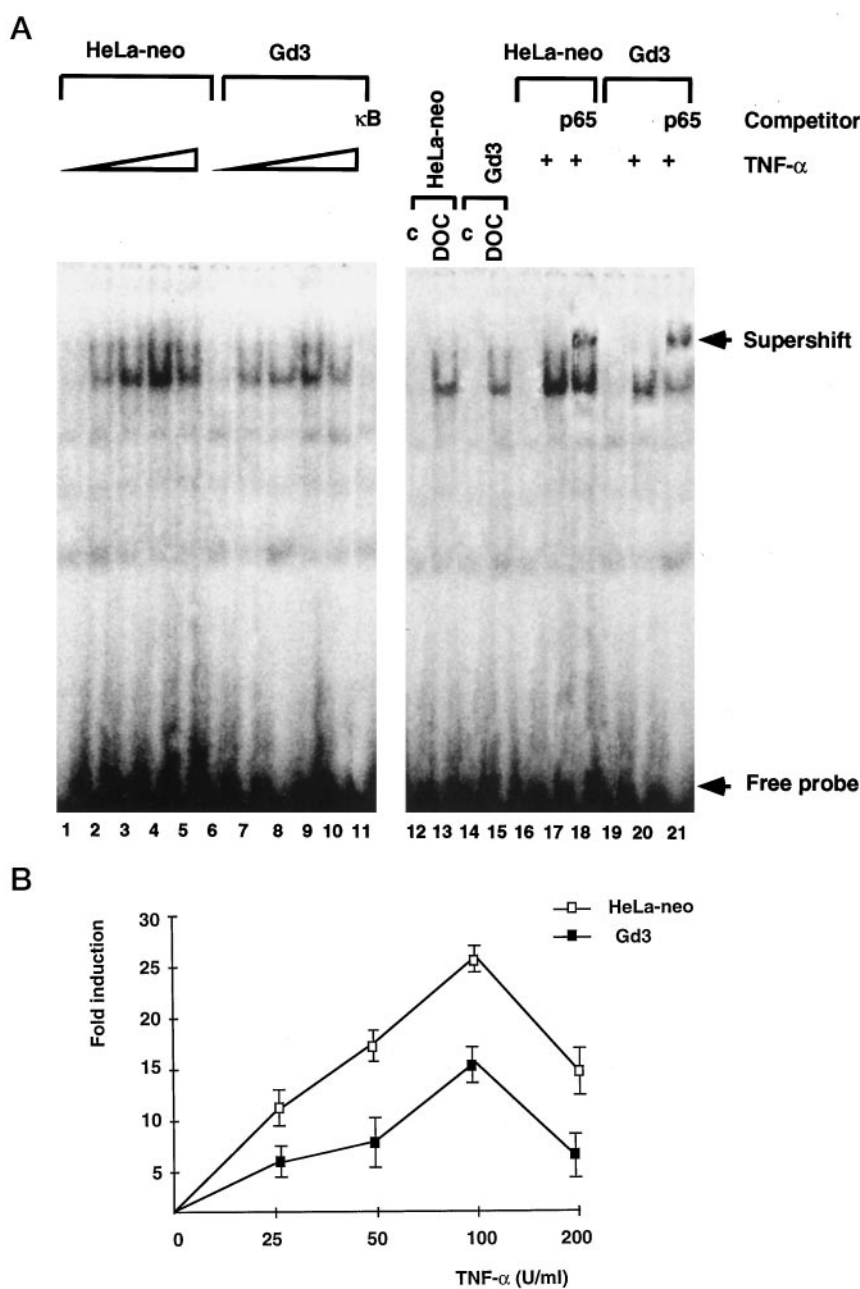
FIG. 4. G6PD overexpression in HeLa cells decreases the level of reactive oxygen species by acting on the glutathione metabolism. *In vivo* estimation of intracellular ROSs level by fluorescent-activated cell sorting analysis using DCFH-DA probe. DCF was measured as described under "Experimental Procedures." In *A* the results are presented as fluorescence histograms determined in a representative experiment. In *B* data are presented as DCF fluorescence index that was calculated as the ratio between the DCF fluorescence index of each sample and that measured in control HeLa-neo cells. Results are given as mean \pm S.D. of three independent experiments. *a*, control HeLa-neo cells; *b*, HeLa-Gd3 clones; *c* and *d*, control HeLa-neo cells and HeLa-Gd3 clone treated for 24 h with 1 mM BSO before intracellular ROSs determination. Similar results were obtained using HeLa-Gd4 clone (not shown).



cated concentrations. In this experiment, we also used parent HeLa cells as control. 24 h after treatment, the rate of apoptotic death was measured on cells fixed in 70% ethanol, stained with

propidium iodide, and subsequently analyzed for DNA content by flow cytometry. The data shown in Fig. 6 clearly demonstrate that cell death was strongly decreased in G6PD-overex-

FIG. 5. G6PD overexpression attenuates TNF- α -mediated NF- κ B binding activity. Nuclear extracts were prepared from HeLa-neo cells (lanes 1–5) and HeLa-Gd3 clones (lanes 6–10) treated with increased quantity (25–200 units/ml) of TNF- α . NF- κ B binding activity was analyzed by electrophoretic mobility shift assay as described under “Experimental Procedures.” In A, an autoradiograph of a typical experiment is presented. Unlabeled competitor (lane 11) or antiserum that recognize the p65 subunit of NF- κ B (lane 12) were added to cell extracts to control the specificity of the binding. In lanes 12–15, equal amounts of cytoplasmic extracts of either HeLa-neo and HeLa-Gd3 were incubated with κ B probe. The mixtures were either left untreated (lanes 12 and 14) or treated for 15 min with 0.8% DOC in the presence of 1% Nonidet P-40 before electrophoretic mobility shift assay (lanes 13 and 15). In B, results are reported as fold induction over the control (untreated cells). Results are given as mean \pm S.D. of three independent experiments. Note the parallel but attenuated TNF- α -mediated induction of NF- κ B binding activity in HeLa-Gd3 clone respect to control cells.



pressing cells (Gd3 and Gd4) in comparison to control cells (HeLa and HeLa-neo). Thus, the levels of G6PD have a dramatic effect on the susceptibility to apoptotic cell death induced by both agents.

DISCUSSION

We have already shown that G6PD expression is up-regulated by oxidants through a mechanism acting mainly on the rate of transcription of this gene (16). Here we show that this effect is specifically counteracted by NAC, an antioxidant that replenishes the GSH pool (35). Furthermore, we found that G6PD increase precedes full GSH restoration, through kinetic analysis of G6PD expression after drug treatment. G6PD overexpression in HeLa cells therefore determines an increase in GSH and a consequent decrease of intracellular ROSs. We show that G6PD expression controls intracellular GSH without interfering with the activity of the other enzymes involved in the peroxide/hydroperoxide-detoxifying pathway. As a result, cells overexpressing G6PD have an overall reduced state. Con-

sequently, downstream effects induced by ROSs burst are buffered in these cells. We found that G6PD overexpression interferes negatively with the activation of the transcription factor NF- κ B by TNF- α and decreases susceptibility to apoptotic signals induced by hydrogen peroxide and TNF- α .

It has been recently suggested that the primary physiological role of G6PD in mammalian cells is the defense against oxidative injuries (15, 38). The question remains as to how G6PD exerts this protective effect. Several pieces of evidence indicated that the formation of GSH from its oxidized form, GSSG, is dependent on NADPH produced by the pentose phosphate pathway and that this pathway can be activated in response to GSH depletion (39). In agreement with these observations, we suggest that levels of G6PD may have a dominant role in the control of output of GSH and thus in the maintaining of an intracellular redox potential. An alternative explanation could be that G6PD is involved in a mechanism that promotes glutathione storage. Therefore, the exact biochemical mechanism underlying the antioxidant properties of G6PD enzyme require

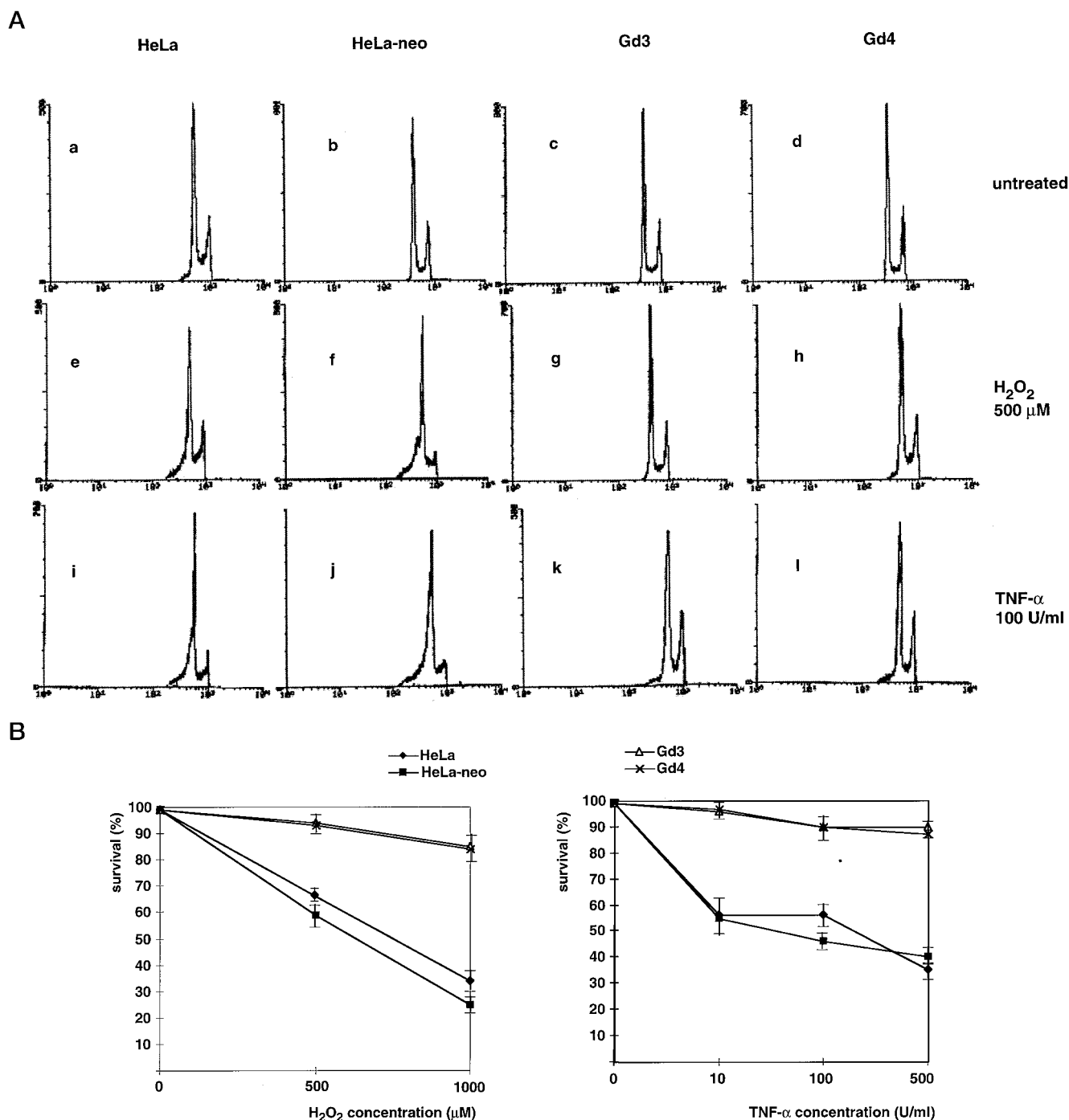


FIG. 6. Flow cytometry analysis of H₂O₂ and TNF- α induced apoptosis in HeLa, HeLa-neo, HeLa-Gd3, and HeLa-Gd4 clones. After incubation with either H₂O₂ and TNF- α for 24 h, control and treated cells were fixed in 70% ethanol, stained with propidium iodide, and subsequently analyzed for DNA content by flow cytometry. **A**, representative flow cytometry determinations of apoptotic cell death. DNA content frequency histograms are reported. *a, e, and i*, HeLa cells; *b, f, and j*, HeLa-neo clone; *c, g, and k*, Gd3-neo clone; *d, h, and l*, Gd4-neo clone. *a-d*, untreated cells; *e-f*, cells treated with 500 μ M H₂O₂; *i-l*, cells treated with 100 units/ml TNF- α . **B**, rate of apoptotic cell death induced by hydrogen peroxide and TNF- α in HeLa, HeLa-neo, HeLa-Gd3, and HeLa-Gd4 cells. Results are given as mean \pm S.D. of three independent experiments.

a more thorough investigation.

Besides the enzymes directly involved in the production and detoxification of ROSs, several proteins have been shown to decrease the net intracellular generation of ROSs and consequently interfere with the downstream effects of oxidative stress. Some proteins, such as bcl-2, have been proposed to control intracellular ROSs levels in a GSH-independent way (40). Other, such as the small heat shock protein hsp27, lead to a decreased production of ROSs as derived from the increased intracellular GSH content (41). In this last case, the hsp27-de-

pendent G6PD enhanced expression was proposed as an explanation of this phenomenon.²

Furthermore, decreased ROSs production has been reported in cells with constitutive decreased expression of transaldolase, the key enzyme of nonoxidative branch of pentose phosphate pathway (42), or in cells overexpressing GSH peroxidase (43). In both cases lower ROSs were derived from an increased

² A. P. Arrigo, personal communication.

GSH/GSSG ratio. In this paper we show that G6PD shares the same properties, but, more interestingly, it is subject to rapid up-regulation in response to oxidative stress. This could be a general rule, since we have already shown that G6PD expression is increased in several human cell lines by drugs that ultimately lead to GSH depletion (16, 17). Furthermore, we have recently demonstrated that, in primary cultures of human and bovine lymphocytes, pesticide-mediated genotoxic effects are coupled to oxidative stress and G6PD up-regulation (44, 45). However, as G6PD enzyme levels were found to be positively regulated by a number of stimuli, such as platelet-derived growth factor (20) and ischemia reperfusion (46, 47), it would be interesting to investigate whether G6PD-enhanced expression can always be related to change in the cellular redox conditions.

It is well known that G6PD is expressed in all cell types, although at varying levels (19). We suggest that G6PD serves as a critical determinant of tissue- and cell type-specific sensitivity to oxidative stress signals. However, we cannot exclude the possibility that other pentose phosphate pathway enzymes, or enzymes involved in the control of peroxide/hydroperoxide scavenging pathway, may have an alternative role to G6PD in different cell types.

Acknowledgments—We thank André Patrick Arrigo and Guido Rossi for the critical review of the manuscript and helpful advice. We also thank Maria Terracciano for skillful technical assistance.

REFERENCES

- Cerutti, P. A. (1985) *Science* **227**, 375–381
- Ames, B. N., Shigenaga, M. K., and Hagen, T. M. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 7915–7922
- Martin, G. M., Austad, S. N., and Johnson, T. E. (1996) *Nat. Genet.* **13**, 25–33
- Buttke, T. M., and Sandstrom, P. A. (1994) *Immunol. Today* **15**, 7–10
- Meister, A. (1988) *J. Biol. Chem.* **263**, 17205–17208
- Meister, A., and Anderson, M. E. (1983) *Annu. Rev. Biochem.* **52**, 711–760
- Kirkman, H. N., and Gaetani, G. F. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **81**, 4343
- Kirkman, H. N., Galiano, S., and Gaetani, G. F. (1987) *J. Biol. Chem.* **262**, 660–666
- Cohen, G., and Hochstein, P. (1963) *Biochemistry* **2**, 1420–1428
- Gaetani, G. F., Galiano, S., Canepa, L., Ferraris, A. M., and Kirkman, H. N. (1989) *Blood* **23**, 334–339
- Luzzatto, L., and Metha, A. (1995) in *The Metabolic Basis of Inherited Disease* (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds) pp. 3367–3398, MacGraw Hill, New York
- Vulliamy, T., Mason, P., and Luzzatto, L. (1992) *Trends Genet.* **8**, 138–143
- Beutler, E., Vulliamy, T., and Luzzatto, L. (1996) *Hum. Genet.* **29**, 49–56
- Nogae, I., and Johnston, M. (1990) *Gene (Amst.)* **96**, 161–169
- Pandolfi, P. P., Sonati, F., Rivi, R., Mason, P., Grosveld, F., and Luzzatto, L. (1995) *EMBO J.* **14**, 5209–5215
- Ursini, M. V., Parrella, A., Rosa, G., Salzano, S., and Martini, G. (1997) *Biochem. J.* **323**, 801–806
- Ursini, M. V., Lettieri, T., Braddock, M., and Martini, G. (1993) *Virology* **196**, 338–343
- Chen, E. Y., Cheng, A., Lee, A., Kuang, W. J., Weigelt, L., Green, P., and Schlessinger, D. (1991) *Genomics* **10**, 792–800
- Battistuzzi, G., D'Urso, M., Toniolo, D., Persico, M. G., and Luzzatto, L. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 1465–1469
- Tian, W. N., Pignatari, N. J., and Stanton, C. R. (1994) *J. Biol. Chem.* **269**, 14798–14805
- Aebi, H. (1984) *Methods Enzymol.* **105**, 121–126
- Carlberg, I., and Mannervick, B. (1985) *Methods Enzymol.* **113**, 485–490
- Mannervick, B. (1985) *Methods Enzymol.* **113**, 490–495
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Town, M., Athanasiou-Metaxa, M., and Luzzatto, L. (1990) *Somat. Cell Mol. Genet.* **16**, 97–108
- Benatti, U., Morelli, A., Meloni, T., Sparatore, B. S. F., Michetti, M., Melloni E., Pontremoli, S., and De Flora, A. (1981) *FEBS Lett.* **29**, 225–229
- Schreck, R., Meier, B., Mannel, D. N., Droge, W., and Baeuerle, P. A. (1992) *J. Exp. Med.* **175**, 1181–1194
- Lee, K. A. W., Bindereif, A., and Green, M. R. (1988) *Gene Anal. Tech.* **5**, 22–31
- Hansen, S. K., Baeuerle, P. A., and Blasi, F. (1994) *EMBO J.* **14**, 2593–2603
- Royall, A. J., and Ischiropoulos, H. (1993) *Arch. Biochem. Biophys.* **302**, 348–355
- Sherwood, S. W., and Schimke, R. T. (1995) *Methods Cell Biol.* **46**, 77–97
- Darzynkiewicz, Z., Bruno, S., Del Bino, G., Gorczyca, W., Hotz, M. A., Lassota, P., and Traganos, F. (1992) *Cytometry* **13**, 795–808
- Kosover, E. M., and Kosover, N. S. (1969) *Nature* **224**, 117–120
- Schreck, R., Rieber, P., and Baeuerle, P. A. (1991) *EMBO J.* **10**, 2247–2258
- Ziegler, D. M. (1991) in *Oxidative Stress: Oxidants and Antioxidants* (Sies, H., ed) pp. 85–97, Academic Press, Orlando, FL
- Nobel, C. S. I., Kimland, M., Lind, B., Orrenius, S., and Slater, A. F. G. (1995) *J. Biol. Chem.* **270**, 26202–26208
- Schreck, R., and Baeuerle, P. (1994) *Methods Enzymol.* **234**, 151–163
- Martini, G., and Ursini, M. V. (1996) *BioEssays* **18**, 1–8
- Clancy, R. M., Levartovsky, D., Leszczynska-Piziak, J., Yegudin, J., and Abramson, S. B. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 3680–3684
- Kane, D. J., Sarafian, T. A., Anton, R., Hahn, H., Gralla, E. B., Valentine, J. S., and Ord, T. (1993) *Science* **262**, 1274–1277
- Mehlem, P., Kretz-Remy, C., Preville, X., and Arrigo, A. P. (1996) *EMBO J.* **15**, 2695–2706
- Banki, K., Hutter, E., Colombo, E., Gonchoroff, N. J., and Perl, A. (1996) *J. Biol. Chem.* **271**, 32994–33001
- Kretz-Remy, C., Mehlen, P., Mirault, M. E., and Arrigo, A. P. (1996) *J. Cell Biol.* **133**, 1083–1093
- Lioi, M. B., Scarfi, M. R., Santoro, A., Barbieri, R., Zeni, O., Di Bernardino, D., and Ursini, M. V. (1998) *Mutat. Res.* **403**, 13–20
- Lioi, M. B., Scarfi, M. R., Santoro, A., Barbieri, R., Zeni, O., Salvemini, F., Di Bernardino, D., and Ursini, M. V. (1998) *Environ. Mol. Mutagen.* **32**, 39–46
- Nishiki, K., Jamieson, D., Oshino, N., and Chance, B. (1976) *Biochem. J.* **160**, 343–355
- Kletzien, R. F., Harris, P. K., and Foellmi, L. A. (1994) *FASEB J.* **8**, 174–181

**Enhanced Glutathione Levels and Oxidoresistance Mediated by Increased
Glucose-6-phosphate Dehydrogenase Expression**
Francesca Salvemini, Annamaria Franzé, Angela Iervolino, Stefania Filosa, Salvatore
Salzano and Matilde Valeria Ursini

J. Biol. Chem. 1999, 274:2750-2757.
doi: 10.1074/jbc.274.5.2750

Access the most updated version of this article at <http://www.jbc.org/content/274/5/2750>

Alerts:

- [When this article is cited](#)
- [When a correction for this article is posted](#)

[Click here](#) to choose from all of JBC's e-mail alerts

This article cites 45 references, 14 of which can be accessed free at
<http://www.jbc.org/content/274/5/2750.full.html#ref-list-1>