Protection against hydrogen peroxide cytotoxicity in Rat-1 fibroblasts provided by the oncoprotein Bcl-2: maintenance of calcium homoeostasis is secondary to the effect of Bcl-2 on cellular glutathione

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The oncoprotein Bcl-2 protects cells against apoptosis, but the exact molecular mechanism that underlies this function has not yet been identified. Studying H_2O_2-induced cell injury in Rat-1 fibroblast cells, we observed that Bcl-2 had a protective effect against the increase in cytosolic calcium concentration and subsequent cell death. Furthermore, overexpression of Bcl-2 resulted in an alteration of cellular glutathione status: the total amount of cellular glutathione was increased by about 60 % and the redox potential of the cellular glutathione pool was maintained in a more reduced state during H_2O_2 exposure compared with non-Bcl-2-expressing controls. In our cytotoxicity model, disruption of cellular glutathione homoeostasis closely correlated with the pathological elevation of cytosolic calcium concentration. Stabilization of the glutathione pool by Bcl-2, *N*-acetylcysteine or glucose delayed the cytosolic calcium increase and subsequent cell death, whereas depletion of glutathione by DL-buthionine-(*S*,*R*)-sulphoximine, sensitized Bcl-2-transfected cells towards cytosolic calcium increase and cell death. We therefore suggest that the protection exerted by Bcl-2 against H_2O_2-induced cytosolic calcium elevation and subsequent cell death is secondary to its effect on the cellular glutathione metabolism.

Key words: glucose, GSH, GSSG, reactive oxygen species, redox balance.

INTRODUCTION

The 26 kDa oncoprotein Bcl-2 has been described as offering protection against various apoptotic and necrotic forms of cell injury, such as those induced by glucocorticoids, interleukin-3 withdrawal, DL-buthionine-(*S*,*R*)-sulphoximine (BSO), calcium ionophores, γ-irradiation, menadione or reactive oxygen species (see [1–3] for reviews), but there is still a great deal of controversy about the mechanism of Bcl-2's action. In some of these injurious processes, disruption of cellular Ca^{2+} homoeostasis, especially elevations of cytosolic Ca^{2+} concentration, is considered to play a decisive intermediary role [4]. Accordingly, effects of Bcl-2 on Ca^{2+} homoeostasis and on Ca^{2+}-mediated cell death have been studied in a number of experimental models [5–12]. These studies, however, led to contradictory results and no clear picture emerged as to how Bcl-2 may affect Ca^{2+}-mediated cell-injurious processes. Above all, it remained unclear whether Bcl-2 regulates intracellular Ca^{2+} compartmentation via direct action on membrane fluxes or by affecting unknown upstream events.

Recent studies have shown that Bcl-2 affects glutathione in several cell types. The effects reported include up-regulation of cellular GSH, increased resistance to apoptosis due to a reductive shift of the cellular oxidation–reduction potential and redistribution of GSH to the nucleus and thus protection against DNA fragmentation [13–18]. Unfortunately, however, in these studies on Bcl-2 and GSH, no consideration has been given to cellular Ca^{2+} homoeostasis, although, in several models of cell injury, links between the cellular glutathione and cellular Ca^{2+} metabolism are well established. The latter especially applies to the model of H_2O_2-mediated cell injury which has been studied extensively for several years [19–21] and in which protection by Bcl-2 has been described too, including inhibition of H_2O_2-induced rise in cytosolic Ca^{2+} and of H_2O_2-induced depletion of the endoplasmic-reticulum Ca^{2+} pool [22–24]. We therefore set out to test the hypothesis that in Bcl-2-transfected Rat-1 fibroblasts treated with H_2O_2 the increase in cytosolic free Ca^{2+} is secondary to the oxidation of cellular glutathione and that Bcl-2 influences intracellular Ca^{2+} fluxes only indirectly by altering cellular glutathione metabolism.

EXPERIMENTAL

Materials

H_2O_2, probenecid, BSO, diethylenetriaminepenta-acetic acid (DTPA), *N*-acetylcysteine, methylglyoxal and glyoxalase I were obtained from Sigma (Deisenhofen, Germany). Monochlorobimane, pluronic F-127 and the acetoxymethyl esters of quin-2 (quin-2-AM) and fura-2 (fura-2-AM) were purchased from Molecular Probes (Eugene, OR, U.S.A.). Peroxidase and glutathione reductase were from Boehringer (Mannheim, Germany), and Dulbecco's modified Eagle medium (low glucose) and fetal-calf serum from Gibco (Eggenstein, Germany). All other chemicals were purchased from Merck (Darmstadt, Germany).

Abbreviations used: BSO, DL-buthionine-(*S*,*R*)-sulphoximine; DTPA, diethylenetriaminepenta-acetic acid; LDH, lactate dehydrogenase; quin-2-AM and fura-2-AM, acetoxymethyl esters of quin-2 and fura-2; HBSS, Hanks' balanced salt solution; 'glutathione' is used where the oxidation state (GSH or GSSG) is not specified.

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Cell culture

The cell lines used in this study were a gift from Dr. G. I. Evan and Dr. T. Littlewood (Biochemistry of the Cell Nucleus Laboratory, Imperial Cancer Research Fund, London, U.K.). To study the function of Bcl-2, we used two individual clones of Rat-1 fibroblasts stably transfected with a retroviral vector directing constitutive expression of human Bcl-2 (together with a puromycin-resistant marker; Rat-1/Bcl-2 #1 and Rat-1/Bcl-2 #2), as described in [25]. Similar results were observed with both clones and the results referred to are those obtained with clone #2. Untransfected Rat-1 fibroblasts were employed as a control cell line, and as the vector control we used a cell line of Rat-1 fibroblasts that constitutively expressed a chimaeric protein, comprising a full-length c-Myc polypeptide fused to part of a mutated human oestrogen receptor together with a puromycin-resistant marker (Rat-1/cMycER cells), which show demonstrable c-Myc activity only in the presence of 4-hydroxytamoxifen. Rat-1/cMycER cells were cultured in the presence of 5 μg/ml puromycin, but in the absence of 4-hydroxytamoxifen, the transfected c-Myc was therefore inactive.

The cells were cultured in Dulbecco's modified Eagle medium (low glucose) containing 10 % (v/v) fetal-calf serum and penicillin/streptomycin (100 units/ml and 100 μg/ml respectively). Subcultures were obtained by trypsin treatment [0.5 % (w/v) trypsin/0.2 % (w/v) EDTA]. Six-well tissue-culture plates were used for viability assays and 25 cm^2 culture flasks for determining cellular ATP or glutathione. For measurements of cytosolic Ca^{2+} concentrations, cells were plated on glass coverslips (28 mm diameter). Cells used for one experiment were subcultured on the same day. Experiments were started 20–24 h after subculturing.

Experimental set-up

At the beginning of the experiments, cells were washed three times with Hanks' balanced salt solution (HBSS) and then covered with Krebs–Henseleit buffer (115 mM NaCl/25 mM $NaHCO_3$/5.9 mM KCl/1.2 mM $MgCl_2$/1.2 mM NaH_2PO_4/1.2 mM Na_2SO_4/2.5 mM $CaCl_2$/20 mM Hepes, pH 7.4) containing 100 μM DTPA; to each well [9.6 cm^2, $(9.6–11.5)\times10^5$ cells] 2.5 ml, to each coverslip [6.2 cm^2, $(6.2–7.4)\times10^5$ cells)] 1.6 ml and to each 25 cm^2 culture flask [$(25–30)\times10^5$ cells] 6.5 ml of Krebs–Henseleit buffer were added. Incubations were performed at 37 °C in an atmosphere of CO_2/air (1:19). H_2O_2 was added from a freshly prepared 100 mM stock solution, containing 100 μM DTPA, at the beginning of the experiments. In some cases, BSO (0.5 mM) was added to the cells 10 h and quin-2-AM (10 μM) or *N*-acetylcysteine (40 mM) 1 h before starting the experiments.

Determination of the cytosolic calcium concentration

The cytosolic calcium concentration was measured by digital fluorescence microscopy using the calcium-sensitive fluorescent dye fura-2. The dye was loaded into the cells as the membrane-permeable acetoxymethyl ester fura-2-AM, and retention of the dye in Rat-1 fibroblasts was improved by the addition of probenecid. For loading, the cells were incubated for 30 min at 37 °C in Krebs–Henseleit buffer containing 2.8 mM probenecid, 0.06 % (v/v) pluronic F-127 and 5 μM fura-2-AM. After this incubation the coverslips were rinsed three times with HBSS and again covered with Krebs–Henseleit buffer. Fluorescence measurements were performed on an inverted microscope (IM-35, Zeiss, Oberkochen, Germany) equipped with the ARGUS-50/CA imaging system (Hamamatsu Photonics, Hamamatsu-City, Japan). Cytosolic free calcium was determined by ratio imaging of fura-2 fluorescence using excitation filters of 340 ± 10 and 380 ± 10 nm and monitoring the emission at 500–530 nm. *In situ* calibration of the calcium concentration was performed by the method of Grynkiewicz et al. [26]. Note that, in these experiments, cells died much earlier than the cells used for the viability assays or glutathione measurements. This is presumably due to the fact that the cells were grown on glass coverslips and were exposed to UV light during the experiments.

Measurement of GSH and GSSG

Samples were deproteinized by the addition of $HClO_4$ (7 %, w/v, containing 2 mM EDTA) to the culture flasks, followed by immediate immersion of the culture flasks into liquid nitrogen. After thawing, the cells were scraped off, and the samples were centrifuged. The pellet was assayed for protein content using the Bradford method [27]. The supernatant was neutralized using 2 M KOH containing 0.3 M Mops. The $KClO_4$ precipitate was removed by centrifugation and the supernatant was used for the measurements. To minimize autoxidation of GSH, samples were kept at 4 °C during preparation and centrifugation and were assayed immediately. GSH was determined by the glyoxalase I method [28,29] and GSSG by the GSSG reductase assay [30].

Determination of cellular glutathione at the single-cell level with monochlorobimane

After various incubation times under experimental conditions, the cells were washed three times with HBSS and incubated for 25 min at 37 °C in Krebs–Henseleit buffer containing monochlorobimane at a final concentration of 50 μM. Retention of the product was improved by the addition of probenecid (2 mM). After this incubation the coverslips were rinsed three times with HBSS and again covered with Krebs–Henseleit buffer. Fluorescence measurements were performed on an inverted microscope (Axiovert 135 TV; Zeiss) equipped with the Attofluor imaging system (Atto Instruments, Rockville, MD, U.S.A.) using an excitation wavelength of 380 ± 10 nm and monitoring the emission at 460–490 nm.

Further assays

Extracellular, i.e. released, lactate dehydrogenase (LDH) activity was measured using a standard assay. At the end of the incubation period, cellular LDH activity was determined after lysis of the cells with the detergent Triton X-100 (1 %, v/v, in HBSS, 30 min at 37 °C). LDH activity was given as a percentage of total LDH activity. Cellular ATP content was determined by capillary electrophoresis as described in [31].

H_2O_2 degradation was studied by adding H_2O_2 (1 mM) to the cells incubated in Krebs–Henseleit buffer containing 100 μM DTPA. After intervals of 30 min, the H_2O_2 concentration was determined spectrophotometrically in aliquots of the supernatant by the peroxidase-catalysed conversion of 4-aminophenazone and 3,5-dichloro-2-hydroxybenzenesulphonic acid to a pink-coloured substance [32].

Statistics

All experiments were performed in duplicate and repeated three to five times. Data are expressed as means $\pm$ S.D. Data obtained from two groups were compared by means of Student's *t*-test.

RESULTS

When cultured Rat-1 fibroblasts (Rat-1/wild-type) were exposed to 1 mM H_2O_2 in substrate-free Krebs–Henseleit buffer, more than 70% of cells lost viability within 2 h (Figure 1a). By contrast, less than 15% of the Bcl-2-transfected Rat-1 fibroblasts (Rat-1/Bcl-2) were injured, even after 4 h of incubation with 1 mM H_2O_2 (Figure 1b). The addition of glucose (10 mM) strongly protected wild-type fibroblasts against H_2O_2-induced cell death (Figure 1a), whereas glucose, surprisingly, did not protect the Bcl-2 transfectants (Figure 1b). In a pattern similar to that observed with wild-type cells, glucose was also protective in the vector control (results not shown). In the absence of H_2O_2, no loss of viability was observed in any of the cells studied during 6 h of incubation.

Preincubation with the intracellular Ca^{2+} chelator quin-2 (10 μM) strongly protected wild-type fibroblasts (and vector control; results not shown) against H_2O_2-induced cell injury (Figure 2a), while no protection by quin-2 was seen in Bcl-2 transfectants (Figure 2b). In the absence of H_2O_2, the cytosolic Ca^{2+} concentration was constant in each cell clone during 3 h of incubation (results not shown). In the presence of 1 mM H_2O_2, cytosolic Ca^{2+} increased in wild-type fibroblasts from 53 ± 16 nM to 515 ± 32 nM during 35 min of incubation (Figure 3). A similar increase of the cytosolic Ca^{2+} concentration was seen in the vector control (Rat-1/cMycER; results not shown). In Rat-1/Bcl-2 cells, however, H_2O_2-induced cytosolic Ca^{2+} increase did not exceed 170 nM (Figure 3) before the fluorescent dye fura-2 leaked out of the cells, i.e. before the cells died. The addition of glucose delayed the H_2O_2-dependent increase in the cytosolic Ca^{2+} concentration in wild-type fibroblasts (Figure 3) and also in the vector control (results not shown). Therefore Bcl-2, like

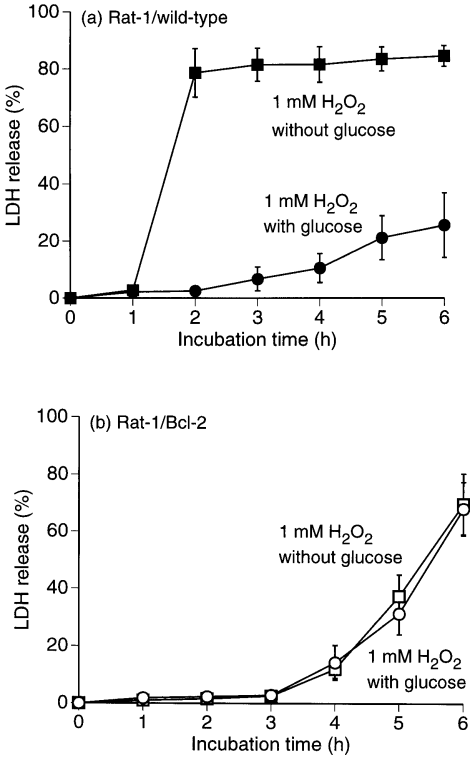

Figure 1 Effects of glucose and of overexpression of Bcl-2 on H_2O_2-induced cell death in Rat-1 fibroblasts

Rat-1/wild-type (**a**) and Rat-1/Bcl-2 cells (**b**) were incubated in Krebs–Henseleit buffer in the presence of 1 mM H_2O_2. Glucose was added at a concentration of 10 mM. Cell injury was assessed by the release of cytosolic LDH. Values shown represent means $\pm$ S.D. for four experiments.

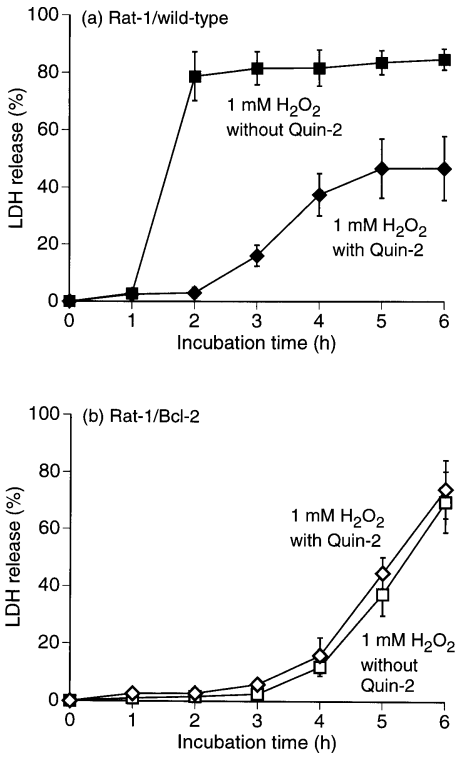

Figure 2 Effect of the calcium chelator quin-2 on H_2O_2-induced cell death in Rat-1 fibroblasts

Rat-1/wild-type (**a**) and Rat-1/Bcl-2 cells (**b**) were incubated in Krebs–Henseleit buffer (without glucose) in the presence of 1 mM H_2O_2. Quin-2-AM (10 μM) was added 1 h before the start of the experiments. Cell injury was assessed by the release of cytosolic LDH. Values shown represent means $\pm$ S.D. for four experiments.

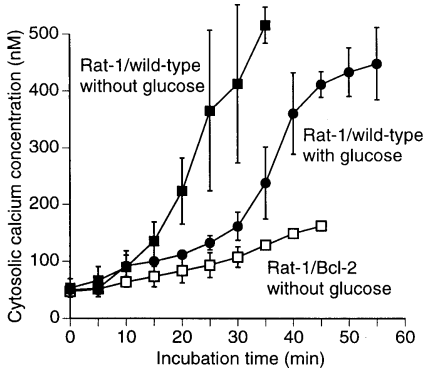

Figure 3 Effects of Bcl-2 and glucose on the H_2O_2-induced increase in the cytosolic Ca^{2+} concentration in Rat-1 fibroblasts

Rat-1/wild-type cells were incubated in Krebs–Henseleit buffer with or without glucose (10 mM) and Rat-1/Bcl-2 cells were incubated in Krebs–Henseleit buffer without glucose, all in the presence of 1 mM H_2O_2. The cytosolic Ca^{2+} concentration was determined by digital fluorescence microscopy using fura-2. Values shown represent means $\pm$ S.D. for four experiments.

Table 1 Effects of H_2O_2 and glucose on the cellular ATP content of Rat-1 fibroblasts

Rat-1/wild-type, Rat-1/cMycER and Rat-1/Bcl-2 cells were incubated in Krebs–Henseleit buffer in the presence of 1 mM H_2O_2 with or without glucose (10 mM). The cellular ATP content was determined by capillary electrophoresis. Values shown represent means ± S.D. for four experiments. *Significantly different from the initial value.

	ATP content (nmol/10^6 cells)		
	Rat-1/wild-type	Rat-1/cMycER	Rat-1/bcl-2
Initial value	5.3 ± 0.2	5.3 ± 0.2	4.9 ± 0.3
30 min			
H_2O_2	4.2 ± 0.7*	4.3 ± 0.5*	3.9 ± 0.3*
H_2O_2 + glucose	3.8 ± 0.6*	4.2 ± 0.5*	3.7 ± 0.4*
60 min			
H_2O_2	3.1 ± 0.5*	3.2 ± 0.3*	2.3 ± 0.5*
H_2O_2 + glucose	2.7 ± 0.4*	3.1 ± 0.6*	2.3 ± 0.3*

glucose, seems to protect against the Ca^{2+}-dependent mode of H_2O_2 toxicity in Rat-1 fibroblasts.

To explore further the protective effect of glucose on cell death in wild-type cells and the vector control (Rat-1/cMycER) and the lack of this effect in Bcl-2 transfectants, we determined the kinetics of H_2O_2 degradation in these cells. However, H_2O_2 (1 mM) degradation was the same in all the cells studied (about 70 % after 60 min and > 90 % after 120 min) and independent of the presence of glucose, thus excluding variations in the rate of H_2O_2 degradation as an explanation for the different time courses in cytotoxicity. To exclude the possibility that the protective effects of Bcl-2 and glucose on H_2O_2 treatment might be due to an improved ATP supply, we measured cellular ATP content during incubation of wild-type cells, Bcl-2 transfectants and the vector control with H_2O_2 (1 mM) in the presence or absence of glucose. In wild-type fibroblasts, cellular ATP content decreased within 60 min by about 40 % (Table 1). Similar results were observed with the vector control (Rat-1/cMycER) and the Bcl-2 transfectants. Glucose did not significantly alter the cellular ATP content either in wild-type fibroblasts, in the vector control or in the Bcl-2 clones.

Although glucose did not affect overall H_2O_2 detoxification or ATP supply, an improved provision of NADPH through the pentose-phosphate pathway and subsequent improved reduction of GSSG might still provide an explanation for the protective effect of glucose. Therefore, we determined whether cellular glutathione levels were altered by glucose and possibly also by Bcl-2. Comparison of wild-type cells and Bcl-2 transfectants showed a 1.6-times-higher GSH level for Bcl-2 cells compared with wild-type cells or vector control (Figure 4a). Furthermore, the marked decrease in GSH levels that was observed for the controls Rat-1/cMycER and Rat-1/wild-type did not occur in Rat-1/Bcl-2 cells, in which the relative decrease in GSH levels was far smaller, and even the absolute decrease appeared to be less marked, although the difference in the absolute values did not reach significance. Moreover, the relative (and absolute) increase of GSSG levels during incubation with H_2O_2 was higher in the controls compared with Bcl-2 transfectants (Figure 4b). The GSSG/GSH ratio was thus far better maintained in Bcl-2 cells (0.15 ± 0.04 in Bcl-2 cells versus 0.64 ± 0.06 in wild-type cells and 0.67 ± 0.19 in the vector control; all values after 30 min incubation with 1 mM H_2O_2). Incubation in the presence of glucose attenuated the decrease in GSH and the increase in

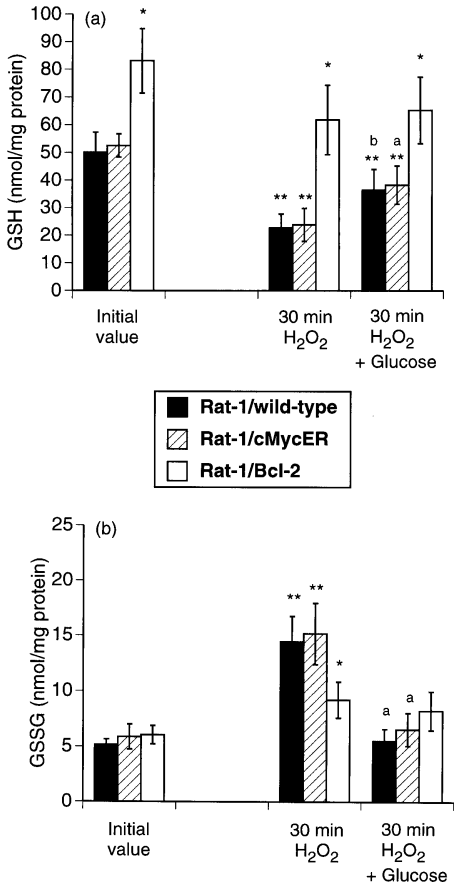

Figure 4 Effects of Bcl-2, H_2O_2 and glucose on the GSH and GSSG content of Rat-1 fibroblasts

Rat-1/wild-type cells, Rat-1/Bcl-2 cells and the vector control Rat-1/cMycER were incubated in Krebs–Henseleit buffer in the presence of 1 mM H_2O_2 with or without glucose (10 mM). At the times indicated, cells were lysed and deproteinized with perchloric acid and (**a**) GSH and (**b**) GSSG were determined enzymically. Values shown represent means ± S.D. for four experiments. Statistical significance: *significantly different from Rat-1/wild-type and Rat-1/cMycER cells, $P < 0.05$; **significantly different from initial values of the respective cell line, $P < 0.05$; [a]significantly different from incubation without glucose, $P < 0.05$; [b]significantly different from incubation without glucose, $P < 0.1$.

GSSG in wild-type cells and in the vector control, but no effect of glucose on either GSH or GSSG was seen in Bcl-2 transfectants (Figure 4). Comparable results were obtained using the GSH-sensitive fluorescent dye monochlorobimane (Table 2). These measurements on the single cell level also showed that there was no compartmentation or significant heterogeneity between the cells. Hence, glucose and Bcl-2 appear to exert their protective functions by the same effect, namely improvement of the glutathione-dependent antioxidative capacity, which also explains why glucose was without effect in those cells already protected by Bcl-2.

To confirm further this key role of the glutathione homoeostasis in the protection achieved by Bcl-2, we manipulated the cellular thiol status. Increasing the thiol-dependent antioxidative capacity by 40 mM *N*-acetylcysteine (which increased initial GSH levels in wild-type cells from 50.2 ± 7.2 to 62.2 ± 6.7 nmol/mg of protein and values after 30 min incubation with H_2O_2 from 22.6 ± 5.1 to 44.6 ± 5.5 nmol/mg of protein) strongly protected Rat-1/wild-type fibroblasts against loss of viability (12 ± 2 versus 78 ± 6 % LDH release after 4 h) and largely prevented the increase

Table 2 Effects of Bcl-2 and H_2O_2 on the GSH content of Rat-1 fibroblasts determined in single cells

Rat-1/wild-type, Rat-1/cMycER and Rat-1/Bcl-2 cells were incubated in Krebs–Henseleit buffer in the presence of 1 mM H_2O_2 with or without glucose (10 mM). The GSH-sensitive fluorescent dye monochlorobimane was used to detect cellular GSH content. Relative fluorescence intensity is given in arbitrary units. Values shown represent means ± S.D. for three experiments. Statistical significance: *significantly different from initial values of the respective cell line, $P < 0.05$; †significantly different from incubation without glucose, $P < 0.05$.

	GSH level (arbitrary units)		
Cells	Initial value	30 min H_2O_2	30 min H_2O_2 plus glucose
Rat-1/wild-type	76.7 ± 1.5	33.0 ± 1.7*	54.0 ± 5.6*†
Rat-1/cMycER	86.6 ± 5.7	36.6 ± 2.1*	59.3 ± 5.5*†
Rat-1/Bcl-2	140.0 ± 7.2	111.7 ± 12.9*	118.3 ± 9.1*

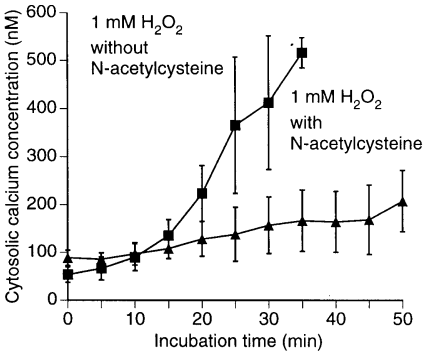

Figure 5 Effect of *N*-acetylcysteine on the H_2O_2-induced increase in the cytosolic Ca^{2+} concentration in Rat-1 fibroblasts

Rat-1/wild-type cells were incubated in Krebs–Henseleit buffer (without glucose) in the presence of 1 mM H_2O_2. *N*-Acetylcysteine was added 1 h before starting the experiments at a concentration of 40 mM. The cytosolic Ca^{2+} concentration was determined by digital fluorescence microscopy using fura-2. Values shown represent means ± S.D. for four experiments.

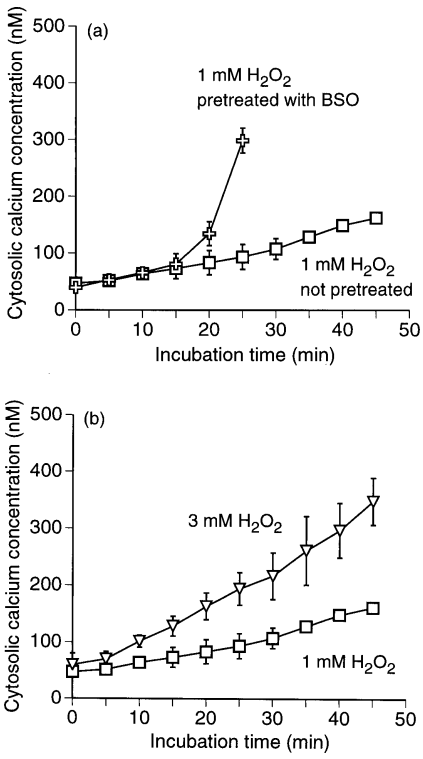

Figure 6 Effects of BSO pretreatment and increased H_2O_2 concentration on the H_2O_2-induced rise in cytosolic Ca^{2+} concentration in Rat-1/Bcl-2 cells

Cells were pretreated (**a**) for 10 h with 0.5 mM BSO or (**b**) not pretreated, and incubated in Krebs–Henseleit buffer (without glucose) in the presence of either 1 or 3 mM H_2O_2. The cytosolic Ca^{2+} concentration was determined by digital fluorescence microscopy using fura-2. Values shown represent means ± S.D. for four experiments.

in the cytosolic Ca^{2+} concentration induced by 1 mM H_2O_2 (Figure 5). When Rat-1/Bcl-2 fibroblasts were incubated for 10 h with 0.5 mM BSO to inhibit γ-glutamylcysteine synthetase, cellular GSH decreased to 33.7 ± 7.4 nmol/mg of protein, i.e. to 41 % of initial levels. These cells showed a rapid increase in cytosolic Ca^{2+} (Figure 6a) and they rapidly died upon addition of 1 mM H_2O_2 (results not shown), in contrast with Rat-1/Bcl-2 cells that were not pretreated with BSO. These results further support the assumption that Bcl-2 does not directly act on Ca^{2+} fluxes, but rather influences an upstream regulator, namely glutathione.

If high glutathione levels and the better maintenance of the glutathione redox balance in Bcl-2 transfectants, and not major differences in calcium homoeostasis, are indeed the reason for protection of these cells against H_2O_2-induced calcium-mediated cytotoxicity, it should be possible to overcome this protection by applying increased oxidative stress, i.e. using a higher H_2O_2 concentration. Therefore we incubated Rat-1/Bcl-2 cells with 3 mM H_2O_2 and we did indeed observe an increase in the cytosolic Ca^{2+} concentration (Figure 6b) paralleled by an increase in cell death (results not shown). At this higher H_2O_2 concentration, quin-2 (10 μM) suddenly became protective, decreasing injury from 65 ± 8 to 33 ± 9 % LDH release after 4 h of incubation. Thus the cytosolic Ca^{2+} increase and subsequent cell death were dependent on cellular glutathione status, and Bcl-2 appears to exert its protective effect through improvement of the anti-oxidative potential provided by glutathione without a direct effect on the cellular Ca^{2+} homoeostasis.

DISCUSSION

H_2O_2, glutathione, and calcium homoeostasis

Disruption of cellular Ca^{2+} homoeostasis and release of Ca^{2+} from intracellular Ca^{2+} stores into the cytosol is a common event in H_2O_2-induced cell death [21,33,34]. An increase in the cytosolic Ca^{2+} concentration obviously plays a decisive role in the cytotoxicity of H_2O_2 towards non-Bcl-2-transfected Rat-1 fibroblasts as well: upon addition of H_2O_2 there was a rapid increase in the cytosolic Ca^{2+} concentration (Figure 3) and the Ca^{2+} chelator quin-2 protected against loss of viability. In the Ca^{2+}-dependent mode of oxidative cell injury, alterations in the cellular glutathione status play a crucial role in raising the cytosolic Ca^{2+} concentration. Glutathione plays an important role in maintaining the redox balance of thiol groups of essential Ca^{2+}-transport systems localized to the endoplasmic reticulum, mitochondria, nucleus and plasma membrane [35,36]. Recent studies have reported decreased glutathione resulting in Ca^{2+}-mediated cell death of PC 12 cells [37] and calcium elevation appearing after glutathione depletion during thymocyte apoptosis [38]. The present results are in line with these, as

addition of H_2O_2 to Rat-1 fibroblasts (wild-type) led to a rapid decrease in GSH, parallelled by an increase in GSSG (Figures 4a and 4b). In addition, stabilization of the cellular GSH pool by *N*-acetylcysteine strongly protected the cells against the increase in cytosolic Ca^{2+} concentration and cell death.

The presence of glucose also significantly delayed the H_2O_2-induced increase in cytosolic Ca^{2+} concentration and thus attenuated cell injury. As already reported for L929 fibroblasts [39], this protective effect of glucose on the Ca^{2+}-dependent mode of H_2O_2 cytotoxicity mostly results from its effect on the glutathione redox cycle. GSSG, formed by the catalytic activity of glutathione peroxidase, is readily re-reduced to GSH by glutathione reductase. The NADPH necessary for this glutathione redox cycle can be provided mainly through the metabolism of glucose via the pentose phosphate pathway. The present results are in line with this view, as addition of glucose also delayed the decrease of GSH after addition of H_2O_2 and lowered the cellular GSSG/GSH ratio as well (Figures 4a and 4b). Hence, glucose inhibits the Ca^{2+}-dependent mode of H_2O_2 cytotoxicity by its effect on the glutathione redox cycle.

The protective effect of glucose has been mostly considered to be due to an increased degradation of H_2O_2 by glutathione peroxidase [40]. In our study, however, the degradation of H_2O_2 was independent of the presence of glucose, suggesting that it was mainly due to the action of catalase. This would be in line with previously published findings that at high concentrations of H_2O_2, as used in the present experiments, the bulk of H_2O_2 is degraded by catalase even in the presence of glucose [41,42]. In addition, it has been shown that catalase and glutathione peroxidase are able to substitute for each other to some extent [43]. Even if only a small part of the total H_2O_2 added is degraded by glutathione peroxidase, the associated GSSG formation results in an altered GSSG/GSH ratio that then initiates secondary alterations such as disturbance of cellular calcium homoeostasis. In addition to the lack of effect on overall H_2O_2 degradation (and thus on the amount of H_2O_2 available for participation in chemical reactions/hydroxyl-radical formation), glucose did not improve ATP supply. An altered H_2O_2 degradation and/or an improved energy supply could thus not explain the protective effect of glucose.

Protection exerted by Bcl-2

The protective effect of Bcl-2 overexpression against injury induced by 1 mM H_2O_2 shows a remarkable analogy to the protection afforded by glucose: loss of viability was largely delayed in the Bcl-2-transfected cells, and there was only a slight increase in the cytosolic Ca^{2+} concentration (Figure 3). In accordance with the latter result, the calcium chelator quin-2 did not show any protection. Furthermore, overexpression of Bcl-2 led to a markedly increased amount of total glutathione and decreased the relative loss of GSH during oxidative stress induction (Figures 4a and 4b). Thus Bcl-2 exerts its protection via inhibition of the Ca^{2+}-dependent mode of H_2O_2 cytotoxicity, and maintenance of the cellular glutathione homoeostasis can completely account for this protective effect. Further evidence of a close relationship between calcium and glutathione homoeostasis, H_2O_2 cytotoxicity and protection by Bcl-2 is provided by the observation that depletion of cellular glutathione by BSO and subsequent treatment with 1 mM H_2O_2 also triggered a pathological Ca^{2+} increase in the Bcl-2 transfectants (Figure 6a). In addition, overcoming the increased antioxidative capacity by increased oxidative stress, i.e. 3 mM instead of 1 mM H_2O_2, reveals a Ca^{2+}-dependent component of cell injury in the Bcl-2 transfectants as well: the cytosolic Ca^{2+} concentration markedly increased (Figure 6b) and quin-2 became protective.

The observation that Bcl-2 does not directly regulate cellular Ca^{2+} compartmentation and fluxes but modulates sensitivity to Ca^{2+}-dependent injurious processes through the intermediary glutathione may also help to explain the contradictory results reported for the action of Bcl-2 on cellular Ca^{2+} homoeostasis and on Ca^{2+}-mediated cell death [5–12]. According to the results obtained here with Rat-1 fibroblasts, Bcl-2 should only affect those Ca^{2+}-mediated processes that are triggered by alterations in cellular glutathione homoeostasis, but should leave non-glutathione-dependent processes unaffected. This proposal, however, has to be verified in cells other than Rat-1 fibroblasts and with inducers of cell death different from H_2O_2.

Bcl-2 and cellular glutathione

Effects of Bcl-2 on cellular glutathione have already been described for a variety of other cell types, although they were not considered with respect to cellular Ca^{2+} homoeostasis [13–18]. Despite these data on Bcl-2 and glutathione, little is known to date about the precise mechanism by which Bcl-2 alters cellular glutathione metabolism. Bcl-2 has been reported as acting to inhibit opening of the mitochondrial permeability transition pore [44]. Since mitochondrial permeability transition may lead to pronounced cellular GSH depletion due to uncoupling of the respiratory chain and an increased formation of reactive oxygen species, such an effect of Bcl-2 may explain the smaller decrease in GSH and increase in GSSG (and thus the better maintenance of the GSSG/GSH ratio) in the Rat-1/Bcl-2 cells upon addition of H_2O_2. An alternative explanation for a smaller decrease in GSH might be that Bcl-2 prevents GSH translocation out of the cells during the oxidative damage as has been reported for Bcl-x_L transfected prolymphocytic cells upon interleukin-3 withdrawal [45]. However, neither of these protective mechanisms can explain the fact that the basal level of GSH was itself markedly increased in the Bcl-2-transfected cells. Since this high level of GSH appears to contribute substantially to the increased resistance of the Rat-1/Bcl-2 cells, direct effects of Bcl-2 on glutathione metabolism, such as inhibition of a methionine-dependent GSH efflux [15], are the more likely explanation for the protection exerted by this oncogene on H_2O_2 cytotoxicity to the Rat-1 fibroblasts. Further investigations, however, are needed to support this assumption, but what seems to be clear from the present study is that, in a model of massive, external oxidative stress induction, Bcl-2 influences intracellular Ca^{2+} fluxes only indirectly by increasing the GSH-dependent antioxidative capacity of the cell.

We thank Dr. G. I. Evan and Dr. T. Littlewood for Rat-1, Rat-1/Bcl-2 and Rat-1/cMycER cell lines. This work was supported by a grant from the Medical Faculty of the University of Essen (IFORES Programm) and the Fonds der Chemischen Industrie.

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Received 4 January 1999/26 February 1999; accepted 9 March 1999