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Enhanced expression of glucose-6-phosphate dehydrogenase in human cells sustaining oxidative stress

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Recent reports have demonstrated that glucose-6-phosphate dehydrogenase (G6PD) activity in mammalian cells is necessary in order to ensure cell survival when damage is produced by reactive oxygen intermediates. In this paper we demonstrate that oxidative stress, caused by agents acting at different steps in the biochemical pathway controlling the intracellular redox status, determines the increase in G6PD-specific activity in human cell lines of different tissue origins. The intracellular level of G6PD-specific mRNA also increases, with kinetics compatible with the induction of new enzyme synthesis. We carried out experiments in which cells were exposed to oxidative stress in the presence of inhibitors of protein or RNA synthesis. These demonstrated that increased G6PD expression is mainly due to an increased rate of transcription, with a minor but significant contribution of regulatory mechanisms acting at post-transcriptional levels. These results provide new information on the defence systems that eukaryotic cells possess in order to prevent damage caused by potentially harmful oxygen derivatives.

INTRODUCTION

Reactive oxygen intermediates (ROIs) are produced inside the cell during oxidative metabolism, and they are probably involved in human diseases such as atherosclerosis, amyotrophic lateral sclerosis, Down's syndrome, reperfusion shock syndrome and cancer, as well as in ageing [1–3]. ROIs are scavenged by both enzymic and non-enzymic antioxidant pathways. The enzymic pathway consists, essentially, of two steps: the dismutation of O_2^- to H_2O_2 catalysed by superoxide dismutases and the conversion of H_2O_2 into H_2O catalysed by glutathione peroxidase and catalase. The second step requires NADPH for both the production of GSH through glutathione reductase [4], and the formation of active catalase tetramers [5,6]. An important source of NADPH is glucose-6-phosphate dehydrogenase (G6PD), the first and rate-limiting enzyme of the pentose phosphate pathway [7].

The role of G6PD in the cell response to oxidative stress is well established in human erythrocytes [7]. Within these cells, which have a very limited biochemical apparatus and where gene expression cannot be regulated because a nucleus is lacking, G6PD is the only source of NADPH; furthermore, an impaired production of this reductive agent directly affects the production of both GSH and catalase [8,9], thus inhibiting both pathways that dispose of hydrogen peroxide. As a result, genetically determined deficiency of G6PD activity is associated with haemolytic anaemia upon exposure to oxidative stress [10].

Recent genetic analysis of G6PD mutations also indicates a protective role against ROIs for nucleated eukaryotic cells possessing alternative routes for the production of NADPH. Indeed, in the lower eukaryote *Saccharomyces cerevisiae*, mutants in the G6PD gene are sensitive to oxidants that specifically deplete the intracellular pool of GSH [11]. Furthermore, mouse ES cells containing G6PD null mutation are uniquely sensitive to oxidants [12].

However, although the *Escherichia coli zwf* gene coding for G6PD is part of a well-studied regulon activated in response to oxidative stress [13], very little is known about the effect of oxidative stress on G6PD expression in eukaryotic cells. Therefore in this paper we have looked into the effects of oxidants on G6PD expression in human cell lines originating from different tissues. We found that, after briefly exposing the cells to hydrogen peroxide, both G6PD-specific enzyme activity and mRNA are rapidly and transiently enhanced. Transient induction of G6PD expression is also observed after treatment with agents that either increase the intracellular concentration of O_2^- or reduce the GSH intracellular content. In contrast, treatment with aminotriazole (AMT), a drug that completely inhibits catalase activity, does not affect G6PD expression. The mechanism regulating G6PD expression appears to affect the rate of transcription initiation. These observations lead us to suggest that G6PD is part of an inducible mechanism protecting eukaryotic cells against oxidative damage.

EXPERIMENTAL

Cell lines and culture conditions

The human hepatoma HepG2 and Hep3B cell lines were grown in Dulbecco's modified minimal essential medium; the human Jurkat T-cells were grown in RPMI1640 medium. Media were supplemented with 10 % (w/v) foetal-calf serum and 1 % (w/v) penicillin/streptomycin (GIBCO).

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 PBS containing the indicated concentrations of H_2O_2 (Merck), phenazine methosulphate (PMS), menadione, AMT, buthionine sulphoximide (BSO), *N*-

Abbreviations used: G6PD, glucose-6-phosphate dehydrogenase; ROI, reactive oxygen intermediate; AMT, aminotriazole; PMS, phenazine methosulphate; BSO, buthionine sulphoximide; NAC, *N*-acetylcysteine; PDTC, pyrrolidine dithiocarbamate; 6PGD, 6-phosphogluconate; MBCl, monochlorobimane; HO, haem oxygenase.

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acetylcysteine (NAC), pyrrolidine dithiocarbamate (PDTC) (Sigma) and diamide (Calbiochem); the concentration of each oxidant used was empirically determined. The conditions chosen for the compounds in each cell line were the best for G6PD induction and the least life-threatening to the cultures. After 30 min incubation at 37 °C, cells were rinsed with PBS and fresh medium was added. Cell viability was determined at the end of each treatment using the Trypan Blue exclusion assay; cell survival was at least 95 % in all tested cell lines. In the case of cells growing in suspension (Jurkat), living cells were purified by centrifugation on a Ficoll Hypaque (Pharmacia) density gradient generated at 400 ***g*** for 30 min. Actinomycin D and cycloheximide (both from Sigma) were added to a final concentration of 10 μg/ml or 20 μg/ml respectively as previously reported [14,15].

G6PD activity was determined, as already described [16], by measuring the rate of production of NADPH. Since 6-phosphogluconate (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, in order to obtain accurate enzyme activity for G6PD [17]. G6PD activity was calculated by subtracting the activity of 6PGD from total enzyme activity.

Catalase activity and total protein concentration were determined according to Aebi [18] and Bradford [19] respectively.

Intracellular GSH levels were determined as described previously [20]. Cells were stained with 1 mM monochlorobimane (MBCI; Molecular Probes) for 1 h at 37 °C and in the absence of light; the staining reaction was stopped by the addition of cold PBS. Samples were then analysed using a FACS Star^{Plus} (Becton Dickinson).

Analysis of mRNA

Total RNA was prepared by the single-step acid guanidinium thiocyanate/phenol/chloroform method [21]. Northern-blot analysis was performed according to standard methods [22]. G6PD and β-actin human cDNA probes used for the hybridization have been previously described ([23] and [21] respectively); a human haem oxygenase (HO) probe was obtained by reverse transcriptase PCR amplification [24] of a 150 bp cDNA fragment whose sequence has been previously reported [25]. Quantitative data were obtained by analysis of Northern-blot experiments with a Phosphor-Imager (Molecular Dynamics).

RESULTS AND DISCUSSION

In order to assess whether oxidants have any effect on G6PD expression in eukaryotic cells, a pilot experiment was performed on Jurkat, a lymphoma-derived human cell line. We exposed aliquots of Jurkat cells to increasing H_2O_2 concentrations, as described in the Experimental section. After 30 min the PBS was replaced with fresh medium and the cells were further incubated at 37 °C for variable times (6, 12 and 24 h). Since high doses of H_2O_2 are toxic to the cells, cell viability in each sample was determined at the end of the incubation period. In the untreated control sample, 95 % of cells were viable, and viability decreased to 75 % in the sample of cells collected 24 h after treatment with 1 mM H_2O_2 (Table 1). As a further precaution to avoid the interference of regulatory circuits dealing with cell death, the cell suspensions collected at each time/concentration point were separated on a Ficoll gradient to purify living cells. The yield of this procedure was > 99 % Trypan Blue-negative cells. G6PD specific activity was then determined on these highly enriched preparations of living cells. As shown in Table 1, an increase in G6PD-specific activity was detected 6 h after treatment with

Table 1 Effect of hydrogen peroxide on G6PD activity and cell viability

G6PD specific activity was determined on viable cells purified on Ficoll gradient. Units of G6PD are defined as μmol of $NADP^+$ reduced/min per mg of protein. The percentage of viable cells was determined from aliquots removed prior to Ficoll purification.

Time ...	6 h		12 h		24 h	
H_2O_2 added	G6PD activity	Cell killing (%)	G6PD activity	Cell killing (%)	G6PD activity	Cell killing (%)
None	0.23	6	0.23	6	0.24	14
50 μM	0.23	6	0.25	6	0.23	14
200 μM	0.51	11	0.24	9	0.23	16
600 μM	0.35	10	0.24	14	0.28	14
1 mM	0.33	15	0.20	10	0.19	25

200 μM H_2O_2 and this increase declined with higher peroxide concentrations and longer incubations in fresh medium after H_2O_2 treatment.

Other pilot experiments, similar to the one described above, were performed with several human cell lines in order to determine the H_2O_2 doses capable of increasing G6PD activity without reducing cell viability. In all cases 200 μM H_2O_2 was found to be optimal.

The kinetics of induction of G6PD activity was determined in HepG2, Hep3B and Jurkat cells after treatment with 200 μM H_2O_2. As shown in Figure 1, we found an increase in G6PD activity as early as 1 h after H_2O_2 addition in all cases; elevated levels of G6PD activity persisted for at least 6 h.

We went on to investigate whether the increase in G6PD activity after H_2O_2 treatment is a consequence of increased G6PD mRNA accumulation, or whether it is due to some post-translational control of G6PD activity. Consequently, we prepared total RNA [21] from HepG2, Hep3B and Jurkat cells at various times after H_2O_2 treatment and we determined the levels of G6PD and β-actin mRNA by Northern-blot analysis. To assess the effectiveness of H_2O_2 treatment and to compare the kinetics of induction with a well-characterized stress gene transcriptionally induced by H_2O_2, the same filters were also hybridized with a probe encoding human HO [26].

A substantial enhancement in the level of G6PD-specific mRNA was observed as a consequence of H_2O_2 treatment, but no detectable increase was observed in the cells treated only with PBS and incubated for the same time (results not shown). The increase in the H_2O_2-treated samples ranges between 2- and 3-fold; it is detectable as early as 1 or 2 h after treatment, and starts to decline at 8 h (Figure 2).

As shown in Figure 2, G6PD and HO are co-ordinately induced by H_2O_2, with the specific mRNAs reaching approximately similar levels of induction, although the expression of β-actin is not affected by H_2O_2 treatment. Induction of G6PD mRNA accumulation with comparable kinetics was observed in a human fibroblast cell line (WI38) as well as in HeLa cells treated with H_2O_2 (results not shown).

We then considered whether drugs that act at different steps in the biochemical pathway of ROI detoxification are equally active in the induction of G6PD expression. We used either diamide or BSO, which are both known to decrease the cellular GSH content [27,28], or menadione and PMS, that both result in the generation of O_2^- (superoxide anions) that rapidly undergo either spontaneous or enzymic (mediated by superoxide dismutase) dismutation to H_2O_2 [29]. Hep3B, HepG2 and Jurkat cells were

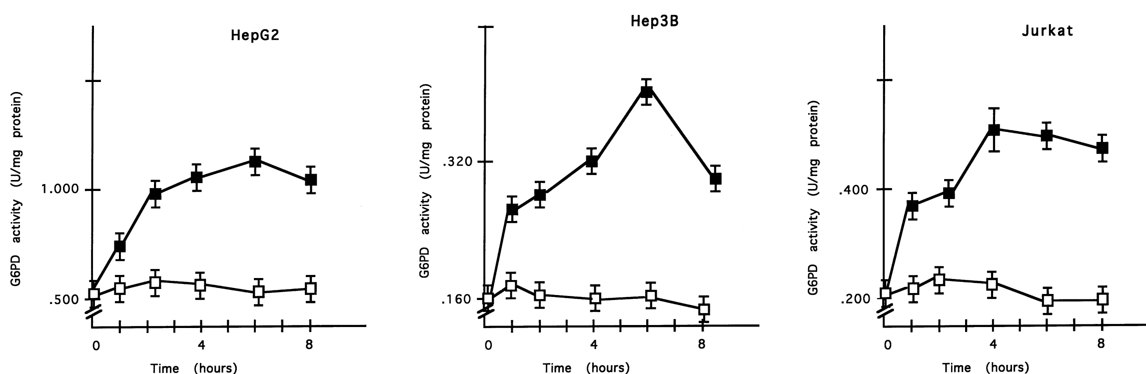

Figure 1 Time course of G6PD activity after H_2O_2 treatment

HepG2, Hep3B and Jurkat cell lines were treated (■) or left untreated (□) for 30 min with 200 μM H_2O_2 in PBS. G6PD activity was determined at various times after treatment as described in the Experimental section. Units (U) of G6PD are defined as μmol of $NADP^+$ reduced/min. Results are means ± S.D. of two or more independent determinations.

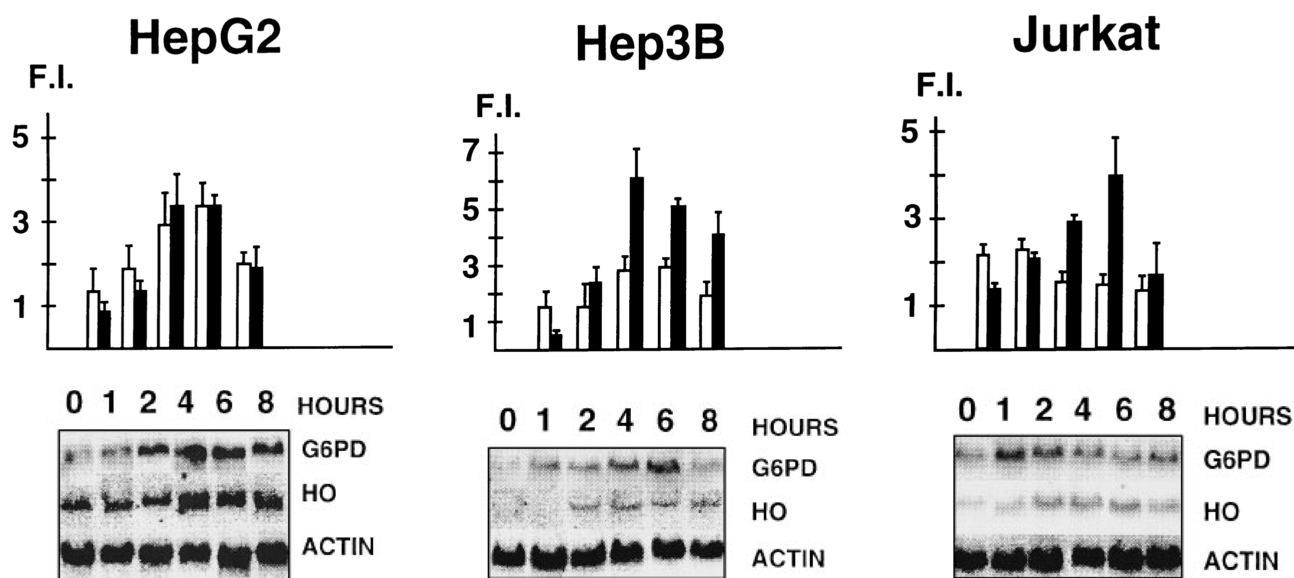

Figure 2 Time course of G6PD and HO mRNA accumulation after H_2O_2 treatment

The levels of G6PD (open bars), HO (solid bars), and β-actin mRNAs were determined at various times after H_2O_2 treatment by Northern-blot hybridization of total RNA extracted from HepG2, Hep3B and Jurkat cell lines. Total RNA (20 μg) was loaded on to each lane. The values indicated in the graph of each panel are quantitative independent experiments (at least three) in which data were first normalized for each time point relative to the corresponding time 0 and then corrected for the β-actin signal of hybridization; F.I., fold induction. A representative Northern blot hybridized with the three probes is shown in the box within each panel. The bands, of the expected size, correspond to G6PD-, HO- and β-actin-specific mRNAs, as indicated.

treated with these compounds and, 2 h later, G6PD activity was measured. At the same time we analysed the levels of intracellular GSH, the major cellular antioxidant. As shown in Table 2, increased G6PD activity was observed in HepG2, Hep3B and Jurkat cells, with a parallel decrease in the intracellular GSH content. Furthermore, we observed that, in Hep3B cells, the diamide and BSO-dependent stimulation of G6PD activity is blocked by cellular pre-treatment with the antioxidant NAC, which is a GSH precursor (results not shown). Instead, when we used the metal chelator PDTC, the diamide-dependent increase in G6PD expression was not affected (results not shown).

Catalase constitutes the alternative cellular pathway to GSH reductase/peroxidase for H_2O_2 scavenging. This enzyme, which is mainly peroxisomal, has a protective effect against oxidative stress, particularly in the avoidance of lipid oxidation [30,31]. Catalase activity can be completely inhibited by AMT; this inhibition predisposes the cell to degenerative phenomena associated with increased cellular oxidation, without reducing the intracellular GSH content [32,33]. We analysed the effect of the catalase inhibitor AMT in Hep3B cells, since, in hepatoma-derived cells, the effect of this drug is well documented [33]. Cells were treated with 20 mM AMT and G6PD activity in extracts of treated cells was determined at different intervals after the addition of the drug. We also measured catalase activity in order to assess the effectiveness of AMT treatment. As reported in Table 3, no detectable increase in G6PD activity was observed in Hep3B cells in which catalase specific activity was reduced to undetectable levels. Incubation of cells with AMT could be continued for up to 24 h without further variations of G6PD activity (results not shown). In accordance with what has already

Table 2 Effect of oxidants on G6PD activity and GSH levels

Hep3B cells were treated for 30 min with 500 μM diamide, BSO, menadione or PMS in PBS. HepG2 and Jurkat cells were treated with 1 mM oxidant concentration. Control cells were only treated with PBS. G6PD activity was determined 2 h after treatment and is expressed as units/mg of protein. Results are means ± S.D. of at least three separate experiments. GSH was measured by fluorescence-activated sell sorting analysis of MBCI-stained cells. Result are given as the percentage of untreated control. N.D., not determined.

	Hep3B		HepG2		Jurkat	
Cells ...	G6PD activity	GSH (% of control)	G6PD activity	GSH (% of control)	G6PD activity	GSH (% of control)
Control	0.18 ± 0.01	100	0.43 ± 0.03	100	0.28 ± 0.05	100
Diamide	0.48 ± 0.01	29	0.94 ± 0.02	50	0.57 ± 0.02	57
BSO	0.43 ± 0.01	47	0.76 ± 0.03	64	0.75 ± 0.03	33
PMS	0.44 ± 0.01	N.D.	0.81 ± 0.01	N.D.	0.61 ± 0.02	N.D.
Menadione	0.47 ± 0.02	44	0.86 ± 0.02	70	0.56 ± 0.02	71

Table 3 Effect of AMT on G6PD and catalase activity

Hep3B cells were incubated in medium containing 20 mM AMT. Control cells were incubated with control solvent. G6PD activity and catalase activity were determined at various times after treatment as described in the text. Units are defined as follow: G6PD, μmol of $NADP^+$ reduced/min; catalase, μmol of H_2O_2 consumed/min. Results are means ± S.D. of three separate experiments.

	G6PD activity (unit/mg of protein)		Catalase activity (units/mg of protein)	
Time (h)	Control	AMT	Control	AMT
1	0.19 ± 0.03	0.24 ± 0.02	39.5 ± 4.8	4.65 ± 1.55
2	0.17 ± 0.01	0.17 ± 0.02	35.5 ± 4.5	< 2
6	0.21 ± 0.03	0.20 ± 0.05	30.1 ± 5.9	< 2

Table 4 Time course of G6PD mRNA levels and G6PD activity in Hep3B cells treated with diamide

Cells were treated as in the legend to Figure 3. Relative G6PD mRNA levels represent arbitrary units normalized to β-actin mRNA levels. G6PD activity was determined as described in the Experimental section and is expressed as unit/mg of protein. Data points are means ± S.D. of three or more independent determinations.

Time (h)	Relative G6PD mRNA	G6PD activity
0	56 ± 30	0.18 ± 0.01
1	128 ± 2	0.44 ± 0.01
2	124 ± 16	0.47 ± 0.01
4	125 ± 5	0.42 ± 0.01

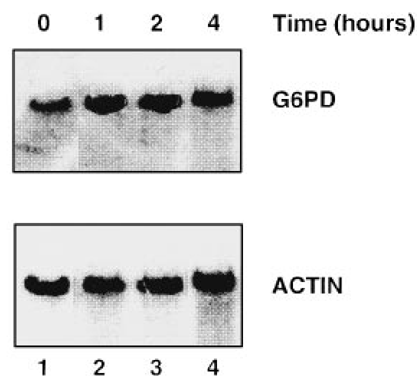

Figure 3 Time course of G6PD mRNA levels and G6PD activity in Hep3B cells treated with diamide

G6PD mRNA levels and G6PD activity were determined at different times after diamide treatment of Hep3B as described in the text. A representative Northern blot hybridized with the two probes is shown. The bands, of the expected size, correspond to G6PD- and β-actin-specific mRNAs, as indicated.

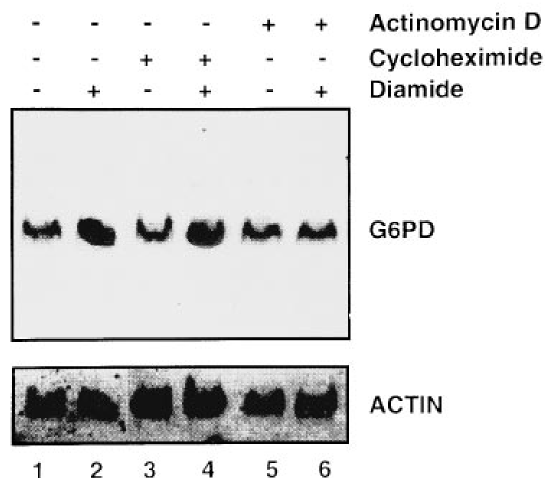

Figure 4 Effect of protein and RNA synthesis inhibitor on diamide-dependent regulation of G6PD mRNA levels and G6PD activity

Hep3B cells were preincubated with or without cycloheximide (20 μg/ml) or actinomycin D (10 μg/ml) for 1 h. Cells were then treated with diamide as described in the text and incubation was continued for 2 h. After diamide treatment total RNA was extracted and analysed by Northern blot and G6PD activity was determined as described in the Experimental section. A representative Northern blot hybridized with the two probes is shown. The bands, of the expected size, correspond to G6PD- and β-actin-specific mRNAs, as indicated.

been reported [32,33], the cellular GSH levels were not reduced by AMT treatment in these experiments (results not shown).

We subsequently analysed the kinetics of induction of G6PD activity and the levels of G6PD-specific mRNA accumulation after diamide treatment in a Hep3B cell line. As shown in Table 4, diamide determines a stimulation of G6PD activity of approx. 2.7-fold (from 0.18 to 0.47 unit) that parallels the increase in G6PD-specific mRNA accumulation. This increase is detectable 1 h after treatment (Figure 3 and Table 4) and it starts to decline after 8 h (not shown).

In order to determine whether the regulation of G6PD expression is dependent upon *de novo* protein synthesis, we treated Hep3B cells with diamide in the presence of the protein synthesis inhibitor cycloheximide. Before the addition of diamide, cells were preincubated for 1 h with cycloheximide, resulting in more than 95% inhibition of protein synthesis (results not shown), and were collected after 2 h of treatment for further analysis. G6PD activity and the levels of G6PD-specific mRNA were measured for each cell aliquot. The effect of diamide on G6PD mRNA accumulation appears not to be affected by

Table 5 Effect of protein and RNA synthesis inhibitor on diamide-dependent regulation of G6PD mRNA levels and G6PD activity

Cells were treated as in the legend to Figure 4. Relative G6PD mRNA levels represent arbitrary units normalized to β-actin mRNA levels. G6PD activity was determined as described in the Experimental section and is expressed as unit/mg of protein. Data points are means ± S.D. of three or more independent determinations.

	Relative G6PD mRNA		G6PD activity	
Treatment	None	+ Diamide	None	+ Diamide
None	64 ± 27	134 ± 26	0.15 ± 0.02	0.38 ± 0.03
Cycloheximide	33 ± 8	79 ± 25	0.17 ± 0.03	0.26 ± 0.02
Actinomycin D	162 ± 48	156 ± 34	0.15 ± 0.01	0.21 ± 0.02

inhibition of protein synthesis (Figure 4, lanes 1–2 and 3–4). On the other hand, the increase of 2.6-fold in G6PD activity occurring in cells treated with diamide was reduced to 1.5-fold when the protein synthesis inhibitor was present (Table 5).

The effect of the RNA synthesis inhibitor actinomycin D on the regulation of G6PD mRNA by diamide was also tested. Cells were treated with actinomycin D for 1 h before diamide treatment; after 2 h of incubation, G6PD activity and G6PD-specific mRNA levels were measured as described in the case of cycloheximide. No detectable increase in G6PD-specific mRNA was observed after 2 h incubation in the presence of diamide and actinomycin D compared with controls, which were only treated with actinomycin D (Figure 4, lanes 5–6). G6PD activity increases (approx. 1.4-fold) from 0.15 to 0.21 unit/mg of protein in cells treated with diamide in the presence of mRNA synthesis inhibitor (Table 5).

In conclusion, we found that a pulse of oxidative stress, obtained by the direct addition of H_2O_2 to the cells, stimulates the expression of G6PD in a rapid and transient manner in the various human cell lines of different tissue specificity that have been tested. When we inhibited the activity of a potent H_2O_2 cellular detoxification agent, catalase, we did not observe any detectable increase in G6PD expression, which suggests that the effect is not due to general degenerative conditions. Indeed, most catalase activity is localized in the peroxisomes, and the protective effect of this enzyme is especially relevant for avoiding lipid peroxidation and subsequent cell damage [30,31,33]. Under conditions in which catalase activity is inhibited, GSH concentration is not affected, since the GSH peroxidase/reductase cycle is functioning. When we used drugs that ultimately lead to cellular GSH depletion, we observed a stimulation of G6PD expression, which was counteracted by NAC, an antioxidant that replenishes the GSH intracellular level. Taken together these observations lead us to suggest that G6PD expression is sensitive to intracellular GSH levels. Additional insights are needed to elucidate the role, if any, of GSSG and to clarify the mechanism underlying such a regulation of G6PD expression.

The increase in G6PD enzyme activity (approx. 2.6-fold) promoted by oxidative stress is associated with an increase in G6PD mRNA in all tested cell lines, and the kinetics of activation are similar to those observed with HO, an enzyme having well-characterized stress-inducible gene. The inhibition of *de novo* transcription by actinomycin D abolishes 70% of the increased accumulation of G6PD-specific mRNA under conditions of oxidative stress, so that the increase in G6PD activity is mainly due to an increase in the rate of G6PD transcription. However, a small increase in G6PD activity is also reproducibly observed when transcription or protein synthesis is inhibited. The results presented here suggest that G6PD enzyme molecules pre-existing in inactive form can be activated according to cellular need. Further evidence for a rapid post-translational mechanism leading to a 1.4-fold increase in G6PD expression in cultured kidney epithelial cells stimulated with platelet-derived growth factor has also been reported [17,34].

Studies of eukaryotic genes regulated by oxidative stress have implicated the NF-κB and AP1 transcription factors in the activating mechanisms [35,36] and SP1 in the down-regulation of other genes [37]. The G6PD promoter is embedded in a CpG island [38] with no canonical NF-κB or AP1 binding site but including several SP1 binding sites, some of which are associated with basic promoter activity [39]. Therefore additional factors must interact with the G6PD promoter under oxidative stress conditions to avoid its down-regulation as a consequence of SP1 inactivation. It is interesting to note that all signals required for regulating the tissue-specific levels of G6PD expression are contained in a segment of DNA including the structural gene and 2.7 kb upstream DNA [40]. The search is now under way to find out which parts of the promoter are necessary in *cis* for the regulation of G6PD by oxidative stress.

To summarize, the findings reported above suggest that eukaryotic cells have evolved a very efficient system to regulate G6PD expression in order to maintain the levels of NADPH required for the enzymic pathway of ROI removal.

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