Metabolic control of resistance of human epithelial cells to H_2O_2 and NO stresses

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The carbon flux through the oxidative branch of the pentose phosphate pathway (PPP) can be viewed as an integrator of the antioxidant mechanisms via the generation of NADPH. It could therefore be used as a control point of the cellular response to an oxidative stress. Replacement of glucose by galactose sensitized the human epithelial cell line HGT-1 to H_2O_2 stress. Here we demonstrate that, due to the restricted galactose flux into the PPP, the H_2O_2 stress led to early cellular blebbing followed by cell necrosis, these changes being associated with a fall in the NADPH/$NADP^+$ ratio and GSH depletion. H_2O_2 cytotoxicity was prevented by adding 2-deoxyglucose (2dGlc). This protection was associated with an increased flow of 2-deoxyglucose 6-phosphate into the oxidative branch of the PPP together with the prevention of the NADPH/$NADP^+$ fall and the maintenance of intracellular GSH redox homoeostasis. Inhibitors of enzyme pathways connecting the PPP to GSH recycling abolished the 2dGlc protection. In carbohydrate-free culture conditions, 2dGlc dose-dependent protective effect was paralleled by a dose-dependent influx of 2dGlc into the PPP leading to the maintenance of the intracellular redox status. By contrast, in Glc-fed cells, the PPP was not a control point of the cellular resistance to H_2O_2 stress as they maintained a high NADPH/$NADP^+$ ratio. Both 2dGlc and Glc inhibited, through the maintenance of GSH redox status, NO cytotoxicity on galactose-containing Dulbecco's modified Eagle's medium (Gal-DMEM)-fed cells. 2dGlc did not prevent the fall of ATP content in NO-treated Gal-DMEM-fed cells, indicating that NO cytotoxicity was essentially due to the disruption of GSH redox homoeostasis and not to the alteration of ATP production by the mitochondrial respiratory chain. The maintenance of ATP content in NO-treated glucose-fed cells was due to their ability to derive their energy from anaerobic glycolysis. In conclusion, Gal-DMEM and 2dGlc-supplemented Gal-DMEM provide a useful system to decipher and organize into a hierarchy the targets of several stresses at the level of intact barrier epithelial cells.

Key words: cultured cells, energy metabolism, NADPH, oxidative stress, pentose phosphate pathway.

INTRODUCTION

Cells have evolved antioxidant responses in order to cope with pro-oxidant conditions arising from normal cellular metabolism and from environmental stresses. Oxidative stress, defined as a shift in the pro-oxidant/antioxidant balance towards oxidants, has been proposed to be associated with the toxicity of many chemicals and with the pathogenesis of many diseases [1]. Barrier epithelia, e.g. digestive epithelia, form the first line of defence against potentially toxic compounds acting via an oxidant stress [2–4]. When maintained in culture, these epithelia, e.g. colonic and gastric cell lines, as well as other barrier cells, e.g. endothelial cells, display a high level of 'intrinsic resistance' to oxidative stress [5–8]. It is therefore of the utmost importance to decipher the intracellular targets of the stressors as well as the mechanisms of response to them.

The redox pair 2GSH/GSSG constitutes the major thiol redox system in the cell, and disruption of GSH redox homoeostasis has been associated with several kinds of oxidant injury [9–11]. It is well known that the nicotinamide nucleotides (NADPH) are a critical source of reducing equivalents needed to maintain several antioxidant defences and GSH homoeostasis. As NADPH supply is dependent on carbohydrate metabolism, one could consider controlling the cellular responses to an oxidant stress by manipulating the metabolism pathways. In a recent study based on the human colonic epithelial cell line HT-29Cl.16E, we have proposed a manipulation of cellular metabolism pathways that could provide a new means for deciphering the mechanisms of cell injury by oxidative stress at a whole-cell level [12].

In the present study, we extend this carbohydrate manipulation to another cultured human epithelial cell line, the HGT-1 cells, by (i) substituting galactose for glucose and (ii) removing carbohydrate from the culture medium. Here we provide deeper insight into the mechanisms underlying this metabolic control of the cellular response to an oxidative stress, i.e. each step of the pathway connecting the carbon flux into the pentose phosphate pathway (PPP) to cell survival. Indeed, we demonstrate here, based on the prototypical oxidant stressor H_2O_2, that it is possible via the manipulation of substrate availability to the oxidative branch of the PPP and the use of specific pharmacological agents to control PPP activity, NADPH/$NADP^+$ ratio, GSH recycling and therefore cell survival.

Moreover, using the NO donor 3-(2-hydroxy-2-nitroso-1-propyl hydrazino)-1-propanamine (PAPANONOate), we point

Abbreviations used: BCNU, 1,3-bis-(2-chloroethyl)-1-nitrosourea; BSO, buthionine sulphoximine; 2dGlc, 2-deoxyglucose; 2dGlc6P, 2-deoxyglucose 6-phosphate; DHEA, dehydroepiandrosterone; DMEM, Dulbecco's modified Eagle's medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Glc6P, glucose 6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; LDH, lactate dehydrogenase; NOx, oxidizing reactive nitrogen species; PAPANONOate, 3-(2-hydroxy-2-nitroso-1-propyl hydrazino)-1-propanamine; PPP, pentose phosphate pathway.

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to two distinct effects of NO in HGT-1 cells: the disruption of GSH homoeostasis by oxidizing reactive nitrogen species (NOx) and a mitochondrial attack. In addition, we establish at the whole-cell level a hierarchy among the mechanisms of response to NO by demonstrating that the maintenance of the antioxidant systems is the primary cellular defence against NO attack.

Finally, our study shows that the metabolic effects of the unrestricted flow of glucose into glucose 6-phosphate (Glc6P), in a medium containing high concentrations of glucose, contributes to the so-called 'intrinsic resistance' of some cell types to oxidative stress. Therefore, it makes such a medium unsuitable for studies aimed at deciphering the cellular targets of an oxidant stress.

MATERIALS AND METHODS

Cell culture and treatment

The HGT-1 cell line, a human gastric epithelial cell line [13], was grown routinely in Dulbecco's modified Eagle's medium (DMEM; Life Technologies)/10 % (v/v) fetal calf serum (Life Technologies). For cellular biology and biochemical experiments, HGT-1 cells were seeded at 100000 cells/well in 6-well culture plates (Costar) and maintained for 48 h in 5 ml of DMEM/10 % fetal calf serum. The medium was replaced 24 h before the experiment by DMEM without glucose (Life Technologies)/10 % dialysed fetal calf serum (carbohydrate-free medium) or DMEM without glucose supplemented with 5 mM galactose (Sigma catalogue no. G-6404; galactose of the highest purity available)/10 % dialysed fetal calf serum (Gal-DMEM). Then, 1 h before the experiments, the medium was replaced with 1 ml of fresh medium: (i) Gal-DMEM or Gal-DMEM supplemented with 100 μM 2-deoxyglucose (2dGlc; 2dGlc-DMEM) or 25 mM glucose (Glc-DMEM), or (ii) carbohydrate-free-DMEM or carbohydrate-free-DMEM supplemented with 100 μM 2dGlc. At time point zero of the experiments, oligomycin (Sigma), H_2O_2 (Sigma) or PAPANONOate (Cayman Chemical) were added at the indicated concentrations, and the cells were cultured for various additional periods of time as mentioned in the Figure legends. When used, 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) or dehydroepiandrosterone (DHEA) were added 1 h before the experiment and buthionine sulphoximine (BSO) 24 h before at the concentrations mentioned in the figure legends.

Quantification of cell death

Cytotoxicity was determined by measuring lactate dehydrogenase (LDH) activity present in the medium (M) and retained in adherent cells (C), using the Enzyline LDH Kit (Biomerieux). Percentage cytotoxicity, represented by percentage LDH release, was calculated as $[M/(M+C)] \times 100$.

Measurement of intracellular ATP

Intracellular ATP content was determined according to Krippeit-Drews et al. [14]. Briefly, cells were lysed in 40 mM NaOH/0.5 mM cysteine solution and stored at -20 °C. For luminescence measurements, aliquots of each sample were dissolved with a buffer containing 20 mM creatine phosphate, 100 mM glycine and 1 mM $MgSO_4$ and neutralized with HCl at pH 7.65. The ATP concentration was measured in a luciferin/luciferase assay using the ATP-lite kit (Packard) with a 1420 multilabel counter (VICTOR^2; Wallac-Perkin Elmer). An aliquot of each sample was assayed for protein content using the Bradford method (Bio-Rad protein assay kit). Results were determined as nmol of ATP/mg of protein.

Measurement of intracellular GSH content

Intracellular GSH and GSSG levels were determined using an enzymic recycling method described by Tietze and modified by Baker et al. [15,16]. Briefly, the cells were harvested by scraping with a rubber policeman in PBS and sonicated at 0 °C. The cell homogenate was centrifuged for 12000 ***g*** at 10 min and the supernatant incubated for 15 min at 0 °C with 5 % sulphosalicylic acid (1:4; v/v). The mixture was centrifuged at 15000 ***g*** for 5 min. The pellet was solubilized in 1 M NaOH and its protein content was assayed using the Bradford method (Bio-Rad protein assay kit). The supernatants were stored at -70 °C until analysis for GSH and GSSG content, using the GSH disulphide reductase/5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) recycling method. Results were determined as nmol of GSH/mg of protein.

Measurement of $^{14}CO_2$ production from [1-^{14}C]2dGlc

$^{14}CO_2$ production from [1-^{14}C]2dGlc was determined using the method described by Bartos et al. [17], by adding 0.2 μCi of [1-^{14}C]2dGlc (specific radioactivity, 57 mCi/mmol; Amersham Life Sciences) to HGT-1 cells cultured in 1 ml of (i) Gal-DMEM containing 100 μM non-radioactive 2dGlc, (ii) carbohydrate-free medium containing increasing doses of non-radioactive 2dGlc (0–500 μM) or (iii) carbohydrate-free medium containing 100 μM non-radioactive 2dGlc and increasing doses of galactose (0–500 μM). Then $^{14}CO_2$ was accumulated for 2 h and cellular metabolism was stopped by adding perchloric acid. Hydroxyhyamine (Sigma) was used to capture $^{14}CO_2$ and its radioactivity was measured in a scintillation counter (Packard Tri-carb 2100 TR). Assessment of CO_2 recovery (95 ± 6 %) was performed by adding 0.125 μCi of $NaH^{14}CO_3$ to separate wells. Cells were solubilized in 1 M NaOH for measurement of protein content (using the Bradford method as described above). Results are expressed as nmol of CO_2/mg of protein.

Measurement of intracellular NADPH and NADP$^+$ content

NADPH and NADP$^+$ concentrations were measured as described by Zerez et al. [18]. Briefly, cells were harvested in a solution containing 10 mM nicotinamide, 20 mM $NaHCO_3$ and 100 mM Na_2CO_3 at 0 °C. The mixture was frozen in liquid N_2, quickly thawed in a water bath at room temperature, chilled to 0 °C and sonicated at 0 °C. To destroy NADP$^+$, an aliquot of the mixture was heated at 60 °C for 30 min and promptly chilled to 0 °C. Both the heated extract (containing NADPH) and the non-heated extract were assayed for NADPH using the spectrophotometric enzymic cycling assay [18].

Assessment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity

GAPDH was assessed as described previously [19] with slight modifications. Briefly, cells were lysed by one freeze/thaw cycle in Hepes buffer (0.05 M, pH 7.2) containing 0.04 mM EDTA. An aliquot (25 μl) was added to 200 μl of Hepes buffer (0.05 M, pH 7.2) containing 0.04 mM EDTA, 25 mM sodium arsenate and 1 mM NAD$^+$. The reaction was started by adding 25 μl of 1 mM D,L-glyceraldehyde 3-phosphate (prepared from the monobarium salt of D,L-glyceraldehyde 3-phosphate diethylacetal as recommended by Sigma) and GAPDH activity was monitored by the reduction of NAD$^+$ to NADH recorded at 340 nm, in a multilabel counter (VICTOR^2; Wallac-Perkin Elmer). An aliquot of each sample was assayed for protein content using the Bradford method. Results are expressed as GAPDH activity, i.e. nmol of NADH/min per mg of protein.

Measurement of lactate accumulation in the culture medium

Trichloroacetic acid (12 %) was added to the incubation medium (2:1) to precipitate the proteins. The mixture was then centrifuged for 10 min at 1500 *g* and aliquots of the supernatant (deproteinized medium) were assayed for lactate using the Test-Combination L-Lactic acid kit (Boehringer Mannheim) according to the manufacturer's instructions. Results are expressed as mg of lactate/ml of medium.

RESULTS

Metabolic control of resistance to H_2O_2 stress

Cells cultured in galactose-containing medium

Replacement of glucose by galactose in DMEM culture medium did not alter cell viability. HGT-1 cells maintained in Gal-DMEM were far more sensitive to the cytotoxic effects of H_2O_2 than those in Glc-DMEM (Figure 1). Basically, the cells were treated with increasing doses of H_2O_2 and the cytoxicity was examined by measuring LDH release after 14 h of treatment. Glc-DMEM-fed HGT-1 cells were remarkably resistant to increasing concentrations of H_2O_2 up to 1 mM (Figure 1). By contrast, Gal-DMEM-fed HGT-1 cells were H_2O_2-sensitive in a dose-dependent manner. This cytotoxicity was significant at a H_2O_2 concentration as low as 50 μM. The H_2O_2 concentration (500 μM) leading to 35 % Gal-DMEM-fed cell death was chosen for further experiments. Microscopic inspection showed that H_2O_2 treatment resulted in the development of plasma membrane blebs (Figures 2A and 2B) followed by cellular detachment associated with LDH release into the medium. Hoechst staining of detached cells showed nuclear condensation without typical features of apoptosis (results not shown). As expected from dying cells, cellular blebbing was paralleled by a decrease in intracellular ATP content (results not shown).

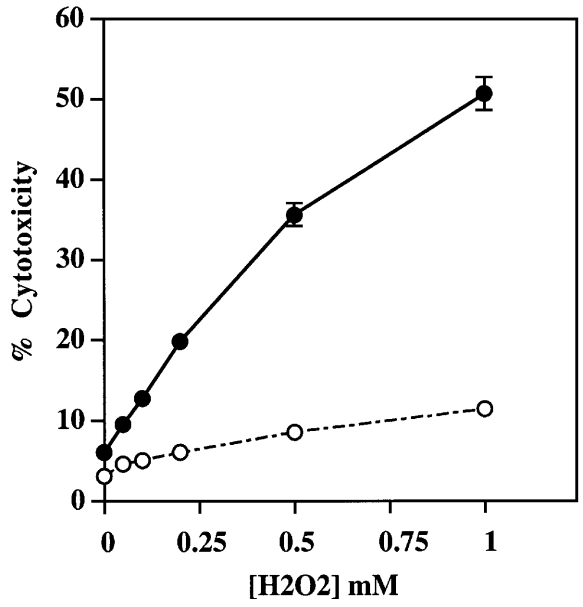

Figure 1 Effect of H_2O_2 on cell viability of Gal-DMEM- or Glc-DMEM-fed HGT-1 cells

Cells cultured in Gal-DMEM (●) or Glc-DMEM (○) were treated for 14 h with increasing concentrations of H_2O_2. Percentage cytotoxicity was determined as described in the Materials and methods section. Each point represents the mean ± S.E.M. from 3–9 determinations. Data points without error bars indicate S.E.M. values less than the symbol size.

Interestingly, 2dGlc addition to Gal-DMEM 1 h prior to H_2O_2 treatment prevented the H_2O_2 cytotoxic effect dose-dependently (Figure 3). The maximal protection (80 % cytotoxicity inhibition) was reached at a 2dGlc concentration as low as 100 μM (Figure 3). Indeed, 2dGlc inhibited cellular blebbing (Figures 2C and 2D), ATP depletion (results not shown), cell detachment and subsequent LDH release into the medium (Figure 3). To gain more insight into the mechanism by which 2dGlc carries out its protection, we measured its decarboxylation through the PPP in control conditions and in response to H_2O_2 stress. H_2O_2 induced a dramatic increase in $^{14}CO_2$ production from HGT-1 cells maintained in 2dGlc-DMEM supplemented with [1-^{14}C]2dGlc (Figure 4). This effect was suppressed by DHEA (Figure 4), an inhibitor of glucose-6-phosphate dehydrogenase (G6PDH), the rate-limiting enzyme of the oxidative branch of the PPP. We then measured the NADPH/$NADP^+$ ratio under three sets of culture conditions, i.e. Gal-DMEM, 2dGlc-DMEM and Glc-DMEM (Figure 5). In these three sets of conditions, the NADPH/$NADP^+$ ratio was not significantly different among control cells. H_2O_2 treatment lowered the NADPH/$NADP^+$ ratio in Gal-DMEM-fed cells, 2dGlc led to a significant inhibition of NADPH/$NADP^+$ ratio decrease and H_2O_2 treatment did not alter the NADPH/$NADP^+$ ratio of Glc-DMEM-fed cells.

As GSH redox homoeostasis is dependent on NADPH, we next examined the effects of 2dGlc on GSH status. The GSH level of H_2O_2-treated 2dGlc-DMEM-fed cells was significantly higher than that of H_2O_2-treated Gal-DMEM-fed cells (Figure 6), indicating that the NADPH provided by 2dGlc catabolism into the oxidative branch of the PPP was sufficient to prevent the disruption of GSH homoeostasis. The fall in GSH level in Gal-DMEM-fed cells upon H_2O_2 treatment was accounted for by the oxidation of GSH into GSSG (results not shown). We used three pharmacological agents, DHEA, BSO and BCNU, inhibiting respectively G6PDH (NADPH supply), γ-glutamyl-cysteinyl transferase (GSH synthesis) and glutathione reductase (GSH recycling). The three agents were able to annihilate the protective effect of 2dGlc against H_2O_2 attack (Figure 7). Together, these findings indicate that 2dGlc, through its metabolism into the PPP upon stimulation by H_2O_2, was able to build up the reducing power required to maintain GSH homoeostasis sufficient to prevent H_2O_2-induced cell death. In the same way, glucose exerted its protective effect against H_2O_2 attack through a GSH-dependent pathway, since both BSO and BCNU blocked its protective effect (Figure 7). DHEA did not suppress the protective effect of glucose (Figure 7). Our observation that the NADPH/$NADP^+$ ratio of Glc-DMEM-fed cells was almost identical with the control after 90 min of exposure to H_2O_2 (Figure 5) is in line with the concept that Glc-DMEM-fed cells are endowed with very efficient mechanisms to restore NADPH from $NADP^+$.

Cells cultured in carbohydrate-free medium

To eliminate an indirect mechanism of 2dGlc protection, e.g. through the diversion of galactose metabolism, we set up carbohydrate-free culture conditions. To this end, HGT-1 cells were adapted for 24 h in carbohydrate-free medium. Under these culture conditions, the cells were assumed to draw their energy from oxidation of a non-carbohydrate source, e.g. glutamine. This medium was then replaced with 1 ml of fresh carbohydrate-free medium for the experiment. As these culture conditions led to rapid loss of cell viability (40 % cell death at 5 h), we de-

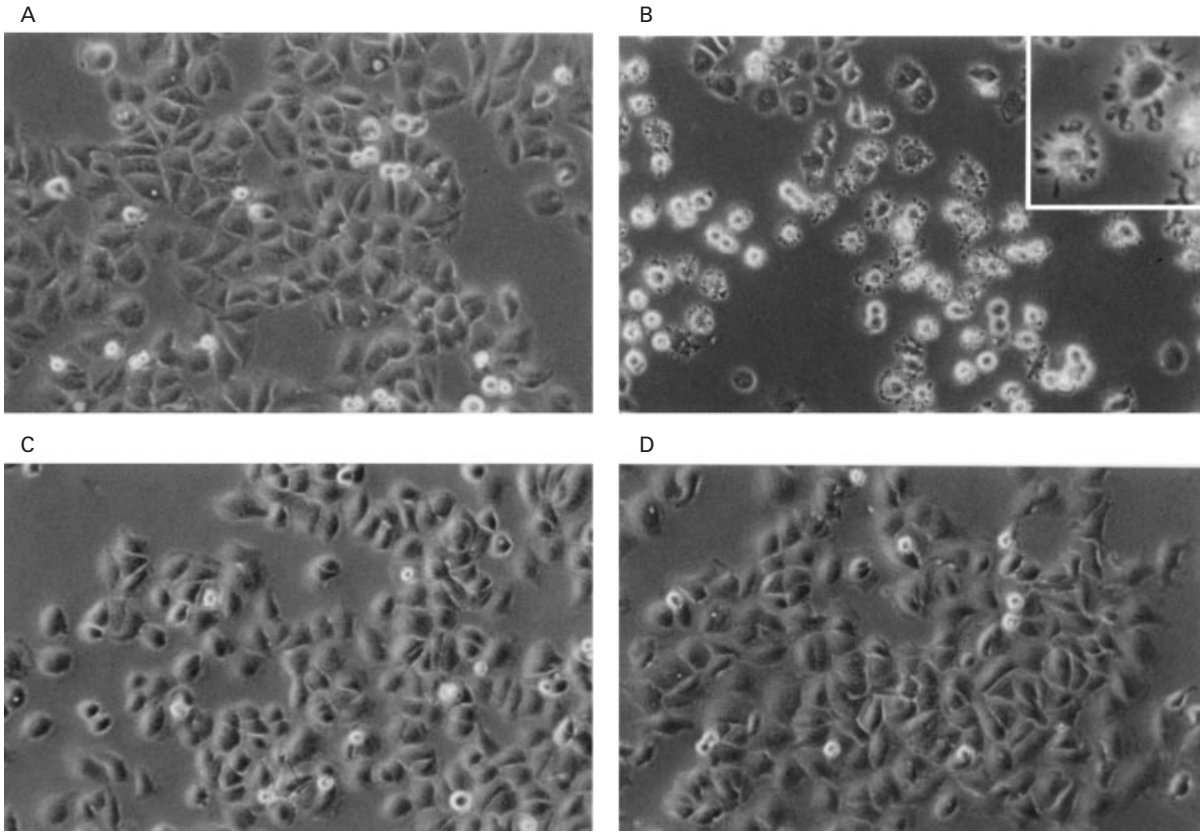

Figure 2 Protective effect of 2dGlc on blebbing induced by H_2O_2 in HGT-1 cells

Cells cultured in Gal-DMEM and 2dGlc-DMEM were incubated for 3 h in the presence or absence of 500 μM H_2O_2 and examined by phase-contrast microscopy. (**A**) Gal-DMEM-fed control cells. (**B**) Gal-DMEM-fed cells treated with H_2O_2. (**C**) 2dGlc-DMEM-fed control cells. (**D**) 2dGlc-DMEM-fed cells treated with H_2O_2. Magnifications, $\times$476; insert, $\times$1190.

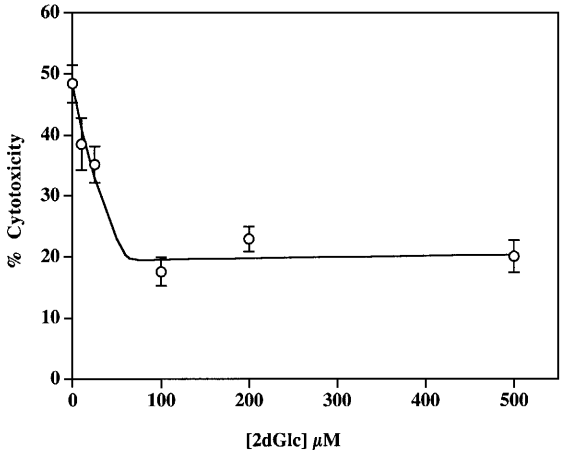

Figure 3 Protective effect of 2dGlc on H_2O_2-induced cytotoxicity in HGT-1 cells

Gal-DMEM-fed cells were treated for 14 h with 500 μM H_2O_2 in the presence of increasing concentrations of 2dGlc added 1 h before H_2O_2 treatment. Percentage cytotoxicity was determined as described in the Materials and methods section. Data are means $\pm$ S.E.M. from 6–9 determinations.

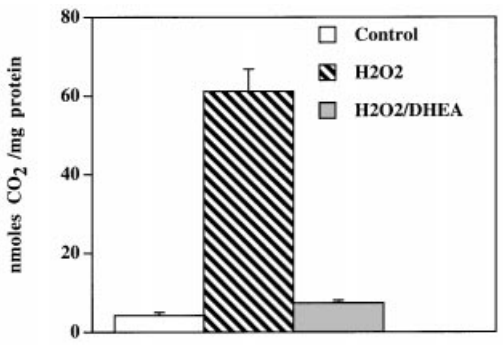

Figure 4 $^{14}CO_2$ production from [1-^{14}C]2dGlc in H_2O_2-treated HGT-1 cells

Cells cultured in 2dGlc-DMEM supplemented with 0.2 μCi of [1-^{14}C]2dGlc were incubated with or without (control) 500 μM H_2O_2 in the presence or absence of 50 μM DHEA. $^{14}CO_2$ production from [1-^{14}C]2dGlc was measured for 2 h as described in the Materials and methods section. Each value represents the mean $\pm$ S.E.M. from 6–9 determinations.

cided to restrict the monitoring of H_2O_2 effects to a period of 3 h, during which viability was not altered in the control cells. H_2O_2 (500 μM) treatment led to early cellular blebbing (results not shown) followed by 50% cytotoxicity (LDH release) after 3 h (Figure 8A). Addition of 2dGlc prevented both early blebbing and a late cytotoxic effect, significantly at a concentration as low as 10 μM and maximally at 100 μM (Figure 8A). This 2dGlc dose-dependent protective effect was paralleled by a dose-dependent influx of 2dGlc into the oxidative branch of the PPP (Figure 8B), which reached a plateau at 200 μM. Interestingly, this concentration was of the same order of magnitude as the one required for maximal protection.

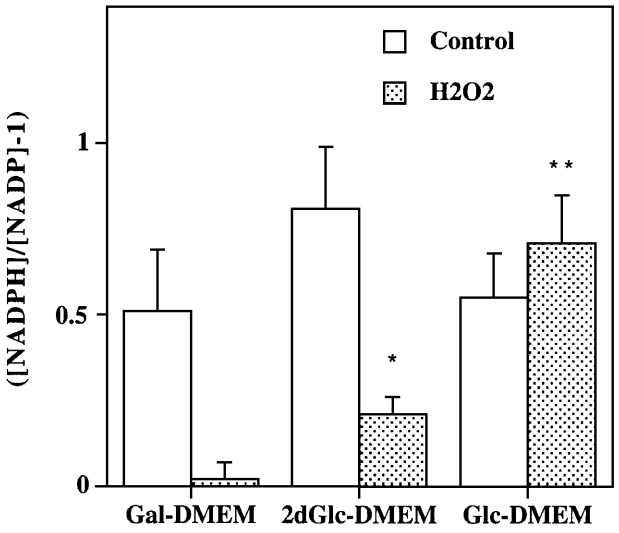

Figure 5 NADPH/NADP$^+$ ratio in control and H_2O_2-treated HGT-1 cells cultured in Gal-DMEM, 2dGlc-DMEM or Glc-DMEM

Cells cultured in Gal-DMEM, 2dGlc-DMEM or Glc-DMEM were incubated for 90 min in the presence of 500 μM H_2O_2. [NADPH] and [NADP$^+$] were measured as described in the Materials and methods section. Results are expressed as [NADPH]/[NADP$^+$] $-$ 1. Each point represents the mean $\pm$ S.E.M. from 3–6 determinations. $^{**}P < 0.01$, H_2O_2-treated Glc-DMEM-fed cells versus H_2O_2-treated Gal-DMEM-fed cells; $^{*}P < 0.05$, H_2O_2-treated 2dGlc-DMEM-fed cells versus H_2O_2-treated Gal-DMEM-fed cells.

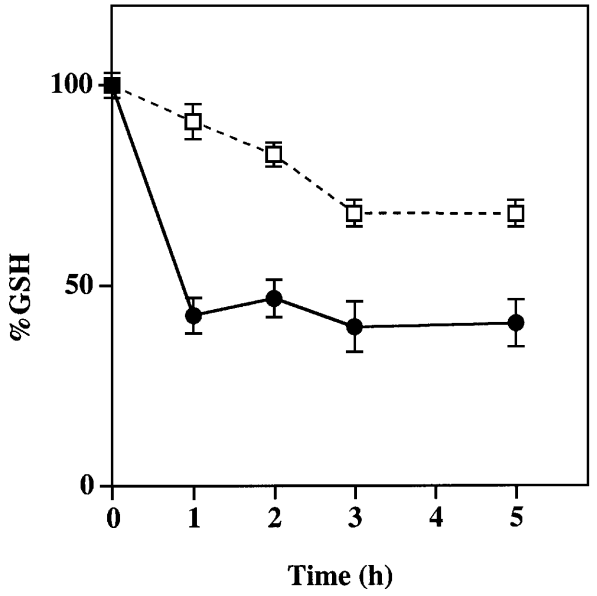

Figure 6 Time course of GSH content upon H_2O_2 treatment of Gal-DMEM- and 2dGlc-DMEM-fed HGT-1 cells

Cells cultured in Gal-DMEM (●) or 2dGlc-DMEM (□) were treated for various periods of time with 500 μM H_2O_2. Intracellular GSH content was measured as described in the Materials and methods section. Results are expressed relative to the initial GSH content (100%), which corresponds to an average of 60 $\pm$ 2 nmol/mg of protein in Gal-DMEM-fed cells and 63 $\pm$ 2 nmol/mg of protein in 2dGlc-DMEM-fed cells. Data are means $\pm$ S.E.M. from three experiments with 3–6 determinations for each experiment.

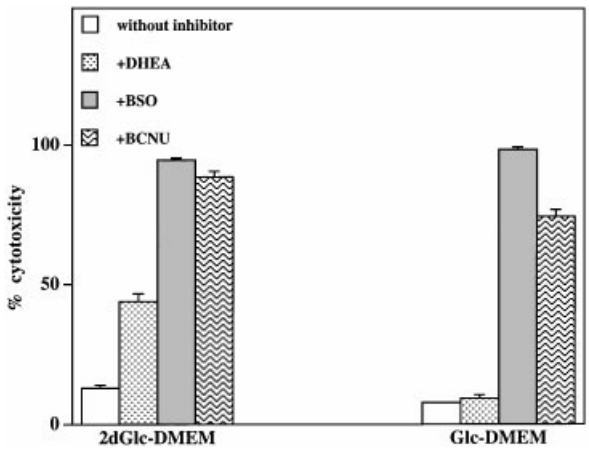

Figure 7 PPP and GSH involvement in Glc-DMEM and 2dGlc-DMEM protection against H_2O_2-induced cytotoxicity in HGT-1 cells

Cells cultured in 2dGlc-DMEM or Glc-DMEM were treated for 14 h with 500 μM H_2O_2 in the presence of 50 μM DHEA, 500 μM BCNU or 1 mM BSO as described in the Materials and methods section. Percentage cytotoxicity was determined also as described in the text. Data are means $\pm$ S.E.M. from 3–9 determinations.

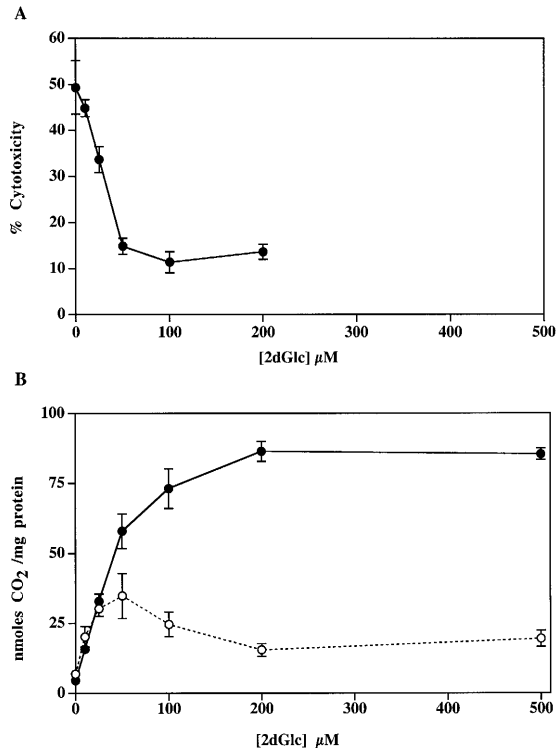

Figure 8 Protective effect of 2dGlc on H_2O_2-induced cytotoxicity in HGT-1 cells maintained in carbohydrate-free medium

Cells cultured in carbohydrate-free medium were treated (●) or not (○) with 500 μM H_2O_2 in the presence of increasing concentrations of 2dGlc added 1 h before H_2O_2 treatment. (**A**) Percentage cytotoxicity was determined as described in the Materials and methods section. (**B**) $^{14}CO_2$ production from [1-^{14}C]2dGlc was measured for 2 h as described in the text. Data are means $\pm$ S.E.M. from 3–6 determinations.

Finally, we tested whether the influx of 2dGlc into the PPP could alter the intracellular redox status. To this end, the activity of a redox-sensitive enzyme, GAPDH, was measured in H_2O_2-treated HGT-1 cells with or without 2dGlc. H_2O_2 treatment led to the loss of 80% of GAPDH activity within 2 h (GAPDH activities for control and H_2O_2-treated cells were 69.3 $\pm$ 3.3 and 15.1 $\pm$ 1.5, respectively; $P < 0.001$; see Table 1), due to reversible enzyme oxidation, as addition of dithiothreitol to the assay

Table 1 2dGlc effect on redox-sensitive GAPDH activity of H_2O_2-treated cells cultured in carbohydrate-free medium

Cells cultured in carbohydrate-free medium supplemented or not with 100 μM 2dGlc were incubated for 120 min in the presence of 500 μM H_2O_2. For each condition, GAPDH activity was measured as described in the Materials and methods section by adding or not 10 mM dithiothreitol to the assay buffer. Results are expressed as absolute GAPDH activity and as the percentage of GAPDH activity without dithiothreitol compared with that in the presence of dithiothreitol (control). Each value represents the mean ± S.E.M. from six determinations.

		GAPDH activity (nmol/min per mg of protein)		
Conditions	H_2O_2	Without dithiothreitol	With dithiothreitol	% Activity reversibly lost by oxidation
Carbohydrate-free medium	−	69.3 ± 3.3	76.4 ± 3.3	9.3 ± 4.3
	+	15.1 ± 1.5	56.1 ± 1.7	74.3 ± 3.2
Carbohydrate-free medium + 2dGlc	−	71.1 ± 2.3	72.1 ± 3.8	1.5 ± 3.2
	+	35.8 ± 1.7	57.9 ± 2.5	38.2 ± 2.9

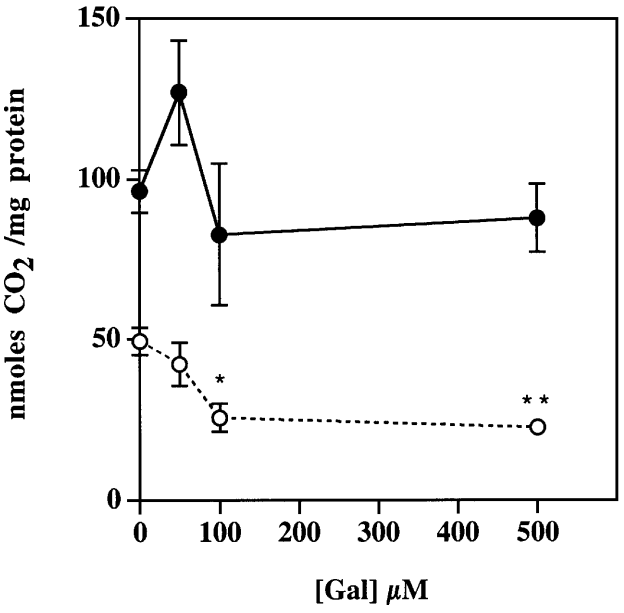

Figure 9 Galactose metabolism in control and H_2O_2-treated HGT-1 cells maintained in carbohydrate-free medium

Cells cultured in carbohydrate-free medium supplemented with 100 μM non-radioactive 2dGlc and 0.2 μCi of [1-^{14}C]2dGlc were treated (●) or not (○) with 500 μM H_2O_2 in the presence of increasing doses of galactose. $^{14}CO_2$ production from [1-^{14}C]2dGlc was measured for 2 h as described in the Materials and methods section. Each value represents the mean ± S.E.M. from three determinations; data points without error bars indicate S.E.M. values less than the symbol size. $^*P < 0.05$ and $^{**}P < 0.01$ indicate significant differences from the control value, i.e. without galactose.

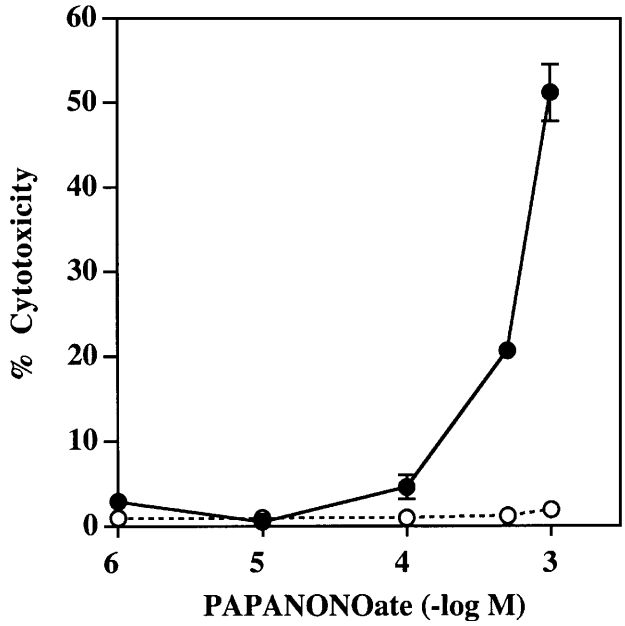

Figure 10 NO cytotoxicity in Gal-DMEM- and Glc-DMEM-fed HGT-1 cells

Cells cultured in Gal-DMEM (●) or Glc-DMEM (○) were incubated for 14 h in the presence of increasing concentrations of the NO donor PAPANONOate. Percentage cytotoxicity was determined as described in the Materials and methods section. Each point represents the mean ± S.E.M. from three determinations. Data points without error bars indicate S.E.M. values less than the symbol size.

buffer recovered enzyme activity. Interestingly, dithiothreitol recovery accounted for 73% of GAPDH activity of untreated control cells (GAPDH activities for control and H_2O_2-treated cells were 76.4 ± 3.3 and 56.1 ± 1.7, respectively; $P < 0.001$; see Table 1), suggesting that H_2O_2 treatment led to an irreversible loss of a fraction of GAPDH. Finally, the GAPDH activity returned upon 2dGlc addition to 50% of the control (GAPDH activities for untreated control and H_2O_2-treated cells were 71.1 ± 2.3 and 35.8 ± 1.7, repectively; Table 1). Altogether these results provide direct evidence that 2dGlc protects HGT-1 cells from H_2O_2 cytotoxicity through the recovery of intracellular redox status via its metabolism into the oxidative branch of the PPP.

Restricted flux of galactose into the PPP upon H_2O_2 stress

We next sought to decipher the metabolic mechanisms underlying the sensitivity of galactose-fed cells to H_2O_2. To this end we monitored the competition of galactose with 2dGlc influx into the oxidative branch of the PPP (Figure 9). Under control conditions galactose (up to 100 μM) dose-dependently competed with 2dGlc metabolism into the PPP. By contrast, galactose was unable to alter the dramatic increase of 2dGlc influx into the PPP under H_2O_2 stress. It is therefore concluded that the metabolism of galactose into Glc6P is a rate-limiting step that prevents reducing power build-up of galactose-fed cells under oxidative stress.

Metabolic control of resistance to NO stress

Using these culture conditions, we next examined the mechanisms of NO-induced stress. HGT-1 cells were treated with increasing concentrations of the NO donor PAPANONOate, whose kinetics of decomposition in culture media are now well known [12]. PAPANONOate was cytotoxic in a dose-dependent manner to Gal-DMEM-fed cells, while it had no effect up to 1 mM on Glc-DMEM-fed cells (Figure 10). 2dGlc (100 μM) partially prevented the NO cytotoxic effect on Gal-DMEM-fed cells (Figure 11). It

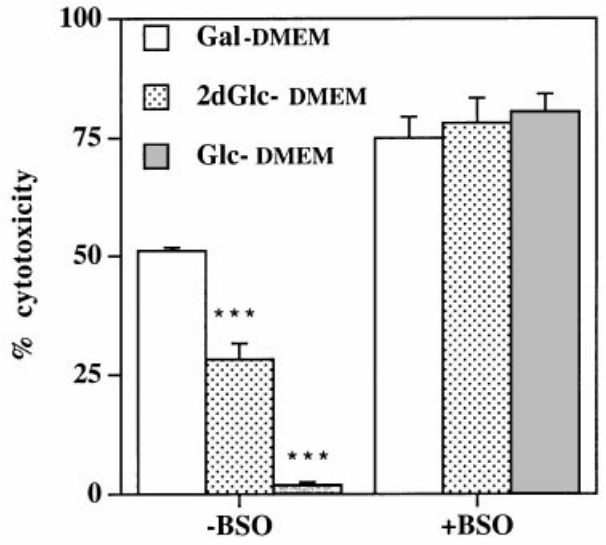

Figure 11 Protective effect of 2dGlc and glucose in NO-treated HGT-1 cells through the maintenance of GSH homoeostasis

Cells cultured in Gal-DMEM, 2dGlc-DMEM or Glc-DMEM, pre-treated or not with 1 mM BSO, were exposed for 14 h to 1 mM PAPANONOate. Percentage cytotoxicity was determined as described in the Materials and methods section. Data are means ± S.E.M. from 6–9 determinations. $^{***}P < 0.001$ indicates a significant difference from the corresponding Gal-DMEM values.

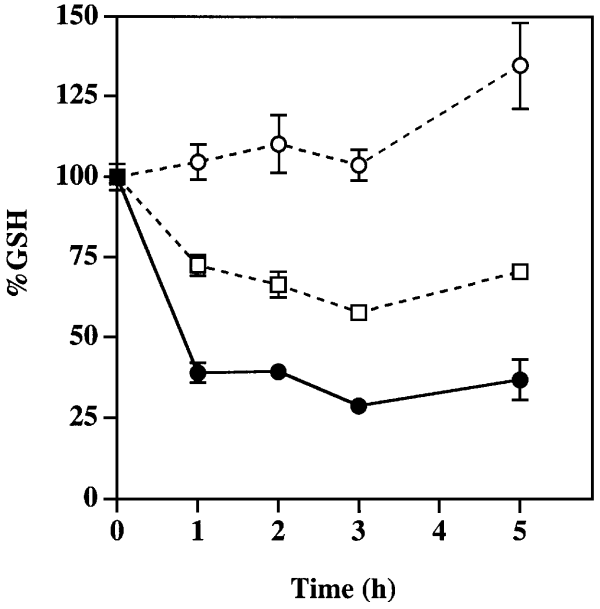

Figure 12 Time course of GSH content of NO-treated Gal-DMEM-, 2dGlc-DMEM- and Glc-DMEM-fed HGT-1 cells

Cells cultured in Gal-DMEM (●), 2dGlc-DMEM (□) or Glc-DMEM (○) were treated for various periods of time with 1 mM PAPANONOate. Intracellular GSH content was measured as described in the Materials and methods section. Results are expressed relative to the initial GSH content (100%), which corresponds to an average of 65 ± 5 nmol/mg of protein in Gal-DMEM-fed cells, 55 ± 2 nmol/mg of protein in 2dGlc-DMEM-fed cells and 87.5 ± 4 nmol/mg of protein in Glc-DMEM-fed cells. Each point represents the mean ± S.E.M. from 1–3 experiments with 3–6 determinations for each experiment; data points without error bars indicate S.E.M. values less than the symbol size.

was therefore concluded that oxidative stress was involved in NO cytotoxicity. This conclusion was supported by two additional experiments. First, in the same way as did H_2O_2, NO induced GSH oxidation in Gal-DMEM-fed cells that was partially but significantly prevented by 2dGlc, and totally prevented by glucose (Figure 12). Second, GSH depletion by BSO pretreatment prevented the 2dGlc protection (Figure 11). Interestingly, BSO pretreatment also abrogated the protective effect of glucose (Figure 11). Together these results suggest strongly that GSH exerted its protective effect by scavenging NO species that are reactive with thiol groups. Consequently, GSH depletion could expose critical cellular thiols to oxidation. As GAPDH is a glycolytic enzyme harbouring a critical thiol in its catalytic site, it is susceptible to inactivation through S-nitrosylation/thiolation/oxidation reactions [19–24] by NOx derived from auto-oxidation of NO released in relatively high concentrations [25]. We therefore chose to measure GAPDH activity on intact cells upon NO treatment. Whereas a 90% inactivation of GAPDH was observed in Gal-DMEM-fed cells, a significantly higher level of GAPDH activity was maintained in cells fed with 2dGlc-DMEM, Glc-DMEM or Gal-DMEM supplemented with GSH ethyl ester (10 mM; Table 2). Together these results argue that 2dGlc provides the reducing power required for the detoxification of NOx through GSH recycling.

Table 2 NO effect on GAPDH activity of HGT-1 cells fed with Gal-, 2dGlc- or Glc-DMEM, or Gal-DMEM supplemented with GSH

Cells cultured in either Gal-, 2dGlc- or Glc-DMEM, or Gal-DMEM supplemented with 10 mM GSH were incubated for 90 min in the presence or absence of PAPANONOate. GAPDH activity was measured as described in the Materials and methods section. Results are expressed as absolute GAPDH activity and as the percentage of GAPDH activity in the presence of NO compared with in the absence of NO (control). Each value represents the mean ± S.E.M. from 6–12 determinations. Values of GAPDH activity in NO-treated cells in Glc-DMEM, 2dGlc-DMEM and Gal-DMEM + GSH were not significantly different.

	GAPDH activity (nmol/min per mg of protein)		
Conditions	Control	PAPANONOate	% Control
Gal-DMEM	83.9 ± 2.6	8.5 ± 1.7	10.1 ± 2.0
2dGlc-DMEM	93.7 ± 6.9	31.5 ± 5.5	33.7 ± 5.9
Glc-DMEM	83.6 ± 8.1	47.8 ± 5.7	57.2 ± 6.8
Gal-DMEM + 10 mM GSH	119.4 ± 0.9	38.8 ± 3.1	32.5 ± 2.6

A 50% fall in ATP content in Gal-DMEM-fed cells was observed within 1 h of 1 mM PAPANONOate treatment (Figure 13). Neither 2dGlc nor GSH ethyl ester addition to Gal-DMEM-fed cells prevented the fall in ATP concentration, whereas ATP content in Glc-DMEM-fed cells remained nearly unchanged (Figure 13). These findings led us to hypothesize that Gal-DMEM- and 2dGlc-DMEM-fed cells derive their energy from a metabolic pathway different from that of Glc-DMEM-fed cells. At this point it was therefore important to determine the energy-metabolism pathways used by the cells under the three sets of culture conditions. Oligomycin, an F_1F_0-ATPase inhibitor, is a useful means to determine whether the cells derive their energy from the mitochondrial respiratory chain. Treatment of Gal-DMEM- or 2dGlc-DMEM-fed HGT-1 cells with oligomycin caused a fall in intracellular ATP content within 1 h (Figure 14), followed by cell death (results not shown). These findings show clearly that Gal-DMEM-fed cells draw their energy from mitochondrial oxidative phosphorylation. In striking contrast, Glc-DMEM-fed HGT-1 cells were totally resistant to oligomycin treatment, in terms of both ATP content (Figure 14) and cell viability (results

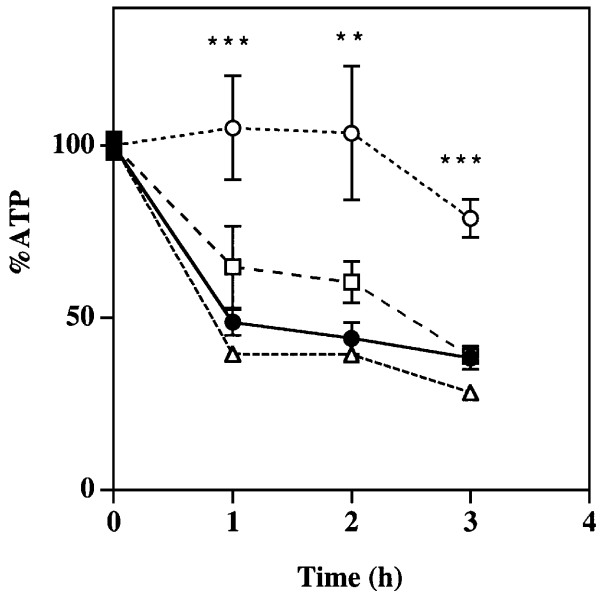

Figure 13 Time course of ATP content upon NO treatment of Gal-DMEM-, Glc-DMEM-, 2dGlc-DMEM- or GSH-supplemented Gal-DMEM-fed HGT-1 cells

Cells cultured in Gal-DMEM (●), 2dGlc-DMEM (□), Glc-DMEM (○) or Gal-DMEM supplemented with 10 mM GSH (△) were treated for various periods of time with 1 mM PAPANONOate. ATP content was measured as described in the Materials and methods section. Results are expressed relative to the initial ATP content (100%), corresponding to an average of 80 ± 7 nmol/mg of protein in Gal-DMEM-fed cells, 54 ± 1.5 nmol/mg of protein in 2dGlc-DMEM-fed cells and 69 ± 0.3 nmol/mg of protein in Glc-DMEM-fed cells. Each point represents the mean ± S.E.M. from 1–3 experiments with three determinations for each experiment. Data points without error bars indicate S.E.M. values less than the symbol size. $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate significant differences from the corresponding values in Gal-DMEM.

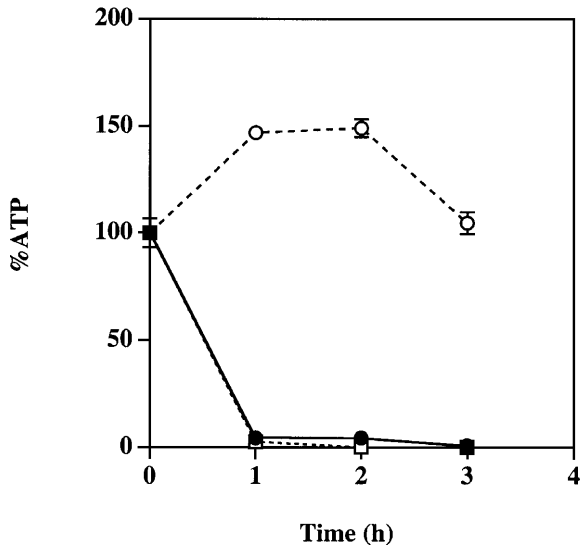

Figure 14 Time course of ATP content upon oligomycin treatment of Gal-DMEM-, Glc-DMEM- and 2dGlc-DMEM-fed HGT-1 cells

HGT-1 cells cultured in Gal-DMEM (●), Glc-DMEM (○) or 2dGlc-DMEM (□) were treated for various periods of time with 1.3 μM oligomycin. ATP content was measured as described in the Materials and methods section. Results are expressed relative to the initial ATP content (100%), which corresponds to an average of 84 ± 6 nmol/mg of protein in Gal-DMEM-fed cells, 53 ± 2 nmol/mg of protein in Glc-DMEM-fed cells and 81 ± 11 nmol/mg of protein in 2dGlc-DMEM-fed cells. Each point represents the mean ± S.E.M. from 3–6 determinations. Data points without error bars indicate S.E.M. values less than the symbol size.

not shown), thus indicating that at high glucose concentrations (i.e. in Glc-DMEM) cells can derive their energy by converting glucose into lactate. Finally, lactate accumulation in the culture medium was significantly increased in PAPANONOate- and oligomycin-treated Glc-DMEM-fed cells compared with control Glc-DMEM-fed cells (Figure 15). This shows clearly that a mitochondrial attack in glucose-fed cells led to an accelerated glycolytic flux that maintained the ATP content. Together these findings led us to conclude that the maintenance of ATP content of NO-treated Glc-DMEM-fed cells is accounted for by a shift in energy metabolism towards glycolytic degradation of glucose into lactate.

DISCUSSION

Here we propose a global mechanistic approach of the cellular response to the prototypical oxidant H_2O_2, based on the use of galactose, whose limited metabolism into Glc6P leads to restricted carbon flux into the PPP (Figures 9 and 16). Under these conditions the cells were sensitized to an H_2O_2 stress. Both morphological and biochemical H_2O_2-induced changes in HGT-1 cells were found to be very similar to those already described by van Gorp et al. [8] in other prototypical barrier cells, i.e. normal endothelial cells. Indeed, both cell types respond to H_2O_2 by marked cellular blebbing associated with a loss of intracellular GSH, followed by nuclear condensation and non-apoptotic cell death.

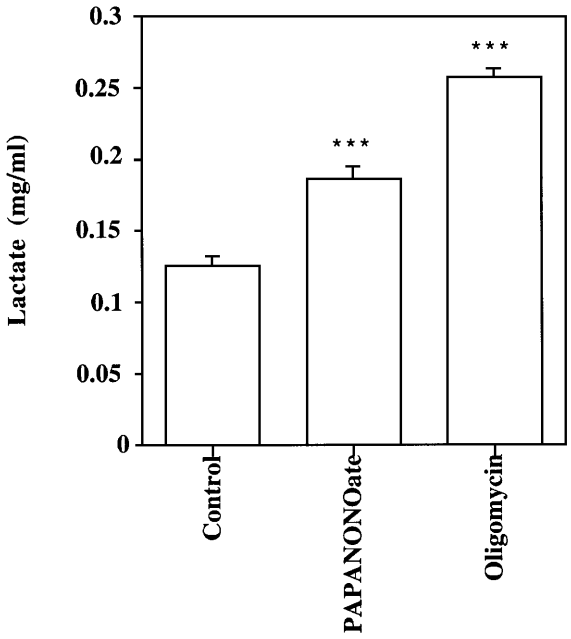

Figure 15 Effects of oligomycin and PAPANONOate on lactate acumulation in the culture medium of glucose-fed HGT-1 cells

Glucose-fed HGT-1 cells were treated or not for 14 h with 1 mM PAPANONOate or 1.3 μM oligomycin. Lactate accumulation in the culture medium was measured as described in the Materials and methods section. Each point represents the mean ± S.E.M. from six determinations. $^{***}P < 0.001$ indicates a significant difference from control cells.

2dGlc is often considered as an anti-metabolite and when used at high concentrations is an inhibitor of glycolysis, as its metabolism in this pathway cannot proceed beyond 2-deoxyglucose 6-phosphate (2dGlc6P) [26]. However, a few studies have shown that 2dGlc6P can be metabolized into the oxidative branch of the PPP [27,28]. It is therefore clear that the measurement of $^{14}CO_2$ production from [1-^{14}C]2dGlc gives a direct assessment of its metabolism into the oxidative branch of the PPP. Thus, it provides a powerful tool with which to selectively increase the substrate availability for the PPP (Figure 16). Here we demonstrate that 2dGlc, through its metabolism into the PPP upon stimulation by H_2O_2, was able to build up the reducing power required to maintain GSH homoeostasis sufficient to prevent H_2O_2-induced cell death. In fact, as 2dGlc was decarboxylated into the PPP, its protective effect resulted from its phosphorylation into 2dGlc6P followed by its catabolism along the oxidative branch of the PPP: from oxidation by G6PDH to decarboxylation by 6-phosphogluconate dehydrogenase. In this process, 2 mol of NADPH must be produced/mol of 2dGlc. In line with this finding was our demonstration that the NADPH/NADP$^+$ ratio was significantly increased in 2dGlc-DMEM-fed cells compared with Gal-DMEM-fed cells in response to H_2O_2 stress. The fact that the GSH redox homoeostasis was maintained only in 2dGlc-DMEM-fed cells and not in Gal-DMEM-fed cells was related directly to NADPH recycling under these culture conditions. These data, together with the results derived from the use of inhibitory agents specific for each step of the pathway connecting the carbon flux into the PPP to GSH recycling (Figure 16), provide a clear demonstration that this metabolic approach allows us to decipher the mechanisms underlying the antioxidant reponse to the H_2O_2 stress. Finally, our findings are in line with the mechanistic concept that in unstressed conditions the relatively high NADPH/NADP$^+$ ratio exerts an inhibitory action on G6PDH [29], as shown by the very low production of $^{14}CO_2$ from [1-^{14}C]2dGlc. H_2O_2 stress leads to GSH oxidation which in turn induces NADPH oxidation. The fall in the NADPH/NADP$^+$ ratio results in the de-inhibition of G6PDH and the activation of PPP as shown by $^{14}CO_2$ production from [1-^{14}C]2dGlc. Concomitantly with studies based on Gal-DMEM-fed cells, we provide direct evidence that in carbohydrate-free medium 2dGlc was able to rescue HGT-1 cells from H_2O_2 cytotoxic effects. The dose-dependency of the protecting effect of 2dGlc paralleled that of its metabolism into the PPP. Finally, as shown by monitoring the redox-sensitive activity of GAPDH, 2dGlc metabolism into this pathway could restore the intracellular reducing power. Therefore, it is clear that the 2dGlc protective effect occurs through the build-up of reducing power.

In sharp contrast, when the cells were cultured at high glucose concentrations they were not amenable to such a mechanistic approach, as they maintained a high NADPH/NADP$^+$ ratio even under H_2O_2 stress. In addition, DHEA, a known inhibitor of G6PDH, did not suppress the protective effect of glucose, a finding suggesting that the NADPH required for GSH recycling in Glc-DMEM-fed cells could also be provided by other metabolic pathways such as malic enzyme or cytosolic isocitrate dehydrogenase pathways [30]. In this context, it is noteworthy that a high-glucose culture medium was found by Sener et al. [31] to augment the NADPH/NADP$^+$ ratio in RINm5F cells. In addition, sustained exposure to high glucose concentrations was found to up-regulate the expression of several genes involved in glucose metabolism [32–35], including malic enzyme [36], an enzyme that serves to recycle NADPH from NADP$^+$. Therefore,

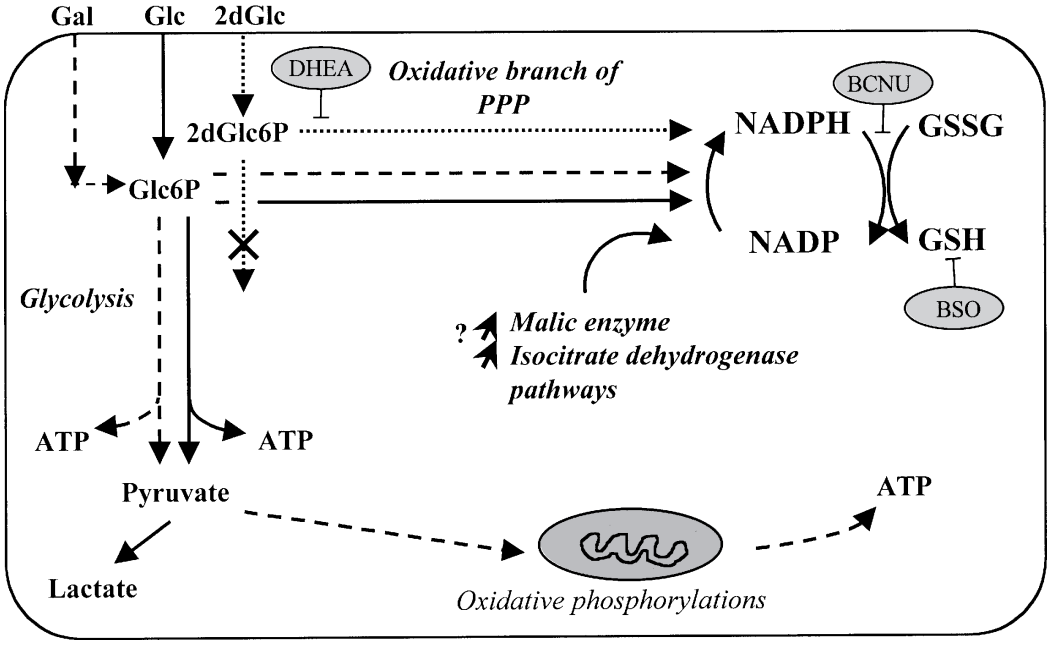

Figure 16 Schematic representation of intracellular redox and energy status under three sets of different culture conditions: Glc-, Gal- and 2dGlc-DMEM

Metabolic control of redox status: cells cultured in Glc-DMEM maintain a high level of intracellular reducing power due to unlimited availability of Glc6P for the PPP and other NADPH-producing pathways, such as the malic enzyme or isocitrate dehydrogenase pathways. In such conditions, NADPH-dependent antioxidant systems are fully powered. Under these conditions, PPP is not a control point of the cellular response to oxidative stress. In Gal-DMEM cells, upon oxidative stress, limited availability of Glc6P for PPP makes the cells unable to maintain NADPH, GSH and cell viability. Addition of low concentrations of 2dGlc to Gal-DMEM, i.e. 2dGlc-DMEM, allows NADPH and GSH supply from NADP$^+$ and GSSG recycling. Activation of the PPP leads to the maintenance of GSH redox homoeostasis and preserves cell viability. Energy metabolism: cells cultured in Glc-DMEM mainly draw their ATP from the glycolytic degradation of glucose into lactate. Gal-DMEM- or 2dGlc-DMEM-fed cells draw their energy solely from mitochondrial oxidative phosphorylation.

it is clear that the PPP is not a control point of the resistance to H_2O_2 in Glc-DMEM-fed cells, as NADPH can be regenerated from other metabolic pathways. This also accounts for the hitherto unexplained remarkable resistance of several epithelial cell lines maintained in glucose-containing medium towards H_2O_2 oxidative stress [5–8].

The restricted metabolism of galactose into Glc6P has two consequences. First it limits the availability of PPP substrate under oxidative stress (this study). Second, it forces the cells to derive their energy from mitochondrial oxidative phosphorylation [12,37]. This concept holds true for HGT-1 cells, as oligomycin induced a rapid ATP depletion from Gal-DMEM- or 2dGlc-DMEM-fed cells. Interestingly, oligomycin treatment was unable to alter the ATP content from cells maintained in high glucose concentrations. This indicates that high glucose concentrations make cells able to derive their energy solely from the anaerobic degradation of glucose into lactate. In fact, this demonstration is in line with previous reports showing that elevated glucose concentrations give rise to enhanced glucose entry and metabolism into Glc6P, leading to an increased rate of glycolysis, an accumulation of lactate, and an inhibition of mitochondrial catabolism of glucose metabolites (Crabtree effect) [37,38]. This also predicts that glucose-fed cells should be insensitive to attacks specifically aimed at disrupting the mitochondrial energy metabolism. Gal-DMEM-based culture conditions provide therefore a unique system to delineate several components of a given stress, i.e. those requiring NADPH recycling through the activation of antioxidant mechanisms and those impacting on the mitochondrial energy metabolism. NO stress is a good candidate to examine this hypothesis as NO has been shown previously to interfere directly with mitochondrial respiration at Complex IV [39–41], resulting in a reversible inhibition of ATP synthesis [42]. Together, our results point to two effects of NO in HGT-1 cells: (i) the disruption of the intracellular redox balance through GSH depletion by NOx derived from autoxidation of NO, leading to oxidative damage as shown previously in the HT29-Cl.16E human colonic epithelial cell line [12], and (ii) mitochondrial attack leading to an inhibition of ATP production. These findings also establish a hierarchy of cellular responses to NO as it is shown that the maintenance of the antioxidant systems is the primary cellular defence against NO attack. These findings are consistent with two recent studies showing that disruption of GSH homoeostasis transforms reversible inhibitory effects of NO donors on DNA synthesis [19] or cell respiration [39,43] into irreversible ones.

Several conclusions can be drawn from this study: cell culture based on high concentrations of glucose induces a shift towards anaerobic glycolysis and maintenance of a high NADPH/$NADP^+$ ratio, supporting cell viability under conditions of strong oxidative stress like the one induced by very high H_2O_2 concentrations. Gal-DMEM-fed cells draw their energy from mitochondrial metabolism and have a limited capacity to recycle NADPH from $NADP^+$, due to a restricted availability of Glc6P as a substrate for the PPP. Under these conditions, adding 2dGlc to the Gal-DMEM-fed cells demonstrates that the oxidant stress is able to activate the PPP. 2dGlc-DMEM is therefore a useful system to decipher and organize into a hierarchy at the level of the intact cell, the mechanisms of response to several stresses that target mitochondrial energy metabolism and antioxidant defences.

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