Enhancement of iron toxicity in L929 cells by D-glucose: accelerated (re-)reduction

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It has recently been shown that an increase in the cellular chelatable iron pool is sufficient to cause cell damage. To further characterize this kind of injury, we artificially enhanced the chelatable iron pool in L929 mouse fibroblasts using the highly membrane-permeable complex Fe(III)/8-hydroxyquinoline. This iron complex induced a significant oxygen-dependent loss of viability during an incubation period of 5 h. Surprisingly, the addition of D-glucose strongly enhanced this toxicity whereas no such effect was exerted by L-glucose and 2-deoxyglucose. The assumption that this increase in toxicity might be due to an enhanced availability of reducing equivalents formed during the metabolism of D-glucose was supported by NAD(P)H measurements which showed a 1.5–2-fold increase in the cellular NAD(P)H content upon addition of D-glucose. To assess the influence of this enhanced cellular reducing capacity on iron

valence we established a new method to measure the reduction rate of iron based on the fluorescent iron(II) indicator PhenGreen SK. We could show that the rate of intracellular iron reduction was more than doubled in the presence of D-glucose. A similar acceleration was achieved by adding the reducing agents ascorbate and glutathione (the latter as membrane-permeable ethyl ester). Glutathione ethyl ester, as well as the thiol reagent *N*-acetylcysteine, also caused a toxicity increase comparable with D-glucose. These results suggest an enhancement of iron toxicity by D-glucose via an accelerated (re-)reduction of iron with NAD(P)H serving as central electron provider and ascorbate, glutathione or possibly NAD(P)H itself as final reducing agent.

Key words: chelatable iron, ferrous iron, nicotinamide adenine dinucleotide, reactive oxygen species, redox state.

INTRODUCTION

Iron is essential for many cellular processes, yet it also has a strong damaging potential. The major part of the cellular iron is safely bound in ferritin as well as in haem- or iron-sulphur-cluster-containing proteins (enzymes). However, a small part (0.2–3 % [1–5]) is loosely attached to proteins or lipids or weakly bound to low-molecular-mass ligands like phosphates or citrate, forming a transit iron pool that keeps iron available for the synthesis of iron-containing proteins [1]. This pool of iron is, because of its methodological definition, also often referred to as ‘chelatable’ iron. In the presence of H₂O₂ this redox-active iron participates in the Fenton reaction, which leads to a decomposition of H₂O₂ to hydroxyl ions (OH[−]) and the highly reactive hydroxyl radicals (•OH). The hydroxyl radicals attack and oxidize lipids, proteins and DNA, and thus lead to cell damage [6].

Reactive oxygen species (ROS)-mediated, iron-dependent tissue injuries are usually thought to be caused by an increased production of O₂^{•−}/H₂O₂, without alterations in the concentration of redox-active iron. In recent years, however, it has become evident that in some pathophysiological processes, such as in ischaemia/reperfusion injury [2,7] or in the toxicity of xenobiotics such as ethanol [8], cell injury is a consequence of both an enhanced production of the primary ROS O₂^{•−}/H₂O₂ and an additional rise in the redox-active iron pool. This rise can be caused by the release of iron from ferritin [9] and/or iron-sulphur clusters [10], for example mediated by O₂^{•−}.

Besides these two pathophysiological settings, a rise in the formation of the primary ROS in the presence of an unaffected concentration of cellular redox-active iron or a rise in O₂^{•−}/H₂O₂ release accompanied by an increase in cellular redox-active iron,

recent studies show that a rise in the chelatable iron pool itself can be the cause of cell damage. Thus during cold-induced apoptosis in rat hepatocytes a pathogenetically decisive increase in the concentration of cellular chelatable iron during cold incubation was observed and caused cell injury although the production of O₂^{•−} and H₂O₂ was even decreased [11].

To further examine and characterize the mechanisms of cell injury elicited by a primary rise in the cellular chelatable iron pool, we experimentally increased the chelatable iron pool of L929 mouse fibroblasts using a membrane-permeable iron(III) complex [12]. Surprisingly, we observed a strong enhancement of iron toxicity by the addition of D-glucose to the incubation medium. This enhanced toxicity appears to be caused by an accelerated initial reduction, and probably also subsequent re-reduction, of the intracellular redox-active iron from the ferric [Fe(III)] to the reactive ferrous [Fe(II)] state (‘redox-cycling’), suggesting that the availability of cellular reducing equivalents could be an important factor in iron-dependent cell injuries.

EXPERIMENTAL

Materials

Eagle’s minimum essential medium, fetal calf serum, FeCl₃, Fe(NH₄)₂(SO₄)₂, 8-hydroxyquinoline (8HQ), L-ascorbic acid, 2-deoxyglucose, L-glucose, reduced glutathione (GSH), glutathione ethyl ester (reduced form), chelex (chelating resin; iminodiacetic acid), L-buthionine sulfoximine (BSO), 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU; carmustine), triethanolamine hydrochloride and *N*-acetylcysteine were obtained from Sigma (Deisenhofen, Germany). Trypsin and penicillin/

Abbreviations used: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; BSO, L-buthionine sulfoximine; DCHF, 2,7-dichlorodihydrofluorescein; λ_{em}, emission wavelength; λ_{exc}, excitation wavelength; 8HQ, 8-hydroxyquinoline; Fe/8HQ, iron(III)/8-hydroxyquinoline; LDH, lactate dehydrogenase; ROS, reactive oxygen species.

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streptomycin were from Gibco (Eggenstein, Germany). Triton X-100, DMSO, D-glucose, NH_4Cl and citric acid trisodium salt (dihydrate) were purchased from Merck (Darmstadt, Germany). Falcon cell culture flasks were from Becton Dickinson (Heidelberg, Germany). The dye precursor 2,7-dichlorodihydrofluorescein (DCHF; as diacetate) and the fluorescent dye PhenGreen SK (as a di-potassium salt and a cell-permeant diacetate) were from Molecular Probes Europe (Leiden, The Netherlands). Glutamate dehydrogenase, α -ketoglutarate, NADH and NADPH were obtained from Roche (Penzberg, Germany). All gas mixtures were delivered by Messer Griesheim (Oberhausen, Germany).

Cell culture

Cells of the murine fibroblast cell line L929 (American Type Culture Collection; NCTC clone 929, strain L) were cultured in Eagle's minimal essential medium supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. Culturing was performed in 75 cm^2 cell culture flasks in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Subculturing was achieved by trypsin treatment [0.25% (v/v) trypsin in citrate saline]. For all measurements, cells were seeded in a density of 2×10^4 cells/ cm^2 and cultured for 48 h. Viability assays were performed in 12.5 cm^2 cell culture flasks; for microscopic fluorescence measurements cells were seeded on glass coverslips (6.2 cm^2) and for the enzymic determination of NAD(P)H Petri culture dishes (175 cm^2) were used.

Cytotoxicity assays

Before starting the experiments, cells were washed twice with Hank's balanced salt solution (HBSS; 137.0 mM NaCl/5.4 mM KCl/1.0 mM CaCl_2 /0.5 mM MgCl_2 /0.4 mM KH_2PO_4 /0.3 mM Na_2HPO_4 /25 mM Hepes, pH 7.4). Experiments were performed in 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 7.35) with an incubation volume of 2.5 ml/flask. Where indicated, the buffer was supplemented with 10 mM D-glucose, L-glucose or deoxyglucose. Iron was added from a fresh stock solution [iron(III)/8HQ (Fe/8HQ) complex with 10 mM iron/20 mM 8HQ in DMSO] 30 min after covering the cells with the respective buffer. N-Acetylcysteine (40 mM), BCNU (150 μM), glutathione ethylester (4 mM) and glutathione (4 mM) were added to the cell culture medium 30 min prior to the start of the experiments and were present during the measurements. Ascorbate (0.2 mM) or BSO (0.5 mM) were added to the cell culture medium 24 h (ascorbate) or 20 h (BSO) before the start of the experiments. Hypoxic conditions were achieved by saturating the incubation solutions with gas mixtures containing 5% CO_2 and 0, 2 or 5% O_2 in nitrogen as indicated before addition to the cells, followed by gentle flushing of the culture flasks with the same gas mixture through cannulae piercing the rubber stoppers of the flasks as described in [13]. The flushing was repeated each time a sample was taken.

Cell viability was determined by measuring extra- and intracellular lactate dehydrogenase (LDH) activity using a standard assay. Released LDH was measured at each time point indicated. At the end of the incubation period, intracellular LDH activity was determined following lysis of the cells using the detergent Triton X-100 (1% in HBSS, 30 min at 37 °C). LDH values were corrected for the change in volume of incubation medium resulting from repetitive sampling, and released LDH activity was given as a percentage of total LDH activity.

NAD(P)H measurements

Fluorescence microscopic measurements

After having transferred the glass coverslips to a modified Pentz chamber, cells were washed twice with warm Krebs–Henseleit buffer and then covered with 1.25 ml of the buffer. As indicated, cells were used either directly after washing or following a preincubation period of 30 min. During the measurements, cells were kept in an atmosphere of 5% CO_2 in air at 37 °C. Autofluorescence [excitation wavelength (λ_{exc}) = 365 ± 12.5 nm and emission wavelength (λ_{em}) = 450–490 nm] of single cells was measured using an inverted microscope (Axiovert 135 TV; Zeiss, Oberkochen, Germany) equipped with the Attofluor imaging system (Atto Instruments, Rockville, MD, U.S.A.). Emission was recorded every 2 min [shorter intervals were avoided to prevent the light-induced oxidation of NAD(P)H]. D-Glucose and iron were added from stock solutions (1 M D-glucose and 20 mM Fe/40 mM 8HQ, respectively) during the measurements.

Enzymic measurements using glutamate dehydrogenase and α -ketoglutarate

Cells were washed twice with warm HBSS buffer and incubated for 30 min in 35 ml of Krebs–Henseleit buffer with or without 10 mM D-glucose under culturing conditions. Cell lysis was performed following a modified protocol described in [14]. In brief, after covering the cells with 1 ml of warm HBSS, 0.5 ml of 1 M alcoholic KOH solution was added and the mixture was incubated for 30 min at room temperature. For protein precipitation pH was adjusted to 7.8 using a phosphate-buffered triethanolamine solution. After another 10 min of incubation at room temperature and centrifugation (10 min, 10000 g) the amount of reduced NAD(P)H was determined in the supernatant according to Klingenberg [14] by measuring the fluorescence decrease due to NAD(P)H oxidation following the addition of glutamate dehydrogenase and α -ketoglutarate/ NH_4Cl . Fluorescence was determined using a Shimadzu RF-1501 spectrofluorometer (λ_{exc} = 365 nm and λ_{em} = 460 nm); a standard curve was recorded using known amounts of NADH and NADPH. Protein content was determined with Bradford's protein assay.

Determination of the rate of iron reduction

PhenGreen SK quenching in a cell-free system

The response of PhenGreen SK fluorescence to ferrous and ferric iron was tested in a chelex-treated cytosolic medium described in detail before [15,16] using a laser-scanning microscope (LSM 510; Zeiss). PhenGreen SK (30 μM ; dipotassium salt) was added to 2 ml of cytosolic medium at 37 °C. An aliquot of 100 μl was placed on a glass coverslip pretreated with chelex and fluorescence was measured with an excitation wavelength of 488 nm through a 505 nm long-pass emission filter in a focal plain 10 μm above the glass surface. The value obtained was set at 100%. To determine the quenching effect of iron, aliquots of either a ferrous iron [1 mM $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ /1 mM citric acid trisodium salt dihydrate/20 mM ascorbate in 10 mM imidazole buffer, pH 7.0] or a ferric iron (1 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 10 mM imidazole buffer) stock solution were added to the thermostat-controlled PhenGreen solution up to a final iron concentration of 10 μM . Fluorescence was measured 2 min after the addition of iron as described above.

Cellular measurements

For cellular measurements, an Axiovert 135 TV inverted microscope equipped with the Attofluor imaging system was used.

After transferring the cells to a Pentz chamber (see above) they were washed twice with warm Krebs–Henseleit buffer. Cells were loaded with 60 μM PhenGreen SK diacetate (in Krebs–Henseleit buffer without supplements in all experiments) for 15 min in the dark. Thereafter, cells were carefully washed three times, and fresh Krebs–Henseleit buffer was added. After an additional incubation for 15 min, the buffer was exchanged, and the chamber was transferred to the prewarmed microscope stage. The fluorescence of each single cell ($\lambda_{\text{exc}} = 488 \pm 10 \text{ nm}$, $\lambda_{\text{em}} = 520 \pm 20 \text{ nm}$) was recorded every minute for 10 min. Thereafter, the microscope shutter was opened allowing a continuous recording of the fluorescence response, and 5 μM iron was added from a fresh stock solution (5 mM Fe/10 mM 8HQ in DMSO). When the rapid fluorescence decrease levelled off, the shutter was closed again and recording was continued in 1 min intervals for another 5–10 min. The slope of the initial linear fluorescence decrease was calculated for each cell.

For some measurements, D-glucose (10 mM), L-glucose (10 mM), BCNU (150 μM), glutathione or glutathione ethyl ester (4 mM each) were added to all buffers except during cellular loading with the dye. For other measurements, cells were preincubated with ascorbic acid (0.2 mM in cell culture medium) for 24 h or with BSO (0.5 mM) for 20 h before start of the experiment; neither ascorbate nor BSO were present during the measurement itself.

DCHF oxidation

Cells cultured on glass coverslips were loaded with the oxidation-sensitive fluorescent-dye precursor DCHF (10 μM in Krebs–Henseleit buffer without any additive, 1 h at 37 °C), washed twice and subsequently incubated for 15 min in Krebs–Henseleit buffer with or without D-glucose as indicated. After another washing step, 2.5 ml of the respective buffer (Krebs–Henseleit with or without D-glucose) was added and the cells were transferred to the prewarmed microscope stage. Iron (20 μM , as Fe/8HQ) was added after recording the fluorescence ($\lambda_{\text{exc}} = 488 \pm 10 \text{ nm}$, $\lambda_{\text{em}} = 520 \pm 20 \text{ nm}$) for 10 min and fluorescence was measured for 2000 s.

Statistics

Fluorescence measurements were repeated at least three times with 10–20 single cells/experiment. Cytotoxicity experiments and NAD(P)H measurements were repeated three or four times in duplicates. Data presented are means $\pm$ S.D. Data obtained from two groups were compared by means of Student's *t* test and a *P* value of < 0.05 was considered significant.

RESULTS

Enhancement of iron toxicity by D-glucose

Incubation of L929 fibroblasts with 10–30 μM of the membrane-permeable complex Fe/8HQ caused a concentration-dependent loss of viability during 5 h of incubation in substrate-free Krebs–Henseleit buffer (Figures 1 and 2A). The ligand alone did not exert any toxicity (results not shown). Unexpectedly, the cellular damage caused by Fe/8HQ was dramatically increased by the addition of 10 mM D-glucose to the incubation medium (Figures 1 and 2B). In contrast, the non-metabolizable glucose analogues/derivatives L-glucose (Figure 1) and 2-deoxyglucose (results not shown) did not significantly enhance Fe/8HQ toxicity, implying that metabolic transformation of glucose is essential for the enhancement of toxicity, and ruling out extra-cellular, chemical and osmotic effects.

Figure 1 Effects of D- and L-glucose on iron-induced cell damage

L929 cells were incubated in Krebs–Henseleit buffer without glucose (○), with 10 mM D-glucose (●) or with 10 mM L-glucose (□). Iron (20 μM) was added as Fe/8HQ complex and cell injury was assessed by the release of cytosolic LDH. Data represent means $\pm$ S.D. of four experiments. *, Significantly different from incubation without glucose; +, significantly different from incubation with L-glucose.

Hypoxic conditions protected cells from the iron-induced loss of viability in the absence as well as in the presence of D-glucose (Figure 3), implying an involvement of ROS in this iron-induced cytotoxicity. Nevertheless, also in the presence of lower oxygen concentrations (2 and 5%), i.e. under oxygen partial pressures in the physiological range, D-glucose strongly enhanced iron toxicity (compare Figures 3B and 3A).

The enhancement of iron-induced oxidative stress by D-glucose was confirmed by fluorescence microscopic measurements with the dye precursor DCHF (loaded into the cells as diacetate), a probe that shows an increase in fluorescence when the fluorescent oxidized compound, dichlorofluorescein ('DCF') is formed from the non-fluorescent parent compound DCHF [17,18]. Upon iron addition to L929 cells loaded with DCHF and incubated in the absence of glucose the fluorescence increased slowly (Figure 4) until cell death (results not shown), suggesting the formation of oxidizing species following the addition of iron. In the presence of D-glucose, the fluorescence increase (and thus the iron-dependent oxidation of the dye) was much faster (Figure 4).

Effects of D-glucose on the cellular reductive state

Iron is generally assumed to mediate ROS-dependent cell damage mainly in the reduced ferrous state, hence a potential link between glucose metabolism and iron toxicity might be the cellular redox state. Metabolism of D-glucose leads to formation of NADH and NADPH during glycolysis, the pentose phosphate pathway, the citrate cycle etc., thereby increasing the availability of reducing equivalents. NAD(P)H is known to be the main cellular electron donor for redox reactions and to provide electrons for the regeneration of different cellular antioxidants, which themselves are electron donors (reducing agents), such as

Figure 2 Dependence of cell injury on iron concentration in the absence and presence of D-glucose

L929 cells were incubated in Krebs–Henseleit buffer in the absence (A) and presence (B) of 10 mM D-glucose. Iron was added as Fe/8HQ complex in the indicated concentrations. Cell injury was assessed by the release of cytosolic LDH. Data represent means $\pm$ S.D. of four experiments.

glutathione and ascorbate. Thus cellular NAD(P)H content is a marker for the general reducing capacity of cells [19]. To monitor a possible influence of D-glucose on the NAD(P)H content, we measured the cellular autofluorescence at $\lambda_{\text{exc}} = 365 \pm 12.5$ nm and $\lambda_{\text{em}} = 450\text{--}490$ nm, which is predominantly caused by NADH and NADPH [20].

Without D-glucose, there was an intermediate fluorescence intensity (Figure 5A) that remained on a stable level for at least 1 h (results not shown). In line with the assumption made above, the addition of D-glucose caused a rapid rise in fluorescence intensity, which reached a plateau about twice as high as the initial value after approx. 5 min (Figure 5A).

Figure 3 Dependence of iron-induced cell injury on oxygen partial pressure in the absence and presence of D-glucose

L929 cells were incubated in Krebs–Henseleit buffer with 20 μ M iron (as Fe/8HQ complex) in the absence (A) and presence (B) of 10 mM D-glucose at different oxygen partial pressures as indicated. Cell injury was assessed by the release of cytosolic LDH. Data represent means $\pm$ S.D. of three experiments.

For confirmation we determined the amount of reduced NAD(P)H in cell lysates using an enzymic assay (glutamate dehydrogenase). In cells incubated for 30 min in glucose-free Krebs–Henseleit buffer an NAD(P)H content of 1.16 ± 0.18 nmol/mg of protein was found; in the presence of D-glucose a 1.5-fold higher amount of reduced NAD(P)H (1.74 ± 0.14 nmol/mg of protein) could be observed.

Influence of D-glucose on iron reduction

The results described above suggest that the enhanced iron toxicity in the presence of D-glucose might be due to an enhanced reduction of iron to the damaging ferrous state caused by an

Figure 4 Effect of D-glucose on the iron-dependent oxidation of DCHF

Cells cultured on glass coverslips were loaded with 10 μ M DCHF (applied as the diacetate) for 1 h and fluorescence was recorded on a digital fluorescence microscope ($\lambda_{\text{exc}} = 488 \pm 10$ nm, $\lambda_{\text{em}} = 520 \pm 20$ nm) in the absence and presence of 10 mM D-glucose. After 10 min 20 μ M iron was added as Fe/8HQ complex (arrow) and oxidation of the dye (fluorescence increase) was monitored for another 2000 s. Data are presented in arbitrary units (a.u.) and represent means $\pm$ S.D. from four experiments with at least 20 single cells/measurement.

increased availability of NAD(P)H and, subsequently, other reducing equivalents. Unfortunately, the determination of the redox state of intracellular chelatable iron is highly problematic. To estimate a possible influence of D-glucose (or other reducing agents) on redox state and/or reduction rate of intracellular iron we therefore established a new application of the fluorescent probe PhenGreen SK, which has been used as an intracellular iron indicator [15,16]. Its response to ferrous and ferric iron is very different: in the presence of only 2 μ M ferrous iron, the fluorescence of 30 μ M PhenGreen SK was already clearly decreased in a cell-free system, whereas even 10 μ M ferric iron did not significantly influence PhenGreen SK fluorescence (Table 1). Based on these different responses, a new kinetic approach was taken here, using this dye not as an indicator for the presence of cellular chelatable iron but to monitor the reduction of iron, in single, viable cells and 'online', by recording the decrease in cellular PhenGreen SK fluorescence caused by ferrous iron.

Figure 6(A) shows the fluorescence of cells loaded with PhenGreen SK as recorded by digital fluorescence microscopy. After 10 min, 5 μ M Fe/8HQ was added, which caused a sudden decrease in cellular PhenGreen SK fluorescence, indicating that the entering ferric iron is reduced to the ferrous state. In the presence of D-glucose there was a marked increase in the rate of the fluorescence decline as compared with the values obtained in cells incubated in substrate-free buffer (without glucose, 0.88 ± 0.34 arbitrary units/s; with D-glucose, 2.44 ± 1.05 arbitrary units/s), whereas this acceleration of iron reduction could not be found in the presence of L-glucose (results not shown).

As D-glucose affects the cellular reductive state via NAD(P)H (Figure 5A), iron reduction is likely to be linked, directly or via other reducing agents, to the oxidation of NAD(P)H. This assumption was confirmed by the finding that the addition of Fe/8HQ decreased the cellular content of NAD(P)H signifi-

Figure 5 Effects of D-glucose and iron on cellular NAD(P)H content

Cells grown on glass coverslips were incubated in Krebs–Henseleit buffer and cellular autofluorescence ($\lambda_{\text{exc}} = 365 \pm 12.5$ nm, $\lambda_{\text{em}} = 450\text{--}490$ nm) was recorded on a digital fluorescence microscope. Data are presented in arbitrary units (a.u.). (A) Effect of D-glucose. Cells were preincubated in glucose-free Krebs–Henseleit buffer for 30 min and fluorescence was measured every 2 min. After 10 min, 10 mM D-glucose (arrow) was added. (B) Effect of iron. After 30 min of incubation in Krebs–Henseleit buffer with or without D-glucose, 20 μ M iron/40 μ M 8HQ (Fe/8HQ) was added. Values directly before and 2 min after iron addition are shown. Data represent means $\pm$ S.D. from four (A) or three (B) experiments. *, significantly different from incubation without glucose (A) or before iron addition (B).

cantly, in both the absence and presence of D-glucose (Figure 5B).

Influences of ascorbate and glutathione on iron reduction

As NAD(P)H may mediate its effect on iron reduction partially or mostly via the reducing agents glutathione and ascorbate, which are well-known reductants for iron [6], we assessed whether it was possible to accelerate iron reduction not only by a rise in the cellular NAD(P)H content via D-glucose, but also by in-

Table 1 Effects of ferrous and ferric iron on the fluorescence of PhenGreen SK in a cell-free system

PhenGreen SK (30 μM) was dissolved in a cytosolic medium (at 37 °C) designed to simulate the composition of the cytosol. Ferrous and ferric iron were added from freshly prepared stock solutions [Fe(II), 1 mM $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ /1 mM citric acid trisodium salt dihydrate/20 mM ascorbate in 10 mM imidazole buffer, pH 7.0; Fe(III), 1 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 10 mM imidazole buffer] up to a final concentration of 10 μM . After the addition of each aliquot, the medium was rapidly mixed and incubated for 2 min at 37 °C. An aliquot (100 μl) of the medium was placed on a chelex-treated glass coverslip and fluorescence was determined at $\lambda_{\text{exc}} = 488 \text{ nm}$ and $\lambda_{\text{em}} \geq 505 \text{ nm}$ using a laser-scanning microscope (see the Experimental section). The relative emission intensities shown are expressed as a percentage of the initial fluorescence intensity (set at 100%). Values shown represent means $\pm$ S.D. from three experiments.

Iron concentration (μM)	PhenGreen SK fluorescence (% of initial fluorescence)	
	Fe(II)	Fe(III)
0	100	100
2	81.8 $\pm$ 2.1	100.7 $\pm$ 1.8
5	52.4 $\pm$ 2.1	100.9 $\pm$ 1.4
8	27.5 $\pm$ 3.0	103.6 $\pm$ 3.8
10	19.9 $\pm$ 1.1	98.6 $\pm$ 3.7

creasing the amount of cellular glutathione or ascorbate, respectively. Indeed, in the absence of D-glucose, addition of the membrane-permeable glutathione ethyl ester in its reduced form and preincubation of the cells with ascorbate accelerated the iron reduction (Figure 6B), indicating that both glutathione and ascorbate can act as the direct reducing agents.

On the other hand, if the cells were supplemented with ascorbate in addition to D-glucose, no further increase in the rate of iron reduction could be found (results not shown). Similarly, glutathione depletion by preincubation with BSO did not significantly influence iron reduction, in neither the presence nor absence of D-glucose (results not shown). The lack of any effect of these variations on iron reduction suggests that the decisive factor for the reduction is NAD(P)H and that the final mediator/reducing agent is variable.

Effects of reducing agents on iron toxicity

If accelerated iron reduction is the reason for the enhancement of iron toxicity by D-glucose, other cellular reductants, especially the NAD(P)H-dependent reductants glutathione and ascorbate, which were also capable of accelerating iron reduction, might have a similar effect on iron toxicity. A preincubation of the cells with glutathione ethyl ester or the unspecific thiol-donor *N*-acetylcysteine did indeed increase iron toxicity to a similar extent as D-glucose (Figures 7A and 7D). Glutathione itself, which is not membrane permeable and served as a control for extracellular effects, did not have any effect on the cellular viability (results not shown). However, neither depletion of glutathione by BSO preincubation nor inhibition of glutathione reductase by BCNU eliminated the toxicity enhancement caused by D-glucose (Figures 7B and 7C), a finding that is in line with their lack of effect on iron reduction (see above).

In contrast to glutathione ethyl ester and *N*-acetylcysteine, preincubation of the cells with ascorbate (24 h) did not increase iron toxicity in the absence of D-glucose (Figure 7E), despite the fact that iron reduction was accelerated (Figure 6B). When applied in addition to D-glucose, a preincubation with ascorbate exerted a (partial) protective effect against the strong toxicity enhancement induced by D-glucose. In contrast, addition of D-glucose to cells loaded with ascorbate markedly enhanced iron toxicity (Figure 7E). These findings may suggest a dual role for

Figure 6 Effects of D-glucose, ascorbate and glutathione on the rate of intracellular iron reduction

L929 cells cultivated on glass coverslips were loaded with 60 μM PhenGreen SK diacetate for 15 min and fluorescence was recorded on a digital fluorescence microscope ($\lambda_{\text{exc}} = 488 \pm 10 \text{ nm}$, $\lambda_{\text{em}} = 520 \pm 20 \text{ nm}$). After 10 min, 5 μM iron/10 μM 8HQ (Fe/8HQ) was added (arrow in A) and the fluorescence decrease was monitored. Data are presented in arbitrary units (a.u.). (A) Typical time course of a measurement performed with cells incubated in glucose-free Krebs–Henseleit buffer and in Krebs–Henseleit buffer supplemented with 10 mM D-glucose. (B) Effects of ascorbate [24 h preincubation (PI) at 0.2 mM] and glutathione ethyl ester (1 h preincubation at 4 mM) on the initial iron reduction rate in the absence of D-glucose. The initial linear slope of the decrease ($-\Delta \text{Fluorescence/s}$) is plotted. In each experiment 10–15 single cells were measured. All experiments were repeated 3–4 times. Data shown represent means $\pm$ S.D. *, Significantly different from incubation without reducing agent.

ascorbate as both an anti- and pro-oxidant, and show that the effect of D-glucose overrules the effects of ascorbate preincubation.

DISCUSSION

D-Glucose normally benefits cultured cells by providing them with a substrate for energy generation and by improving their

antioxidative defence capacity. This was shown for various kinds of cell injury in different cell types, including alloxan-induced hamster β -cell damage [21], NO-caused injury to a human colonic endothelial cell line [22], MPP⁺ (1-methyl-4-phenylpyridinium)-mediated apoptosis in PC 12 cells [23] and H₂O₂-induced cell damage in L929 and Rat-1 cells, where the supplementation of the incubation buffer (Krebs–Henseleit) with D-glucose strongly decreased H₂O₂ toxicity [24,25]. Surprisingly, a completely different picture evolved using the present system: iron toxicity exerted by Fe/8HQ was dramatically enhanced by the addition of D-glucose to the incubation medium (Figures 1 and 2). The evidence presented here strongly suggests that this increase in toxicity is mediated by an enhanced reduction of the ferric iron entering the cell via an increased availability of cellular reducing equivalents.

To exert iron toxicity not by an extracellular attack on the plasma membrane but by a manipulation of the cellular chelatable iron pool, cells were loaded with iron using the iron complex Fe/8HQ. The chelation to the fairly small and lipophilic 8HQ made the iron membrane permeable and thus allowed us to bypass the highly regulated cellular uptake of iron, whereas iron salts such as FeCl₃, FeSO₄ and Fe(NH₄)₂(SO₄)₂ or iron bound to nitrilotriacetic acid did not enter the cells with comparable ease (experiments in isolated hepatocytes; [16] and F. Petrat and U. Rauen, unpublished work) and did, in contrast to the membrane-permeable iron complex, not exert considerable toxicity in L929 fibroblasts (I. Lehn-Beyel and U. Rauen, unpublished work).

The determination of intracellular chelatable iron (reviewed in [26]) is already very difficult and the determination of its redox state is even more problematic. EPR spectroscopy, the only method described so far which allows unequivocal differentiation between ferric and, with the addition of specific chelators (shifting the equilibrium), ferrous iron, has been used for whole-cell measurements only in bacteria [10] and yeast [27]. In mammalian cells, however, these measurements have only been performed in homogenates and not in whole cells. During sample preparation for these measurements (homogenization followed by freezing in liquid nitrogen as described in [3,28–30]), an artificial oxidation of ferrous iron is very likely. In addition, neither the observation of rapid changes in iron valence nor measurements in living cells are possible with EPR spectroscopy. Similarly, almost all methods based on the use of specific chelators for ferrous and ferric iron (ferrozine assay, etc.) can only be applied to cell homogenates in which the Fe(II)/Fe(III) ratio present in viable cells is no longer maintained. Fluorescent indicators present an attractive alternative as they are sensitive and can be applied to viable cells. The fluorescent probe calcein was suggested to be suitable for the determination of ferrous chelatable iron some years ago [31,32]; however, more recent studies cast doubts on this specificity [26,33], and even suggested calcein as an indicator for ferric iron [33]. We here developed another methodological approach, based on the different response of the fluorescent dye PhenGreen SK to ferrous and ferric iron which allows the monitoring of the intracellular reduction of iron in viable cells ‘online’ and at the single-cell level.

Using this new approach, we could show that the ferric iron entering the cells is reduced to ferrous iron intracellularly (Figure 6). This finding is in line with observations in the literature describing that, under physiological conditions, the major part of the cellular chelatable iron pool is in the ferrous state [10,34]. The reduction of iron was strongly accelerated in the presence of D-glucose. Since the subsequent increased availability of ferrous iron probably enhances the production of reactive and damaging species during Fenton-like reactions and thus also the re-oxidation of the ferrous iron to the ferric state, it is likely that D-

glucose accelerates not only the initial reduction of the entering iron but also enhances the subsequent ‘redox turnover’ of iron (illustrated in Scheme 1).

The compounds generally thought to be responsible for cellular iron reduction are the NAD(P)H-dependent reducing agents ascorbate and glutathione. Ascorbate is known to be a very potent extra- and intracellular reducing agent for ferric iron. In combination with ferric iron, it is widely used to produce extracellular oxidizing ferrous systems (e.g. the ‘Udenfriend system’ [6]) and it is usually considered to be the prime reductant for iron intracellularly. When L929 cells were loaded with ascorbate, iron reduction was also accelerated in our system (Figure 6B). This cell line, however, normally contains only very small amounts of ascorbate [less than 0.1 nmol of ascorbate/mg of protein (C.-D. Badrakhan, M. Kirsch and H. de Groot, unpublished work), equivalent to < 12 μ M, assuming approx. 8.5 μ l of cell volume/mg of protein [35–37]], as it is also described for other cell lines [38,39]. Thus despite its high reducing potential, ascorbate is very unlikely to be the main iron-reducing compound in our experimental system under normal culturing conditions. However, the acceleration of the iron reduction observed in the presence of D-glucose could still be due to an enhanced regeneration of cellular ascorbate, although this is not very likely as a simultaneous provision of ascorbate and D-glucose did not further increase the reduction rate.

Reduced glutathione is also able to reduce iron bound to various intracellular ligands. We could show here that (intracellular) glutathione is able to accelerate iron reduction (Figure 6B) as well as increase iron toxicity (Figure 7A). D-Glucose has previously been shown to increase the GSH/GSSG ratio and to strongly improve its maintenance under oxidative conditions in L929 cells [24], effects which are probably due to the enhanced availability of NADPH. With a GSH content of 1.35 ± 0.5 nmol/10⁵ cells (equivalent to a concentration of about 4 mM, assuming an approx. 3.5 pl cell volume [35–37]) GSH is likely to be the predominant reducing agent responsible for the intracellular iron reduction in our system. However, glutathione depletion did not influence the reduction rate of iron in the presence of D-glucose, and neither glutathione depletion via BSO nor the inhibition of the glutathione re-reduction by BCNU eliminated the enhancing effect of D-glucose on iron toxicity (Figures 7B and 7C), suggesting that at least one electron donor independent of glutathione contributes to the enhancing effect of D-glucose.

Both reducing agents, GSH and ascorbate, finally depend on NAD(P)H as an electron donor, and iron reduction seems to be closely connected with NAD(P)H oxidation. Furthermore, a direct electron transfer from NAD(P)H to iron also needs to be considered. The one-electron reduction potential of NAD(P)H is (in aqueous solution and under standard conditions) more positive than that of the glutathione couple [6,40], but still in the range to allow the reduction of Fe(III) to Fe(II), as described for *in vitro* systems investigating Fenton-driven DNA damage [41,42]. In cellular systems, reduction potentials are modified by different factors, but a direct reduction of iron by NAD(P)H also appears possible and has been suggested to be an important factor in H₂O₂ toxicity in *Escherichia coli* [43]. A potential role of NAD(P)H as direct reducing agent in our system is supported by the finding that the addition of D-glucose and the subsequent increase of the NAD(P)H content is sufficient to accelerate the iron reduction and strongly enhance iron toxicity, irrespective of whether the other cellular reducing agents are increased (addition of ascorbate) or decreased (glutathione depletion by BSO). Besides direct reduction, one could imagine electron transfer from NAD(P)H via enzymes or cofactors.

Figure 7 For legend, see facing page.

Scheme 1 Proposed mechanism for the D-glucose-induced enhancement of iron toxicity

After entering the cell, ferric iron, applied as the highly membrane-permeable complex formed from FeCl_3 and 8HQ [Fe(III)/8HQ], is reduced to ferrous iron, reoxidized by participation in ROS-forming reactions and subsequently re-reduced to the ferrous state. This redox cycling is accelerated by D-glucose, the metabolism of which increases the amount of the central electron provider NAD(P)H. The final reduction of the ferric iron can be exerted via glutathione (GSH), ascorbate (Asc) or possibly by NAD(P)H itself.

Although all three reducing agents can participate, the relative contribution of each reducing agent to intracellular iron reduction is likely to be influenced by changes in the cellular environment as well as by the cellular growth state and experimental conditions, because especially in complex cellular systems redox potentials depend on many different factors like the amount of reducing agent, metal concentration or the intracellular chemical environment, including pH and, most importantly, ligands bound to the metal. Thus it is possible that ascorbate, which due to its low concentration is the more unlikely primary reducing agent in our system, is the most important one in other cell types. However, all possible settings involve NAD(P)H as the central electron 'provider' (summarized in Scheme 1) and thus iron reduction is influenced by D-glucose metabolism irrespective of the final reducing agent.

While we here studied the cytotoxic effects of an increased availability of chelatable iron (without a primary rise in the formation of ROS, i.e. a type of cell injury that might be caused by a direct reaction of iron with dioxygen, as proposed by Qian and Buettner [44]), a similarly altered iron reduction depending on the availability of NAD(P)H could also influence other kinds of iron-dependent or iron-mediated injuries. H_2O_2 toxicity, for example, can be mediated by a calcium/thiol-group-dependent mechanism and/or an iron-dependent pathway [24,25]. Whereas under substrate-free conditions both mechanisms probably con-

tribute to the cell damage (with the calcium-dependent pathway being more pronounced and faster), in the presence of D-glucose the calcium-dependent route is inhibited (by improvement of the availability of reduced glutathione), yet the damage caused by the iron-dependent mechanism seems to be enhanced in Rat-1 as well as in L929 cells. This could well be explained by the findings described here.

In experimental models of oxidative cell injury like the present one, an enhanced antioxidant capacity is not only no advantage for the cell but can even be the cause for severe and rapid damage. When antioxidants can act as reducing agents and thus increase the redox turnover of catalytically active transition metals, whose reduced form participates in reactions that form oxidizing species, a pro-oxidant effect can be exerted by a rise in the redox capacity of the cell, as summarized in Scheme 1. This is a process that has also been described for ascorbate in combination with iron [6,45] and for thiol reagents, especially glutathione and cysteine, which have been described to increase iron cytotoxicity [46], selenium toxicity (reviewed in [47]) and copper-dependent DNA damage in cell-free systems [48], probably also via accelerated redox turnover. Similar to the antioxidants acting as pro-oxidants, it appears at first view paradoxical that more reducing equivalents enhance oxidative stress; this, however, can be explained by the accelerated generation of the injurious ferrous iron and increased redox cycling of iron as described above (see Scheme 1).

The data presented here clearly show that iron toxicity is strongly enhanced by addition of D-glucose, which exerts its effect via an increase in the cellular NAD(P)H content and a subsequently accelerated (re-)reduction of the iron. These findings suggest that not only the increase in the amount of redox-active iron but also the cellular redox state, and thus the availability of ferrous iron, are important determinants for iron-dependent oxidative cell injuries.

This study was supported by the Deutsche Forschungsgemeinschaft (RA 960/1-1).

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Figure 7 Effects of modifications in the availability of reductants on iron toxicity

L929 cells were incubated in Krebs–Henseleit buffer without (open symbols) and with (filled symbols) D-glucose in the presence of 20 μM iron/40 μM 8HQ. The availability of reducing equivalents was influenced, besides by D-glucose, by the following compounds ($\blacktriangle$, $\triangle$): (A) 4 mM glutathione ethyl ester (preincubation for 1 h and presence during the experiment); (B) 0.5 mM BSO [20 h preincubation (PI)]; (C) 150 μM BCNU (1 h preincubation and presence during the experiment); (D) 40 mM N-acetylcysteine (1 h preincubation and presence during the experiment) or (E) 0.2 mM ascorbate (asc; 24 h preincubation). Cell injury was assessed by LDH release. Data shown represent means $\pm$ S.D. of three (B and C) or four (A, D and E) experiments. *, Significantly different from incubation without addition of reductant; +, significantly different from incubation with D-glucose. The effect of D-glucose is shown for comparison.

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Received 22 April 2002/14 August 2002; accepted 15 August 2002

Published as BJ Immediate Publication 15 August 2002, DOI 10.1042/BJ20020639