Newly delivered transferrin iron and oxidative cell injury

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Abstract Cell iron status was assessed in terms of its capacity to mediate cell injury by pro-oxidants. Cultured K562 cells, which maintain a stable cytosolic labile iron pool (LIP) of <0.5 μM, underwent distinct changes after short exposures to transferrin (Tf) followed by *t*-butyl hydroperoxide (TBHP): (a) rise in LIP, detectable fluorimetrically; (b) increased lipid peroxidation and (c) eventual cell death. All of these effects were inhibited by weak bases or iron chelators. Similarly, hydrogen peroxide caused rises in both LIP and oxidant species detectable with 2′,7′-dichlorofluorescin diacetate, which were enhanced by preincubation with Tf. The Tf-delivered iron disappeared from LIP and the TBHP-reactive pool with a $t_{1/2}<30$ min. The results indicate that the catalytic potential of iron is highest while in transit between endosomes and cytosolic ligands.

Key words: Iron; Oxidative stress; Free radical; Calcein; Chelator; Hydrogen peroxide; Lipid peroxidation; Cell death

1. Introduction

Several forms of cell injury induced by pro-oxidant drugs involve catalysis by transition metals present in the cell cytosol [1,2]. Iron is the most likely candidate, being the most abundant and among the most reactive of those metals. The potential for inducing cell damage depends to a large extent on factors which affect the availability of particular forms of iron needed for conversion of otherwise stable and poorly reactive substances (e.g. drugs and metabolites) into highly reactive radicals. These include: (a) the chemical forms and levels of catalytically active iron, (b) accessibility of those forms to the alleged substances and (c) the proximity of the biochemical targets to the sites of radical formation. The major cell iron-containing compartments comprise (i) ferritin, the principal cytoplasmic iron storage protein [3], (ii) mitochondria, the site of synthesis of heme and the primary location of heme proteins [4,5], and (iii) the labile pool of cytoplasmic iron (LIP), which is associated with low-affinity ligands and is accessible to chelators [1,5–7]. Relatively smaller quantities of iron are presumably distributed among various organelles, some electrostatically bound to numerous negatively charged species, including phospholipids and other anionic substances [8,9]. Most of these iron fractions can potentially participate in Fenton reactions leading to production of noxious species. Even ferritin, in spite of its function as a safe iron(III) repository, can release reactive iron(II) upon exposure to reducing agents or superoxide and other radicals [2,11], or participate directly in radical mediated protein damage [12]. However, the most readily available cell source of reactive iron is apparently LIP. This is based primarily on the requirement of partially coordinated and weakly bound iron for promoting Fenton reactions [1,2] and on the proven capacity of iron chelators to prevent cell damage by pro-oxidants [5,6,10] or by ischemia/reperfusion [13–15]. LIP constitutes a heterogeneous fraction of iron bound to a variety of ligands [1,5] and entails various degrees of catalytic potentials. Using cultured human erythroleukemia K562 cells, we show here that resting cell LIP levels do not compromise cellular ability to cope with the generation of reactive oxygen species by lipophilic pro-oxidants while a fraction of cytosolic iron, which has been newly acquired from Tf and is in transit to various cell compartments, has enhanced reactivity toward pro-oxidants, leading to cell damage.

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Abbreviations: BSA, bovine serum albumin; CA, calcein; CA-AM, calcein acetoxymethyl ester; DTPA, diethylenetriamine-pentaacetic acid; FCS, fetal calf serum; HBS, 150 mM NaCl, 20 mM HEPES, pH 7.3; LIP, labile iron pool; LPP, lipid peroxidation products; MDA, malondialdehyde; PI, propidium iodide; SIH, salicylaldehyde isonicotinoyl hydrazone; Tf, transferrin; TBA, thiobarbituric acid; TBHP, *t*-butyl hydroperoxide; DCF, 2′,7′-dichlorofluorescein

2. Materials and methods

2.1. Materials

CA, its acetoxymethyl ester (CA-AM) and 2′,7′-dichlorofluorescin diacetate were obtained from Molecular Probes (Eugene, OR). The divalent metal ionophore A23187, *t*-butyl hydroperoxide (TBHP), malondialdehyde bisdimethylacetal and thiobarbituric acid (TBA) were from Sigma Chem. Co. (St. Louis, MO). All other materials were of the highest available grade. The iron chelators, salicylaldehyde isonicotinoyl hydrazone (SIH) (a generous gift from Dr. P. Ponka, Lady Davis Institute for Medical Research, Montreal, Canada), 2,2′-bipyridyl (Fluka, Buchs, Switzerland) and DFO (Ciba-Geigy, Basel, Switzerland) were prepared as 50 mM stock solutions in dimethylsulfoxide. Chromatographically pure Tf, $>70\%$ iron-saturated, was from Kama-Da Industries (Kibbutz Kama, Israel).

2.2. Cell treatments

Human erythroleukemia K562 cells in suspension were propagated by daily 1:1 dilution in α-MEM medium containing 7% fetal calf serum supplemented with L-glutamine and antibiotics. Prior to treatments, the growth medium was removed by centrifugation of the cells. Pretreatments with Tf were carried out at a density of 1×10^6 cells/ml in bicarbonate-free α-MEM medium containing 20 mM HEPES, pH 7.3 (αMEM-HEP). Tf was added from a concentrated 25 mg/ml stock solution of 150 mM NaCl, 10 mM HEPES-Tris, pH 7.3 (HBS buffer) to reach the indicated concentration, and removed after the preincubation, unless otherwise indicated. All treatments with TBHP were carried out at 37°C on cells suspended in HBS buffer at 1×10^6 cells/ml.

2.3. Determination of cellular LIP

1 ml of cell suspension (1×10^6 cells) was loaded with 0.25 μM CA-AM for 5 min at 37°C in α-MEM-HEP medium containing 2 mg/ml BSA. Excess CA-AM was removed by centrifugation and resuspension in 1 ml of the same medium lacking CA-AM. The cells were maintained at room temperature until used, within 60 min. Just prior to measurements, 1 ml of the CA loaded cell suspension was centrifuged in a microcentrifuge and the cells were resuspended in 2 ml of

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prewarmed HBS buffer containing 2 mM DTPA. The cell suspension was transferred to a stirred, thermostatted (37°C) cuvette and CA fluorescence was measured as described previously [16–18]: excitation 488 nm, emission 517 nm, 10 nm slits, on a Perkin-Elmer LS-5B fluorometer equipped with a temperature controlled cuvette holder and magnetic stirrer.

A baseline fluorescent signal was obtained for a suspension of CA-loaded cells and the signal was adjusted to 60 (on a scale of 0–100). The increase in fluorescence produced by the addition of 100 μM of the highly permeable iron chelator SIH was recorded. The presence of the impermeant chelator DTPA (2 mM) ascertained that the response due to SIH was entirely due to its chelation of intracellular CA-bound iron.

Calibrations to determine the relationship between changes in fluorescence and intracellular iron concentration were carried out in HBS lacking DTPA but containing an anti-calcein antibody to quench all extracellular fluorescence [16,17]. Ionophore A23187 (10 μM) was added (this produced $<5\%$ change in the signal), followed by 1.0 μM iron(II) added as ferrous ammonium sulfate, and the corresponding change in the fluorescence was determined. This calibration relies on the assumption that in the presence of A23187, iron(II) is fully equilibrated across the cell membrane, so the CA-detectable concentration of intracellular iron is 1.0 μM.

2.4. Lipid peroxidation (LPP)

LPP was determined by an adaptation of the thiobarbituric acid method described by Esterbauer and Cheeseman [20]. Following TBHP treatments, the cells (2×10^6 per sample) were centrifuged, the cell pellet was resuspended in 0.2 ml of cold HBS containing 2 mM DTPA and 0.01% butylated hydroxytoluene, and 0.5 ml of 10% TCA was added. After 5 min on ice, the TCA precipitate was removed by centrifugation (12000 rpm, 2 min), 0.5 ml of the clear supernatant was mixed with an equal volume of 0.67% thiobarbituric acid and incubated in a boiling water bath for 10 min. The samples were cooled and fluorescence was measured (excitation 522 nm, emission 550 nm, slits 10 nm, Perkin-Elmer LS-5B fluorometer). Calibration of LPPs was done with malondialdehyde (MDA) obtained fresh by acid hydrolysis of malondialdehyde bisdimethylacetal [20]. Since MDA's reaction product with thiobarbituric acid had the same fluorescence spectrum as cell-derived LPPs, we assume that the LPPs were mainly cellular malondialdehyde.

2.5. Determination of intracellular oxidant species with 2′,7′-dichlorofluorescin diacetate

Cells were suspended at a density of 0.5×10^6 cells/ml in HBS buffer containing 10 μM 2′,7′-dichlorofluorescin diacetate and incubated for 15 min at 37°C, with or without H_2O_2. The reaction was stopped by rapid centrifugation of the cells and resuspension in ice-cold HBS buffer containing anti-fluorescein antibody, and the fluorescence of the cell suspension was recorded (excitation 488 nm, emission 525 nm, slits 10 nm, Perkin-Elmer LS-5B fluorometer). The antibody was present in excess and quenched all extracellular fluorescence, thus ensuring that only intracellular oxidant species-mediated conversion of the non-fluorescent 2′,7′-dichlorofluorescin to the fluorescent 2′,7′-dichlorofluorescein was being recorded. The values of intracellular 2′,7′-dichlorofluorescein concentration ($[DCF]_{in}$) were derived from known values of total intracellular volume in each sample (0.5 μl volume per sample of 0.5×10^6 cells) and calibration of 2′,7′-dichlorofluorescein concentration vs. fluorescence.

2.6. Cell viability

Cell viability was determined by Trypan blue exclusion and by uptake of propidium iodide (PI). In the former, cell suspensions were mixed with 1/10 volume of 0.4% Trypan blue and the live/dead ratio was determined by counting in a hemocytometer. The second assay is based on the relative impermeability of living cells to PI. Following its entry into dead cells, PI intercalates into DNA causing a large increase in fluorescence. Samples of 1 ml (1×10^6 cells) suspended in HBS in 24-well plates were incubated in the presence or absence of TBHP for 90 min at 37°C. The cell suspensions were then mixed with 1 ml of 5 μM PI in HBS at room temperature, and fluorescence at emission 520 nm, excitation 605 nm was measured after 2 min, when the signal became stable. Then, 0.1 ml of 10% Triton X-100 was added to fully permeabilize all cells in the system and give an estimate of the maximal binding of PI. This reading was taken as 100% cell death. The percentage of dead cells was calculated (after background subtraction) from the ratio of the readings before and after Triton X-100 addition. The cultures routinely contained ≥95% viable cells, as determined by Trypan blue exclusion. The results obtained with the PI assay correlated well with those obtained with Trypan blue.

CA has also been used extensively as a vital dye, since it is retained only by viable cells and leaks out of non-viable ones. Although we did not use CA here as a viability indicator due to its capacity to chelate iron, its retention in the cells during the LIP assays was an additional indicator of viability. We ascertained that the CA-loaded cells used in the LIP assays had an intact permeability barrier, by carrying out the assays in the presence of the non-permeable chelator DTPA (2 mM). In the experiments shown, this chelator did not cause a gradual dequenching of CA which would be indicative of membrane leakiness. This was also confirmed by use of an anti-CA fluorescence quenching antibody which causes quenching of CA fluorescence in leaky cells.

3. Results

3.1. Effect of TBHP and H_2O_2 on cellular LIP, MDA production and viability

Previously we have shown that K562 cells exposed to TBHP and H_2O_2 show an increase in LIP as detectable with

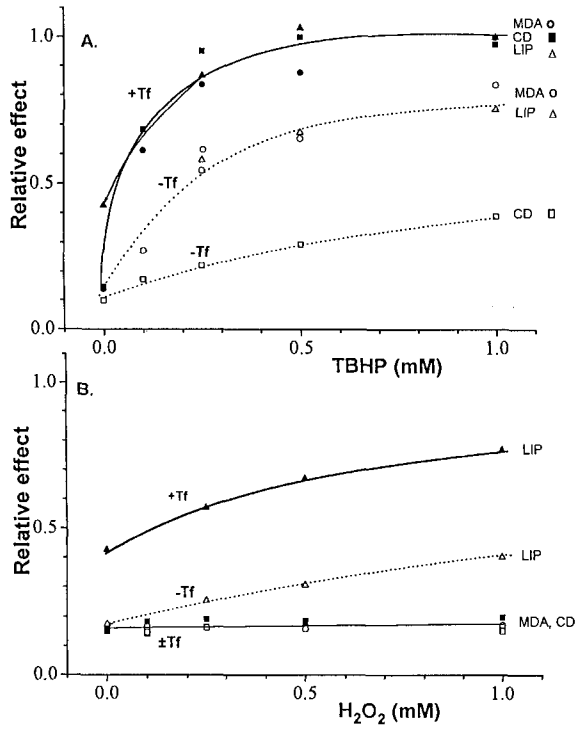

Fig. 1. Effect of TBHP and H_2O_2 on cellular LIP, MDA production and viability. K562 cells were preincubated for 20 min at 37°C in serum-free medium in the presence (filled symbols) and absence (empty symbols) of 100 μg/ml Tf. After removal of the Tf by centrifugation, the cells were treated with various concentrations of TBHP (A) or H_2O_2 (B) in HBS solution for 15 min at 37°C. The reaction was stopped by centrifugation of the cells and resuspension in α-MEM-HEP medium containing 2 mg/ml BSA. LIP levels (triangles) were determined after loading cells with 0.25 μM CA-AM for 5 min at 37°C, as described in Section 2. Cellular MDA levels (circles) were determined immediately after the treatments. For measurements of cell death (CD, squares), cells were suspended in HBS solution with and without 100 μg/ml Tf, and incubated for 90 min at 37°C in the presence of either TBHP or H_2O_2. Cell viability was determined using the PI method as described in Section 2. Data are given as relative to the maximal value attained in all the experiments (set as 1.0). The value of 1.0 corresponds to: 2 μM for LIP, 5 pmol/10^6 cells for MDA and 40% cell death for CD.

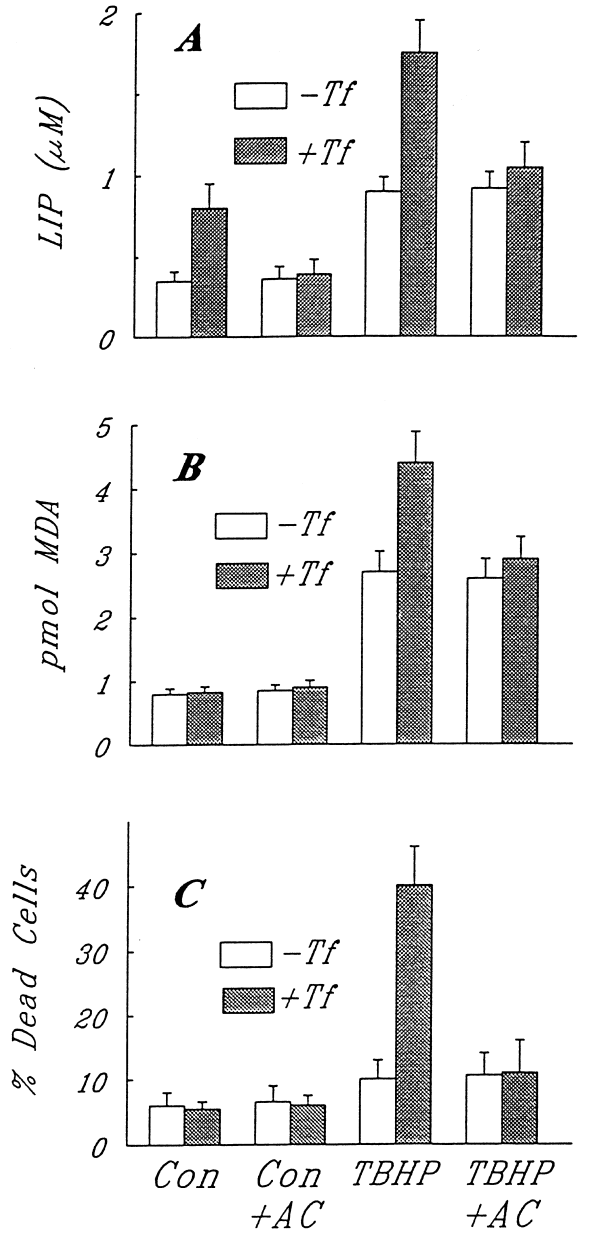

Fig. 2. The effect of blockade of Tf-iron release on TBHP-mediated LIP increase, MDA production and cell viability. K562 cells were preincubated for 20 min at 37°C in serum-free medium in the presence and absence of 100 μg/ml Tf, with and without 25 mM ammonium chloride (AC). After removal of the Tf by centrifugation, the cells were treated with 0.25 mM TBHP, with or without AC as indicated, for 15 min at 37°C. The reaction was stopped by centrifugation of the cells and resuspension in α-MEM-HEP medium containing 2 mg/ml BSA. The cells were then analyzed for LIP levels (A) and for MDA production, in pmol MDA/10^6 cells (B), as described. C: Cells taken from culture were centrifuged and resuspended in HBS solution with the indicated additions. Cell viability was determined after 90 min at 37°C, as described. Empty bars: no Tf; hatched bars: 100 μg/ml Tf; Con: control; AC: 25 mM ammonium chloride; TBHP: 0.25 mM TBHP.

CA [18]. Accordingly, the LIP of cells incubated for 15 min with increasing concentrations of pro-oxidants rose from a basal level of 0.35 μM to maximally 3.6-fold with 1 mM TBHP (Fig. 1A) and 2.15-fold with 1 mM H_2O_2 (Fig. 1B). When the cells were preincubated with Tf (0.1 mg/ml) for 20 min, their basal LIP rose by 2.3-fold (from 0.35 to 0.80 μM, Fig. 1). A subsequent 15 min exposure to TBHP or H_2O_2 caused a further 2.5- and 1.9-fold increase in LIP, respectively. The TBHP-generated production of MDA followed the same pattern as LIP (Fig. 1A). Increasing concentrations of TBHP caused a gradual rise in cellular MDA from a basal level of

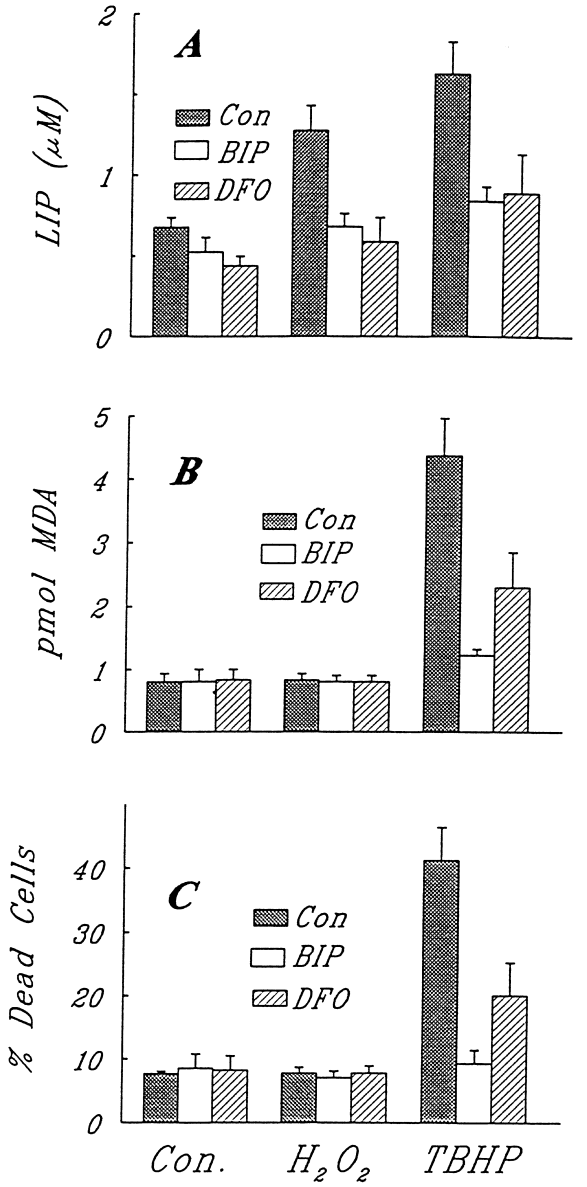

Fig. 3. Effect of iron chelators on H_2O_2- and TBHP-mediated LIP increase, MDA production and cell viability. K562 cells were preincubated for 20 min at 37°C in α-MEM-HEP medium containing 100 μg/ml Tf. After removal of the Tf by centrifugation, the cells were resuspended in HBS solution containing 0.1 mM BIP (empty bars), 0.1 mM DFO (hatched bars) or no chelator (cross-hatched bars). They were then treated with 1.0 mM H_2O_2 or 0.25 mM TBHP for 15 min at 37°C. The reaction was stopped by centrifugation of the cells and resuspension in α-MEM-HEP medium containing 2 mg/ml BSA. The cells were then analyzed for LIP levels (A) and for MDA production in pmol MDA/10^6 cells (B), as described. C: Cells taken from culture were centrifuged and resuspended in HBS solution containing 100 μg/ml Tf with the indicated additions. Cell viability was determined after 90 min at 37°C, as described. Cross-hatched bars: no chelator; empty bars: 0.1 mM BIP; hatched bars: 0.1 mM DFO; Con: untreated control; H_2O_2: 1.0 mM H_2O_2; TBHP: 0.25 mM TBHP.

0.65 pmol/10^6 cells in controls lacking Tf up to 4 pmol/10^6 cells (i.e. 6-fold) at 1 mM TBHP. After a 20 min preincubation with Tf, the MDA production in response to TBHP was augmented by 1–1.5 pmol MDA/10^6 cells (Fig. 1A). In contrast to TBHP, H_2O_2 failed to cause any detectable MDA formation (Fig. 1B).

The 15 min exposure of cells with basal LIP levels to either TBHP or H_2O_2 caused no detectable change in the cell permeability barrier, as measured by accumulation of either Trypan blue or PI, or by leakage of calcein from CA-AM-loaded cells (data not shown). However, a significant level of cell death became detectable after >45 min incubations with TBHP. As shown in Fig. 1A, a 90 min exposure of cells to TBHP in the absence of Tf caused only ≤16% cell death, while in the presence of Tf it rose to 41%. H_2O_2, at concentrations up to 1 mM (and even 5 mM, not shown), caused no detectable toxicity within 90 min, with or without Tf (Fig. 1B).

3.2. *The effect of blockade of Tf-iron delivery on TBHP-mediated LIP increase, MDA production and cell viability*

In order to show that the TBHP-mediated increases in LIP, MDA production and cell death required the prior release of iron from Tf, we tested the effect of the weak base NH_4Cl, which is known to alkalinize endosomes and inhibit the release of iron from Tf [17]. As shown in Fig. 2, NH_4Cl (25 mM) prevented the Tf-mediated increase in LIP, both in the presence and in the absence of TBHP, but had no effect on the basal LIP, i.e. in cells not preincubated with Tf (Fig. 2A). A similar pattern of inhibitory effects of NH_4Cl was obtained for MDA production (Fig. 2B) and cell death (Fig. 2C). In both cases, NH_4Cl did not affect MDA production and cell death due to TBHP in the absence of Tf, but prevented their augmentation by Tf. Chloroquine (0.2 mM), another weak base which also alkalinizes acidic compartments, blocked the action of Tf similarly to NH_4Cl, although it was only 35% as potent (not shown).

The inhibitory effect of NH_4Cl indicates that in order for the iron to be TBHP-reactive, it must first be released from Tf, presumably in the acidic environment of the endosome.

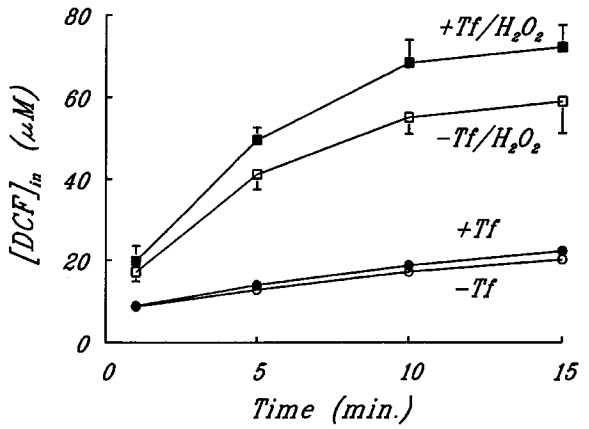

Fig. 4. Generation of intracellular oxidant species by exogenous H_2O_2 as determined with 2′,7′-dichlorofluorescin diacetate. K562 cells were preincubated for 20 min at 37°C in α-MEM-HEP medium in the presence (filled symbols) and absence (empty symbols) of 100 μg/ml Tf. After removal of the Tf by centrifugation, the cells were resuspended in HBS solution containing 10 μM 2′,7′-dichlorofluorescin diacetate. They were then incubated at 37°C in the presence (squares) and absence (circles) of 50 μM H_2O_2 for the times indicated, and the fluorescence of intracellular 2′,7′-dichlorofluorescein ($[DCF]_{in}$) was determined as described in Section 2.

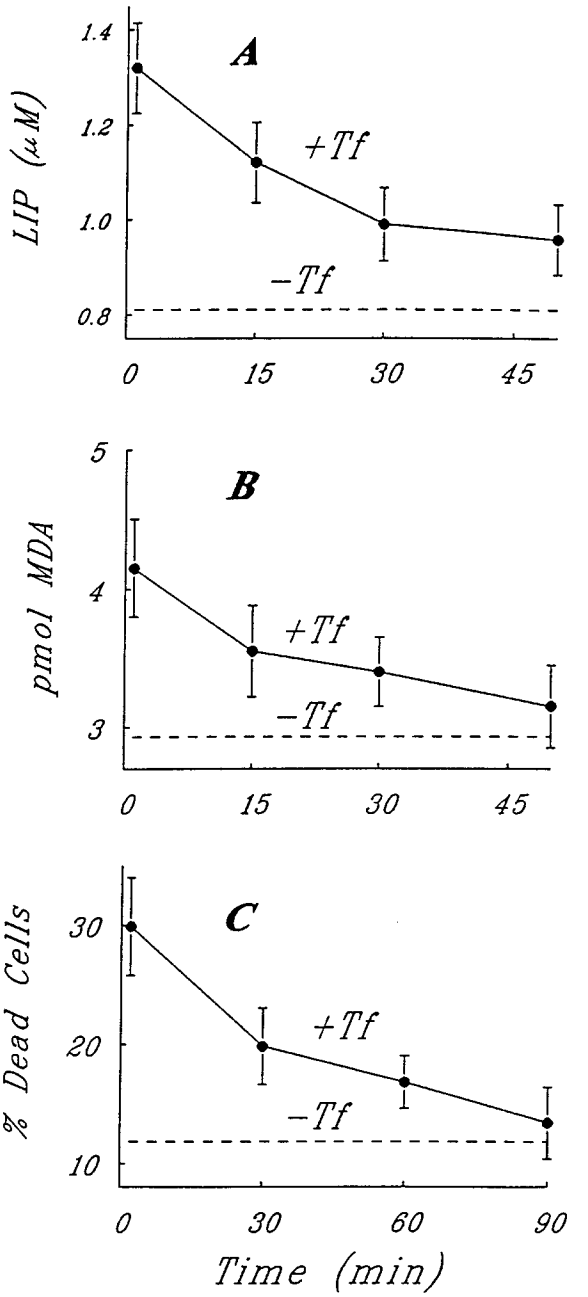

Fig. 5. Removal of newly acquired iron from the TBHP-reactive pool. K562 cells were preincubated in α-MEM-HEP medium containing 100 μg/ml Tf for 10 min at 37°C, Tf was removed by centrifugation and the cells were further incubated at 37°C in α-MEM-HEP medium for periods of 1–50 min, as shown. They were then treated with 0.25 mM TBHP for 15 min and immediately assayed for (A) LIP and (B) MDA production (expressed as pmol MDA/10^6 cells). C: The cells were first preincubated at 37°C in serum-free culture medium containing 100 μg/ml Tf for 2 h, Tf was then removed by centrifugation, the cells were further incubated at 37°C in culture medium for the times indicated, and then treated with 0.25 mM TBHP in HBS for 90 min prior to viability determinations. Dashed lines indicate the levels observed for control cells treated with TBHP without pre-exposure to Tf.

We showed that neither TBHP nor H_2O_2 release iron from Tf directly, by incubating Tf with the reagents in solution and measuring the release of iron with the fluorescent iron probe methylanthranilic desferrioxamine [17]. Using this method, iron was found to be spontaneously released from Tf at pH < 6.0 but not at pH > 6.0, and this was not affected by 0.5 mM TBHP or H2O2 (data not shown).

3.3. Effect of iron chelators on TBHP-mediated LIP increase, MDA production and cell viability

The iron chelators 2,2′-bipyridyl (BIP) and desferrioxamine (DFO), both at 0.1 mM, were tested for their capacity to decrease LIP levels and concomitantly prevent TBHP-mediated damage to cells which had been preincubated with Tf (Fig. 3). These chelators decreased the LIP levels by 19 and 35% respectively in untreated controls, and virtually abrogated the increase in LIP due to both H_2O_2 and TBHP (Fig. 3A). TBHP-mediated MDA production was inhibited by 88 and 58% by BIP and DFO respectively (Fig. 3B). These chelators also inhibited TBHP-mediated cell death by 96 and 71% (Fig. 3C). Whereas DFO is considerably less hydrophobic than BIP and enters cells more slowly, it apparently reached sufficient intracellular levels during the 15 min preincubation to chelate a portion of the LIP. Moreover, DFO's relatively poor permeability may explain its lower inhibitory activity towards TBHP-mediated MDA production and cell death. Two other permeable iron chelators, *o*-phenanthroline and SIH, at 0.1 mM, had similar inhibitory effects to those of BIP on both MDA production and cell death (data not shown).

3.4. Generation of intracellular oxidant species by exogenous H_2O_2, as determined with 2′,7′-dichlorofluorescin diacetate

Since H_2O_2 failed to cause detectable MDA production or significant cell death, we sought an alternative method for detecting H_2O_2-derived cytosolic oxidant species. The probe of choice was 2′,7′-dichlorofluorescin diacetate, which enters cells readily, is hydrolyzed by esterases to the non-fluorescent 2′,7′-dichlorofluorescin and undergoes conversion to the fluorescent 2′,7′-dichlorofluorescein (DCF) upon reaction with oxidant species. The reaction of 2′,7′-dichlorofluorescin with H_2O_2 alone is relatively slow, but is markedly enhanced in the presence of iron [31]. As shown in Fig. 4, untreated control cells showed a slow accumulation of DCF, indicative of the basal level of endogenous oxidant species. Exogenous H_2O_2 (50 μM) raised the DCF levels by 3.1-fold. Pretreatment with Tf increased DCF accumulation in control and H_2O_2-treated cells by 10 and 22% respectively. Although higher concentrations of exogenous H_2O_2 produced parallel increases in DCF accumulation, the relative increase due to Tf preincubation was most marked at concentrations below 50 μM H_2O_2 (not shown).

3.5. Removal of newly acquired iron from the TBHP-reactive pool

As demonstrated in Figs. 1 and 2, newly acquired Tf-iron shows an enhanced reactivity with TBHP. Therefore it was of interest to determine how rapidly the cells can shift this iron to a less reactive form, and whether this is reflected in parallel changes in LIP levels. The initial rise in LIP, generated in cells by TBHP after a 10 min preincubation with 100 μg/ml Tf, was followed by a time-dependent decline after removal of Tf (Fig. 5A). The half-life, $t_{1/2}$, of the decay toward the level of cells that had not been preincubated with Tf was 21 min. A similar pattern was obtained for MDA production, where the decay $t_{1/2}$ was 16 min (Fig. 5B). The minimum preincubation period with Tf for a detectable increase in TBHP-mediated cell death was approx. 30 min. Therefore we chose an optimal Tf preincubation period of 2 h for cell death determinations. Under this condition, the $t_{1/2}$ of the period required for the cells to become as resistant to TBHP as control cells that had not been preincubated with Tf was 27 min (Fig. 5C).

4. Discussion

4.1. The relationship between LIP levels and oxidant injury

The view that a cytosolic form of iron confers on cells susceptibility to pro-oxidant-mediated damage [1,2,14] is based on two major pieces of evidence: (i) the increased susceptibility to oxidant stress artificially created by prolonged exposures to iron salts [5] and (ii) the protective action conferred by various iron chelators on oxidatively stressed cells and tissues [1,2,5,6,21]. While strongly suggestive, neither of these experimental approaches addresses the question of whether iron acquired by normal physiological pathways can be a catalyst of oxidant-mediated injury. On the one hand, the uncontrolled iron overload generated by exposure to non-Tf-bound iron can result in extremely high intracellular iron concentrations, vastly in excess of the cytoplasmic chelating or buffering capacity. On the other hand, full protection by iron chelators implies that only the chelatable or labile cellular iron (LIP) is potentially reactive and germane to the oxidant damage.

We set out to assess whether the physiological forms of iron delivered to cells are chemically reactive when challenged with pro-oxidants. We used Tf, the in vivo iron carrier, as an iron source, since, in contrast to the facilitated transport of inorganic iron [22,23], endocytic uptake of iron via the Tf receptor-Tf system is relatively slow, highly regulated and furnishes the physiological forms of iron. The levels of the labile or chelatable forms of cytosolic iron were monitored on line as LIP levels with the fluorescent intracellular iron probe, calcein [17–19], and correlated with pro-oxidant-mediated cell damage. A distinct correlation was found between LIP levels and the extent of cellular injury in cells treated with TBHP: (i) when the initial LIP was increased by preincubation with Tf, MDA production rose in parallel, and (ii) the chelators BIP (specific for iron(II)) and DFO (binds both iron(II) and (III)) lowered LIP levels and concomitantly decreased MDA production. Similarly, the capacity of TBHP to cause cell death was found to be determined by iron supply. K562 cells taken from cultures grown in 7% fetal calf serum were resistant to TBHP for up to 45 min, however they underwent a breakdown in the permeability barrier when exposed to TBHP for 90 min in the presence of Tf as an iron source. The actual LIP levels attained in the damaged cells after 90 min of incubation with TBHP in the presence of Tf could not be experimentally determined. We can only surmise that they might be at least as high as those found after short, 15 min incubations shown in Figs. 1–3, i.e. ≥2.0 μM.

4.2. Differential sensitivity of K562 cells to H_2O_2 and TBHP

In contrast to TBHP, H_2O_2 failed to cause any cell damage

detectable by the present assays. This was in spite of the fact that H_2O_2 caused a significant increase in the levels of oxidant species in the cytosol, as shown by the enhanced accumulation of the fluorescent probe DCF (Fig. 4). Furthermore it caused the release of a significant portion of non-chelatable iron into the LIP, detected as a rise in LIP levels in the absence of an external iron source. This indicates that the H_2O_2 was indeed cell-active but either did not have sufficient access to iron targets or did not accumulate to sufficient levels to generate lipid peroxidation products. While several possible mechanisms could explain the quantitative differences between the effects of the two pro-oxidants, we favor the possibility that iron adsorbed to membranes might react with the hydrophobic TBHP, which partitions into lipids and accumulates in cell membranes. These two reactants, when co-localized in membranes, might induce localized damage, principally lipid peroxidation. In contrast, the freely soluble H_2O_2 becomes uniformly dispersed throughout the cell, giving rise to a variety of oxidation products [1,2,20] of which only a small fraction would be MDA arising from lipid peroxidation.

4.3. *Unmasking of weakly bound cytoplasmic iron by its reactivity with pro-oxidants*

The observation that H_2O_2 and TBHP can release cellular iron from a non-LIP, chelator-inaccessible fraction into cytosolic LIP detectable with calcein ([18], and present data), is compatible with the idea that LIP is in dynamic equilibrium with other fraction(s) of weakly bound iron. Previously we have shown that calcein-detectable LIP is composed primarily of iron(II) and about 20% iron(III). Presumably, much of the cytoplasmic iron(III), being tightly bound to various ligands, would not be detected as LIP in this assay. One well defined fraction of iron(III) is bound to ferritin, but it can be dislodged as iron(II) by reducing species [11,24,25]. Other ligands for iron(III) may include nucleotides [4,16] or polypeptides [7,27]. Irrespective of the specific iron-binding moieties, the observed peroxide-mediated increase in LIP levels is explainable by an initial Fenton reaction between the peroxide and iron(II), giving rise to free radicals which then reduce tightly bound iron(III) and release it as the highly soluble iron(II) ion. The free iron(II) is then detected by calcein, whose reactivity with iron(II) is greater than with iron(III) [28]. This mechanism is in line with observations of release of tissue iron following hypoxia/reperfusion [14,15,26] and oxidant stress [29].

4.4. *Inactivation of newly acquired iron*

The principal element of the present studies is the distinction, by means of TBHP reactivity, between newly acquired iron and that which has already been 'processed' in the cytoplasm. This is illustrated in Fig. 5, where newly acquired Tf-iron initially reacts with TBHP (in terms of increases in LIP, MDA production and cell death), but its reactivity is gradually lost with time and virtually disappears after about 60 min. Conceivably, immediately after its exit from the endosome, the newly released iron(II) [17,30] is still closely associated with internal membrane surfaces where it can easily react with TBHP partitioned within the lipid bilayer. This physical proximity between pro-oxidant and metal catalyst would also explain the differences between H_2O_2 and TBHP in causing lipid peroxidation and cell death. The subsequent decrease in LIP and in TBHP-mediated damage likely reflects the binding of this iron(II) to high affinity ligands (such as ferritin), concomitant with oxidation to iron(III). The inactivation kinetics are in reasonable agreement with our previous determination of the transit time of Tf-^{55}Fe from the chelatable iron pool to a chelator-inaccessible fraction in K562 cells, as 1–2 h [17].

In summary, our results indicate that cellular LIP is in dynamic association with non-LIP forms of iron which are potentially reactive. Thus the iron supplied by Tf endocytosis carries high catalytic potential during its transit between endosomes and high-affinity inactivating ligands. The physiological relevance of these findings would depend on the rate of Tf-iron acquisition, i.e. cellular Tf-receptor levels, as well as the concentration and type of pro-oxidant species accumulated by the cells.

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