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Does the cellular labile iron pool participate in the oxidation of 2′,7′-dichlorodihydrofluorescein?

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Abstract

The fluorogenic probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) is widely used for the estimation of oxidative stress in cells. It is known that 2′,7′-dichlorodihydrofluorescein (H_2DCF), product of intracellular hydrolysis of H_2DCF-DA, is oxidized to the fluorescent compound, DCF, mainly by hydrogen peroxide (H_2O_2) in the presence of catalysts. The present study was aimed at answering the question whether the labile iron pool (LIP) may contribute to the oxidation of H_2DCF in cellular systems. The membrane-permeable lipophilic iron chelator salicylaldehyde isonicotinoyl hydrazone (SIH) was found to inhibit oxidation of the probe by H_2O_2 dependent on ferrous ions but not by peroxidase or superoxide dismutase in defined *in vitro* systems. When applied to cells, the probe inhibited considerably oxidation of H_2DCF in V79 Chinese hamster fibroblasts and two murine lymphoma L5178Y(LY) sublines (LY-R, LY-S) differing in LIP level, the extent of inhibition being greater in the LY-R line of higher LIP level. These results demonstrate that LIP is a significant factor determining the rate of intracellular H_2DCF oxidation.

Keywords: *2′,7′-dichlorodihydrofluorescein diacetate, iron chelation, salicylaldehyde isonicotinoyl hydrazone (SIH), labile iron pool*

Abbreviations: *DMSO, dimethyl sulfoxide; HBSS, Hank's buffered salt solution; H_2DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; HPR, horseradish peroxidase; LIP, labile iron pool; ROS, reactive oxygen species; SIH, salicylaldehyde isonicotinoyl hydrazone; SOD, superoxide dismutase*

Introduction

The maintenance of optimal, low stationary levels of reactive oxygen metabolites is an important parameter of cellular homeostasis. Its modulation due to either increased production of reactive oxygen and nitrogen species or decreased level of antioxidants, referred to as oxidative stress, is observed in numerous pathological states. An increasingly popular means of detecting oxidative stress consists of the use of fluorogenic probes, like 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA), for estimation of the rate of production of reactive oxygen species (ROS) [1–5]. The main problem concerning the use of these probes consists in the promiscuity of their reactions making impossible identification of the ROS responsible for the oxidation of the probe. 2′,7′-Dichlorodihydrofluorescein (H_2DCF) has been introduced for determination of peroxides, including hydrogen peroxide. However, the probe reacts also with other

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ROS, especially peroxynitrite [2,6,7], and can be oxidized directly by various compounds, including peroxidase Compound I and analogous forms of other heme proteins, among them cytochrome c, without the intermediacy of ROS [8,9]. Moreover, it has been reported that in the absence of catalysts the probe is unreactive for hydrogen peroxide and is oxidized by hydrogen peroxide only in the presence of peroxidase or transition metal ions [5,10,11].

Although most authors assume that the rate of H_2DCF oxidation reflects mainly the cellular rate of hydrogen peroxide production, it has been argued that in some cells the rate of H_2DCF oxidation may be limited rather by the availability of catalysts, especially the peroxidase activity which is not abundant in cells of certain types [11]. It is intriguing also what role in the oxidation of H_2DCF can be played by the labile (transit) iron pool [12–14], which is present in the cells and can be evaluated on the basis of its availability to chelating compounds and quantified fluorimetrically [15,16]. This pool of iron is thought to catalyze the generation of highly ROS, especially the hydroxyl radical, and can be expected to participate in intracellular H_2DCF oxidation. The "labile iron" can be expected to be important in the oxidation of H_2DCF especially in cells of low peroxidase activity. This study was aimed at an insight into this question using a membrane-penetrating iron chelator, salicylaldehyde isonicotinoyl hydrazone (SIH) which selectively complexes Fe^{3+} and blocks iron-dependent production of hydroxyl radical from hydrogen peroxide [17–19].

Materials and methods

Materials

Cell culture media, fetal bovine serum, calf serum and antibiotics were purchased from Invitrogen (Paisley, UK). H_2DCF-DA was from Molecular Probes (Leiden, The Netherlands). The iron chelator SIH was a generous gift of Dr P. Ponka (Lady Davis Institute for Medical Research, Montreal, Canada). All other materials were obtained from Sigma (Deisenhofen, Germany).

Cell culture

V79 cells (Chinese hamster lung fibroblasts) growing in monolayers were cultured in Dulbecco's modified Eagle medium (DMEM) containing glucose (4500 mg/l), sodium pyruvate and pyridoxine. Growth medium was supplemented with 10% v/v heat-inactivated fetal bovine serum, penicillin (50 U/l) and streptomycin (50 mg/l).

Murine leukemia cells L5178Y-S (LY-S) and L5178YR (LY-R), were cultured in RPMI 1640 medium with Glutamax-I, containing 25 mM HEPES, 8% v/v heat inactivated calf serum and antibiotics: penicillin (50 U/l) and streptomycin (50 mg/l).

Oxidation of H_2DCF-DA in cells after chelator treatment

Estimation of H_2DCF-DA in the cellular systems in the presence of SIH was performed in 96-well microplates. Fluorescence intensity was monitored with a Fluoroscan Ascent FL microplate reader with a filter pair of: $\lambda_{ex} = 485$ nm and $\lambda_{em} = 538$ nm.

V79 cells were seeded in a complete medium onto 96-well plates (40,000 cells/well) and incubated for 20–24 h under cell culture conditions. Then the cells were incubated in the presence of 10–200 μM SIH for 1 h. After the incubation, cell monolayers were rinsed with Hanks's buffered salt solution (137 mM NaCl, 5 mM KCl, 0.8 mM $MgCl_2$, 1.4 mM $CaCl_2$, 1 mM Na_2HPO_4, 10 mM HEPES and 5.5 mM glucose, pH 7.0; HBSS) and added with 5 μM H_2DCF-DA in HBSS. Fluorescence was measured after 30-min incubation at 37°C.

Murine lymphoma cells (LY-S and LY-R) were used for the experiments at a density of 40,000 cells/well and treated in an analogous manner. After incubation with the chelator, the cells were centrifuged (5 min, 300 g), rinsed with HBSS and suspended in buffer containing 5 μM H_2DCF-DA. Intensity of fluorescence was read after 30-min incubation of the cells with the probe.

Hydrolysis of H_2DCF DA

H_2DCF was obtained by alkali hydrolysis of H_2DCF DA. Briefly, 5 μl of 5 mM H_2DCF-DA solution in DMSO were added with 5 μl of methanol and 10 μl of 2 M NaOH and incubated at room temperature for 1 h. Then the mixture was neutralized with 20 μl of 100 mM sodium phosphate buffer, pH 7.0 and 10 μl of 2 M HCl. The H_2DCF produced was kept on ice and used on the day of preparation.

Measurement of H_2DCF oxidation in cellular lysates

V79 cells (10^7/ml) were lysed by three freezing/thawing cycles in 10 mM borate buffer, pH 7.4, supplemented with a mixture of protease inhibitors (0.3 μM aprotinin, 0.1 mM leupeptin, 1.4 μM E-64, 13 μM bestatin and 2 mM AEBEF). Cellular lysates were centrifuged through Centricone (MW cutoff: 10 kDa; 200*g*, 30 min; 4°C). Afterwards, oxidation of the probe was studied in the low-molecular weight fraction.

Cell viability

Viability of V79 cells was estimated by measuring the ability of live cells to reduce MTT to the formazan [20].

The rationale for the assay is that only living cells are able to reduce MTT, mainly due to the activity of mitochondrial dehydrogenases. Cells were seeded onto 96-well plates at a density of 1×10^3 cells/well. After overnight culture part of cells was treated by SIH in a range of concentrations (10–200 μM) for 1 h. At the end of treatment the cell monolayers were rinsed with HBSS, fresh medium was added and incubation was continued for next 72 h. The other part of cells was incubated with the chelator by 72 h. In the next step, MTT-containing medium (final MTT concentration of 333 μg/ml) was added. After 3 h the medium was removed and the formazan crystals were dissolved in DMSO. Absorbance was read at 590 nm.

Data analysis

Unless indicated otherwise, results are expressed as mean ± SD. Statistical analysis of the data was performed using ANOVA and Tukey's *a posteriori* test. Values were considered to be statistically important at $\star P < 0.05$ or $\star\star P < 0.01$.

Results

Preliminary experiments were run in defined *in vitro* systems to examine the effect of SIH on the oxidation of H_2DCF by hydrogen peroxide in the presence of iron salts and horseradish peroxidase (HRP). Hydrogen peroxide alone did not oxidize the probe (not shown). Ferric ions alone brought about some oxidation of H_2DCF (Figure 1(A)), which was only slightly enhanced by H_2O_2 (Figure 1(D)). Ferrous ions induced much stronger oxidation of H_2DCF in the absence of hydrogen peroxide (Figure 1(B),(C)) which was further enhanced by the presence of hydrogen peroxide (Figure 1(E),(F)). The oxidation of H_2DCF by H_2O_2 and ferrous ions was rapid and was almost complete in 10 s, the shortest possible time of measurement (Figure 1(E)). Efficient oxidation of H_2DCF by hydrogen peroxide in the presence of ferrous ions suggests that the probe is oxidized by the product(s) of the Fenton reaction in this system. Ferrous ions alone cannot be expected to oxidize H_2DCF but such oxidation has been observed (Figure 1). A plausible explanation may be that autoxidation of Fe^{2+} generates low amounts of hydrogen peroxide which forms a Fenton system with the remaining ferrous ions, even in the absence of exogenous hydrogen peroxide [21]. Another possibility is that H_2DCF is oxidized by oxidizing species formed by the reaction of Fe^{2+} with dioxygen (ferryl and perferryl ions). These species were demonstrated to be important initiators of free radical reactions under conditions of high excess of dioxygen over hydrogen peroxide [22].

SIH alone induced slight oxidation of H_2DCF; the presence of hydrogen peroxide did not affect this oxidation significantly, decreasing it rather than increasing (Figure 2(A)). The chelator decreased considerably H_2DCF oxidation by Fe^{2+} (Figure 2(B)) and $Fe^{2+} + H_2O_2$ (Figure 2(C)), but only when present in excess with respect to Fe^{2+} (molar ratio of SIH: $Fe^{2+} > 1$). These results are in line with the reported inhibition of hydroxyl radical formation by SIH and its analogs [19]. Our results showed also increased oxidation of probe in the systems where concentration of SIH was lower relative to ferrous ions (Figure 2(B),(C)). This suggests that inhibitory effect of chelator appears only when iron is fully chelated in tridentate complexes with SIH, otherwise enhanced oxidation of H_2DCF is observed.

H_2DCF is known to be oxidized efficiently by Compound I of peroxidases and other hemoproteins [8,9,23]. Since SIH, due to its structure, and especially the presence of phenolic —OH, might be expected to be a substrate for Compound I, we checked whether it could interfere with the oxidation of H_2DCF by HRP. We observed oxidation of the H_2DCF by HRP alone (Figure 3(A)), which was considerably enhanced in the presence of hydrogen peroxide (Figure 3(B)). There was no significant inhibition of the oxidation of the probe by HRP alone or by HRP/H_2O_2 (Figure 3).

We also checked if SIH can inhibit oxidation of H_2DCF mediated by the peroxidative action of CuZnSOD. In agreement with literature data [25,26], we observed an almost 3-fold enhancement of peroxidative activity of CuZnSOD by bicarbonate, but found no significant effect of the chelator on H_2DCF oxidation both in the absence and in the presence of $NaHCO_3$ (data not shown). Bicarbonate did not affect the fluorescence yield of DCF under the conditions of the measurements (data not shown).

In the first step of investigation of the role of cellular labile iron in H_2DCF oxidation we checked the effect of lipophilic chelator (SIH) on cell viability. We found that while long-term (72-h) exposure of V79 cells to micromolar concentrations of SIH indeed decreases considerably cell viability, a short-term (1-h) exposure is without any effect on the viability of these cells (Figure 4).The short-term incubation of cells with SIH was employed in the experiments.

We studied the effect of SIH on H_2DCF oxidation in three mammalian cell lines: a Chinese hamster fibroblast V79 cell line and two murine leukemia cell lines. The latter lines were demonstrated to differ in the level of intracellular labile iron pool (LIP) which is higher in the L5178YR (0.57 μM) than in the L5178YS cell line (0.18 μM) [27]. SIH pretreatment decreased H_2DCF oxidation in all cell lines studied, the extent of the decrease being higher in L5178YR cells (Figure 5).

The inhibition of H_2DCF oxidation by SIH is due mainly to its influence on the reaction dependent on low-molecular weight cellular constituents.

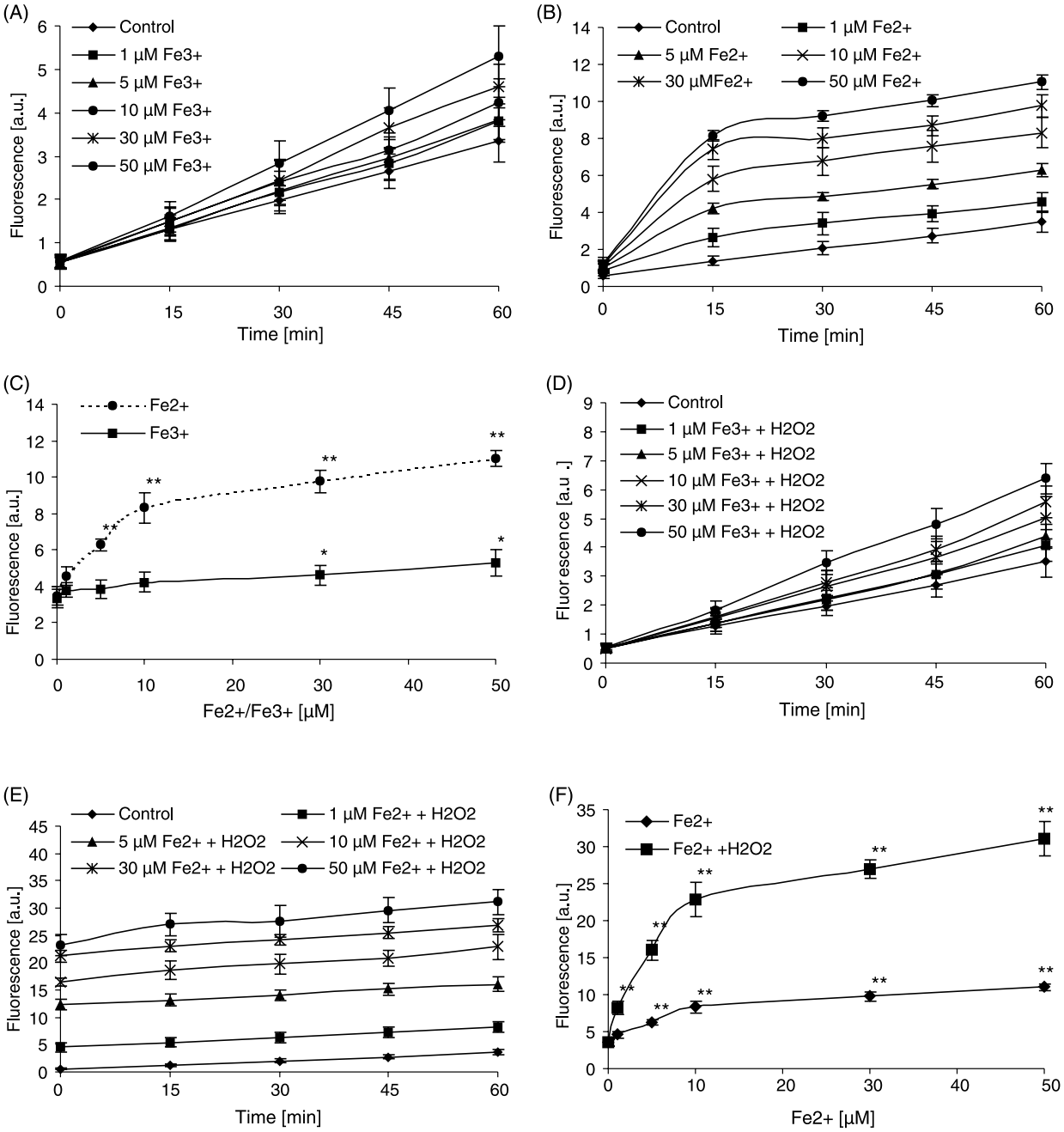

Figure 1. Oxidation of H_2DCF by ferrous/ferric ions and hydrogen peroxide. A and B, kinetics of H_2DCF oxidation by 1–50 μM Fe^{3+} (A) and 1–50 μM Fe^{2+} (B). C, concentration dependence of H_2DCF oxidation by Fe^{2+} and Fe^{3+} (data for incubation time of 60 min). D and E, kinetics of H_2DCF oxidation by 20 μM H_2O_2 and Fe^{3+} (D) and Fe^{2+} (E); iron ions present at concentrations of 1–50 μM iron. (F), concentration dependence of H_2DCF oxidation by Fe^{2+} in the absence and in the presence of H_2O_2 (data for 60-min incubation). Conditions: 5 μM H_2DCF, 100 mM borate buffer, pH 7.4, temperature of 37°C; $n = 3$. Fluorescence measured in a Fluoroscan Ascent FL microplate reader.

We compared the effect of SIH on the oxidation of the probe by V79 cells (Figure 6(A)) and lysates of these cells passed through a Centricon membrane (molecular cutoff of 10 kDa; Figure 6(B)). The inhibition of DCF oxidation was significant in both cases, about 40% in the cells and about 50% in the low-molecular weight filtrate. It was not complete (which could be expected), apparently due to the presence of non-chelatable catalytic iron (e.g. traces or fragments of cytochrome c) (Figure 6(B)).

Discussion

Although the fluorogenic probe H_2DCF has been introduced for a convenient determination of peroxides [1], it has been reported to be not easily oxidized to the fluorescent DCF by hydrogen peroxide in the absence of catalysts [5], an observation confirmed in the present study. Peroxidases and iron salts can act as catalysts of H_2DCF oxidation and there are reasons to suspect that the labile pool of iron

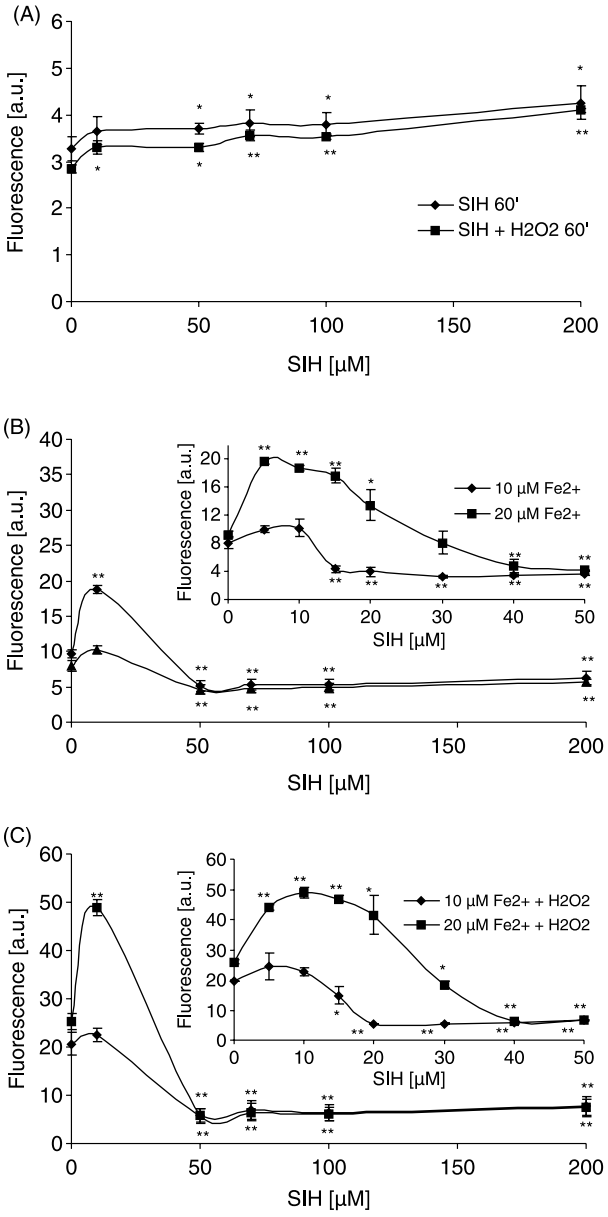

Figure 2. Effect of SIH on the oxidation of H_2DCF by Fe^{2+} and hydrogen peroxide. A, oxidation of H_2DCF by SIH alone and SIH in the presence of H_2O_2 (data for 60-min incubation); B and C effect of SIH on the oxidation of H_2DCF, by Fe^{2+} (B) and by Fe^{2+} plus H_2O_2 (C) Conditions: 5 μM H_2DCF, 100 mM borate buffer, pH 7.4, 5–200 μM SIH, 20 μM H_2O_2, 10 or 20 μM Fe^{2+}, temperature of 37°C; $n = 5$. Fluorescence measured in a Fluoroscan Ascent FL microplate reader.

present within the cells may contribute to intracellular oxidation of H_2DCF.

Experimental proof of this hypothesis in intact cells is not simple due to the low membrane permeability of iron chelators. However, we expected that the hydrophobic membrane-permeant SIH, used to identify and quantify the pool of "loosely bound iron" ("labile iron pool", LIP) in intact cells [12,28] may be an useful tool in this respect.

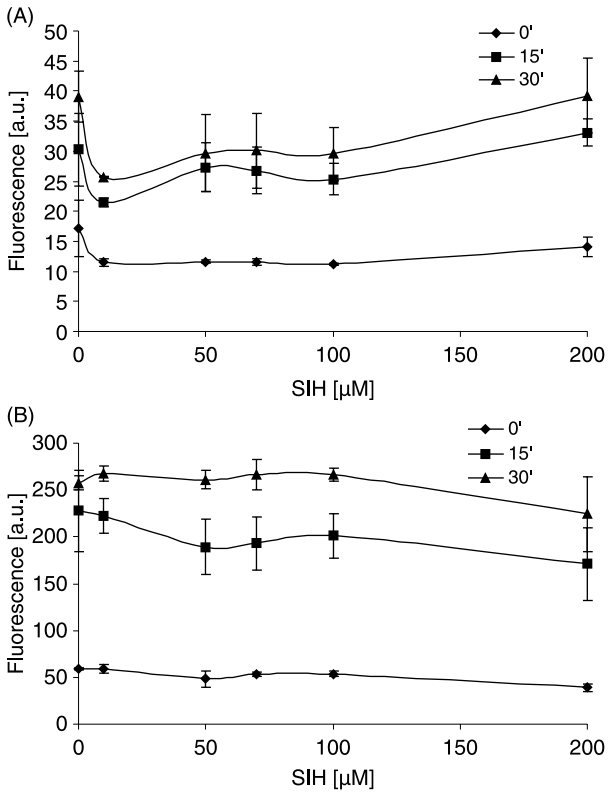

Figure 3. Effect of SIH on the oxidation of H_2DCF by HRP alone (A) and HRP + 2.5 μM hydrogen peroxide (B). Conditions: 5 μM H_2DCF, 100 mM borate buffer pH 7.4, 5 U/ml HRP, temperature of 37°C; $n = 3$. Fluorescence measured in a Fluoroscan Ascent FL microplate reader.

There may be several possible mechanisms of intracellular DCF oxidation. The probe can be oxidized by hydrogen peroxide in reactions catalyzed by ferric/ferrous ions or by peroxidases present in a cell. It has been recently suggested that CuZn-superoxide

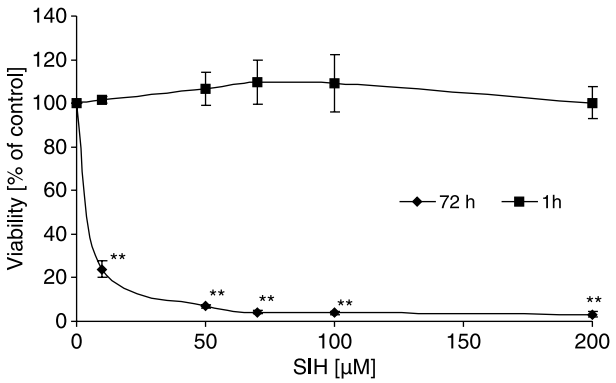

Figure 4. Effect of SIH on the viability of V79 cells. Cells were seeded onto 96-well plates at a density of 1×10^3 cells/well. After overnight culture part of cells was treated by SIH in a range of concentrations (10–200 μM) for 1 h. At the end of treatment the cell monolayers were rinsed with HBSS, fresh medium was added and incubation was continued for next 72 h. The other part of cells was incubated with the chelator by 72 h. Cell viability was estimated on the basis of MTT reduction, as described under Materials and methods; $n = 3$.

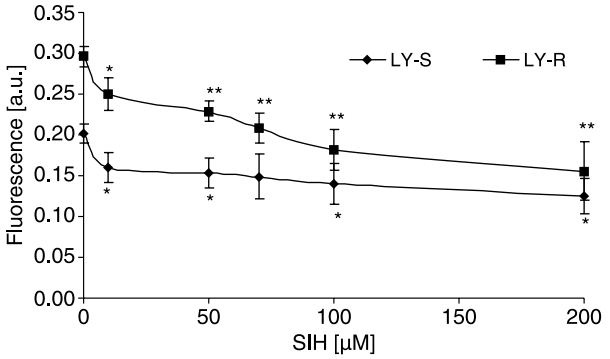

Figure 5. Effect of SIH on H_2DCF oxidation in mammalian LY-S and LY-R cells. The cells were incubated in the presence of 10–200 μM SIH for 1 h, rinsed with HBSS solution and added with H_2DCF-DA in HBSS (final concentration of 5 μM). Fluorescence was measured after 30 min incubation at 37°C; $n = 5$.

dismutase (CuZnSOD) is an important intracellular catalyst of H_2DCF oxidation [24]. In the course of apoptosis, cytochrome c liberated from mitochondria can oxidize DCF [8]. Peroxynitrite may become important in H_2DCF oxidation when formed in significant amounts [2,6,7]. Carbonate and hydroxyl radicals, as well as nitrogen dioxide react very efficiently with H_2DCF [29]. Taking into account steady-state concentration of various ROS and potential catalysts, one can expect that peroxidase, CuZnSOD and "free" iron ions (LIP) should be the most important factors determining the rate of H_2DCF oxidation in intact cultured cells not exposed to sources of reactive nitrogen species. It was therefore critical to demonstrate that SIH interferes selectively with the reactions of "free" iron ions and not with the reactions of H_2DCF oxidation catalyzed by peroxidases or CuZnSOD.

We found that SIH effectively inhibited the oxidation of H_2DCF by hydrogen peroxide catalyzed by ferrous and ferric ions (Figure 2). It can be noted that iron ions were not very efficient catalysts of oxidation of the probe. Although H_2O_2 (20 μM) and ferrous ions (10 or 20 μM) were in excess over H_2DCF (5 μM), oxidation of the probe did not exceed 10% (Figure 1). Interestingly, low concentrations of SIH stimulated rather than inhibited oxidation of H_2DCF in the Fe^{2+}/H_2O_2 system (Figure 2), the inhibitory effect being evident for SIH/Fe^{2+} ratio ≥ 2. It is known that SIH forms a tridentate complex with iron [30] and apparently only such complexation prevents iron involvement in H_2DCF oxidation, 1:1 complexes being even more active than uncomplexed ferrous ions.

SIH did not inhibit H_2DCF oxidation by HRP (Figure 3) or CuZnSOD (data not shown). Thus, the membrane-permeable iron chelator SIH which inhibits oxidation of H_2DCF by iron ions and hydrogen peroxide while not affecting oxidation of the probe by hydrogen peroxide mediated by peroxidase or CuZnSOD, provides a feasible means to examine the contribution of LIP into the intracellular oxidation of H_2DCF. A prerequisite for such an assumption is that the compound used does not disturb the cellular metabolism too seriously. This facet my be of concern since it has been reported that SIH and its analogs caused apoptosis in Jurkat T lymphocytes and K562 cells [17,18]. On the other hand, taking into account the non-monotonous dependence of the extent of DCF oxidation by ferrous ions on the SIH concentration (Figure 2), and the uncertainty about the intracellular distribution of SIH, the use of possibly high concentration of the chelator is necessary to expect a significant molar excess of SIH over the labile iron. We found that 1-h treatment of cells with SIH does not compromise their viability (Figure 4) so significant apoptosis could be excluded under these conditions. These results prove that inhibition of intracellular oxidation of H_2DCF by SIH is a measure of iron-catalyzed oxidation.

We demonstrated that SIH inhibited oxidation of H_2DCF in cell extracts and in intact cells. The magnitude of the inhibitory effect of SIH on the intracellular H_2DCF oxidation attained about 40% in V79 cells, about 43% in LY-R cells and about 55% in LY-S cells (having higher LIP pool [27]) at the highest SIH concentrations used. The extent of inhibition was even greater in the protein-poor low-molecular weight

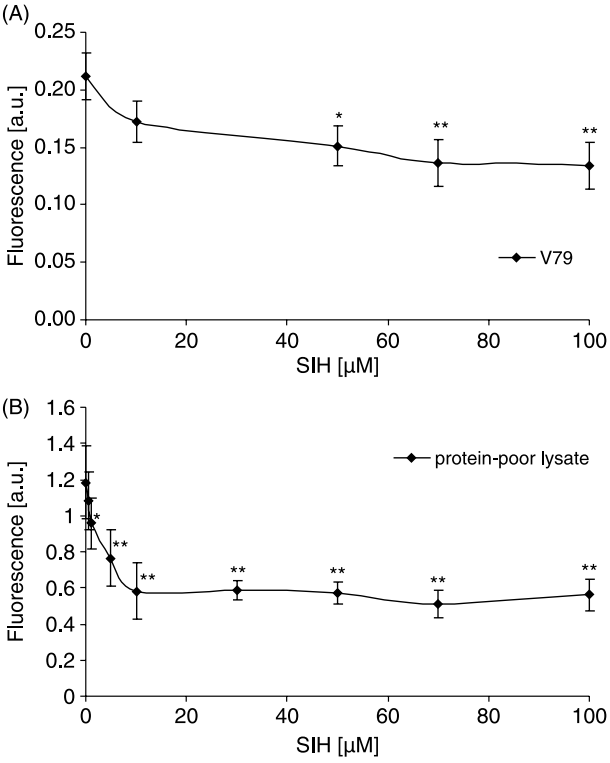

Figure 6. Effect of SIH on the oxidation of H_2DCF in V79 cells (A) and on the oxidation of DCF in protein-poor fraction of lysates of these cells (B). Incubation: 30 min, temperature of 37°C; $n = 3$.

fraction of cell extracts (about 50%). These data indicate that the cellular LIP is a significant factor in the determination of the oxidation of H_2DCF (and seemingly also other fluorogenic probes). Moreover, the size of the labile in pool should be taken into account in the interpretation of H_2DCF oxidation in cellular systems.

The oxidation of H_2DCF is a measure of undesired oxidation reactions proceeding inside the cells. Various reactions of such type can be expected to be influenced by the size of LIP. Indeed, a correlation was found between the cellular level of LIP and the yield of 8-oxodGuo, a typical marker of ROS-induced DNA damage, in human lymphocytes [31] and between the LIP level and DNA breaks estimated by the comet assay in cells treated with H_2O_2 [14]. An important role has been attributed to LIP in the ischemia–reperfusion injury [32]. Therefore, chelation of LIP may be important not only as a research tool but also in therapeutical practice.

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