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RELEASE OF IRON FROM FERRITIN BY 6-HYDROXYDOPAMINE UNDER AEROBIC AND ANAEROBIC CONDITIONS

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6-hydroxydopamine (6-OHDA) proved to be a very effective agent for iron release from ferritin. Iron release was enhanced in the presence of SOD, catalase and under anaerobic conditions. Ascorbic acid, a well known agent able to release iron from ferritin, increased the amount of released iron in more than an additive manner when used in combination with 6-OHDA. Similar to 6-OHDA, 6-hydroxydopa (Topa) and 1,2,4-benzenetriol were also able to release iron in large amounts; in contrast, catecholamines and other benzenediols were comparatively ineffective.

KEY WORDS: 6-Hydroxydopamine, ferritin, iron release, ascorbic acid, neuroblastoma.

INTRODUCTION

6-OHDA, a catecholamine analogue that can be taken up by sympathetic nervous cells, has been widely used for experimental chemical sympathectomy.[1,2] Its cytotoxicity is mainly due to the formation of reactive oxygen compounds during its autoxidation.[3-6] For this process the presence of transition metals -for example iron- is necessary. In combination with ascorbic acid that acts as redox cycler, it has also been proposed for purging of bone marrow from neuroblastoma cells prior to autologous bone marrow transplantation.[7,8] Sine neuroblastoma cells contain iron-rich ferritin,[9] we investigated whether 6-OHDA is able to release iron from ferritin as it has already been described for superoxide anion and a large number of systems forming reactive oxygen species.[10-12]

MATERIALS AND METHODS

Chemicals

Horse spleen ferritin (12% iron saturated), ferrozine, catalase, L-norepinephrine, L-3,4-dihydroxyphenylalanine (Dopa), L-epinephrine, EDTA, glucose oxidase (GOD) and superoxide dismutase (SOD) were obtained from Serva, Heidelberg, FRG.

3-(N-morpholino) propanesulfonic acid (Mops), 6-hydroxydopamine (6-OHDA), 5-hydroxydopamine (5-OHDA), L-ascorbic acid (AA) and 3,4-dihydroxybenzoic acid (protocatechuic acid) were from Sigma, Munich, FRG.

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1,2-benzenediol (catechol), 1,3-benzenediol (resorcinol), 1,4-benzenediol (hydroquinone), 1,2,4-benzenetriol and 3-(2,4,5-trihydroxyphenyl)-DL-alanine (Topa) were purchased from Aldrich-Chemie, Steinheim, FRG.

Chelex® 100 was obtained from Bio-Rad Laboratories, Richmond, USA.

Measurement of Ferritin Iron Release

We used the method previously described by Boyer *et al.*[13] Briefly, ferritin (7.5×10^{-6} mol/l) was incubated for 2 minutes at 37° C in 10^{-1} mol/l Mops buffer (pH 7.0) with ferrozine (10^{-3} mol/l) and the measurement was started by adding the examined substances to a final volume of 2 ml. The appearance of the Fe-ferrozine complex was continously registrated at 562 nm on a photometer (Ultrospect 4050, LKB Biochrom, Cambridge, UK). The rate of iron release was calculated using $\varepsilon_{562\,nm}$ 27,900 $M^{-1}\,cm^{-1}$.[14]

The data obtained with 6-OHDA that autoxidizes to a red 6-OHDA quinone and with 6-OHDA derivatives were corrected by the determination of the absorbance change at 562 nm in an assay containing ferritin, the substance to be investigated, but no ferrozine. To prevent autoxidation previous to the addition, the substances were dissolved in 0.01 M HCl.

Anaerobic Conditions

In order to achieve anaerobic conditions, 1 mg/ml glucose, 1000 U/ml catalase and 10 U/ml GOD were added to the Mops buffer. The absence of oxygen was confirmed by measurements with a Clark oxygen-electrode (Hansatech, UK).

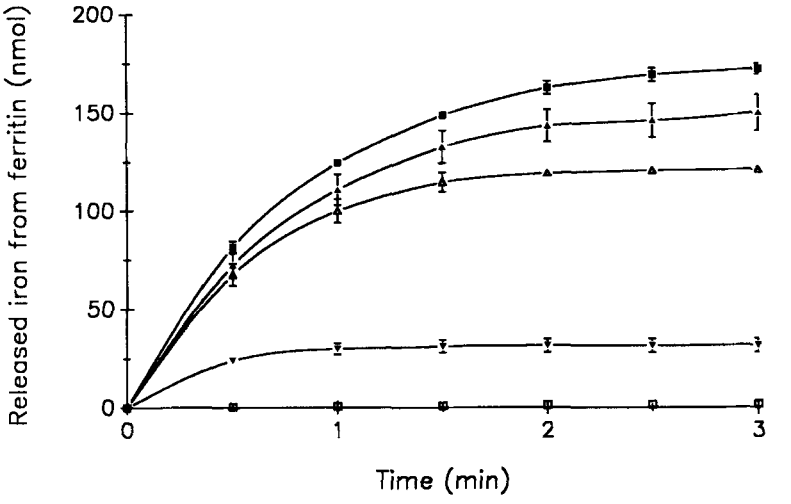

FIGURE 1. Iron release from ferritin by different concentrations of 6-OHDA (-▼- -▼- 10^{-5} mol/l, -△- -△- 5×10^{-5} mol/l, -▲- - ▲- 10^{-4} mol/l) in comparison to 10^{-4} mol/l ascorbic acid (-□- -□-) and the combination of 10^{-4} mol/l ascorbic acid with 10^{-4} mol/l 6-OHDA (-■- -■-). Error bars represent ± SD (n = 3). Only error bars greater than symbols are drawn.

RESULTS

Iron Release from Ferritin

During incubation of 6-OHDA with ferritin (0.75 μmol/l) a concentration-dependent release of iron was observed, visualized by formation of the ferrous complex of ferrozine. In Figure 1 three characteristic curves of 6-OHDA (10^{-4}, 5×10^{-5} and 10^{-5} mol/l) are shown in comparison to 10^{-4} mol/l ascorbate, which is a well-known iron releasing agent.[10,13] Its initial rate (0.56 nmol/min) was about 300 fold less than that of 10^{-4} mol/l 6-OHDA (156.81 nmol/min). These data indicate that 6-OHDA very effectively releases iron from ferritin. When ferritin was diluted 1:10 and 1:100, a concentration-dependent decline of Fe-ferrozine formation was observed. Furthermore dialysis of ferritin for 24 h using 10 g/l chelex 100® and 1 mmol/l EDTA[13] did not alter the saturation kinetics observed with 6-OHDA (data not shown).

The incubation of 10^{-4} mol/l 6-OHDA in combination with 10^{-4} mol/l ascorbate resulted in an enhanced release of iron. This increase may ensue from reduction of the 6-OHDA quinone by ascorbate leading to a redox cycle as previously described,[2] and it is not just an addition of the effects of both compounds.

Influence of Superoxide Dismutase (SOD) and Catalase

When 10^{-4} mol/l 6-OHDA were incubated with 1000 U/ml catalase or 100 U/ml SOD, iron release was elevated (Figure 2). Addition of H_2O_2 (10^{-3} mol/l) to a sample containing 10^{-4} mol/l 6-OHDA reduced iron release, indicating that the stimulatory effect of catalase is possibly due to the removal of H_2O_2 generated by 6-OHDA autoxidation. This inhibitory effect was not caused by the oxidative destruction of the Fe-ferrozine complex. This could be demonstrated by adding 10^{-3} mol/l H_2O_2 to a sample containing 3.3×10^{-5} mol/l $FeSO_4$ and 10^{-3} mol/l ferrozine, where A_{562nm} was not affected (data not shown).

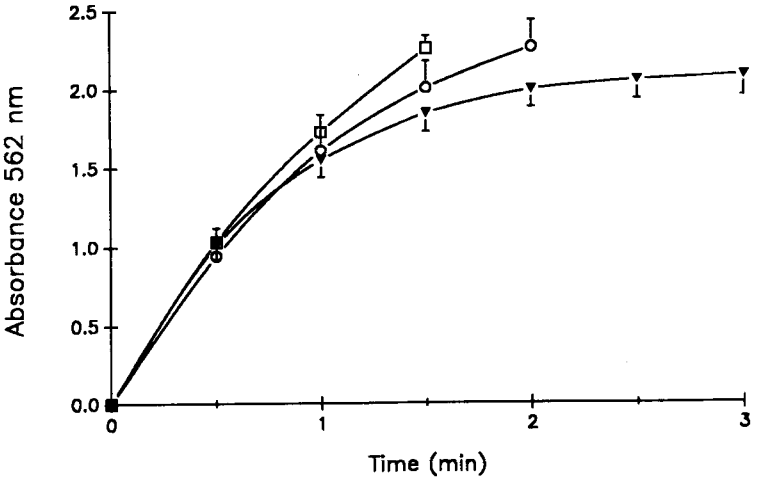

FIGURE 2. Effect of catalase (1000 U/ml, -O- -O-) and SOD (100 U/ml, -□- -□-) on the iron release by 10^{-4} mol/l 6-OHDA (-▼- -▼-) under aerobic conditions (Mean ± 1 SD, n = 3)

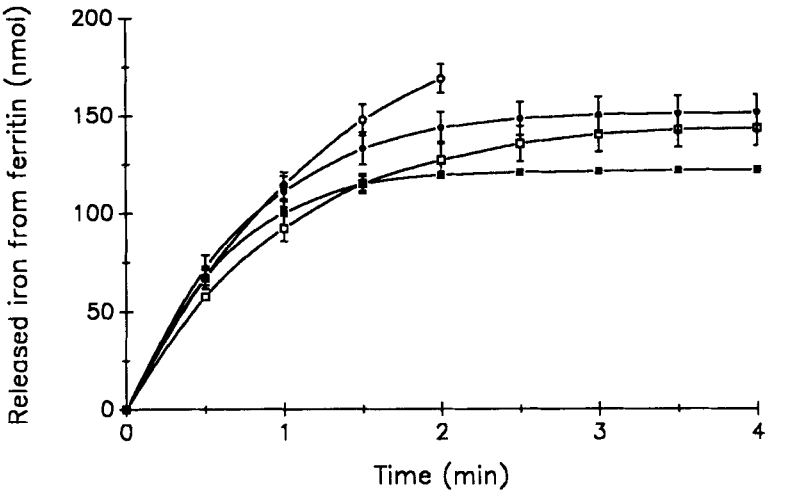

FIGURE 3. Iron release by 10^{-4} mol/l (●-●, -○- -○-) and 5×10^{-5} mol/l (-■- -■-, -□- -□-) 6-OHDA. Open symbols represent aerobic and filled symbols anaerobic conditions. Error bars are 1 SD (**n** = 3). Only error bars greater than symbols are drawn.

SOD retards the formation of 6-OHDA quinone[15] (measured at 490 nm in the absence of ferritin and ferrozine). We therefore suggest that the elevation of iron release in the presence of SOD was due to an enhanced amount of reduced 6-OHDA.

Iron Release Under Anaerobic Conditions

As shown in Figure 3, iron release using 10^{-4} and 5×10^{-5} mol/l 6-OHDA was augmented when oxygen was not present in the incubation system. These data indicate that superoxide anion which also is able to release iron from ferritin[16,17] and which is formed in 6-OHDA autoxidation, is not important for iron release by 6-OHDA. A similar increase under anaerobic conditions could be observed when a $FeCl_3$ solution (3.3×10^{-5} mol/l) was used instead of ferritin (data not shown).

Investigation of 6-OHDA Analogues

Because of the very good iron releasing effect of 6-OHDA we also investigated compounds showing structural similarities to 6-OHDA (benzenediols and 1,2,4-benzenetriols).

The investigated benzenediols included catecholamines, protocatechuic acid, catechol, resorcinol and hydroquinone. They proved to be only poorly effective in release of iron as indicated by the initial rates shown in Table 1. Catecholamines and protocatechuic acid were more effective than benzenediols without side chain.

All 1,2,4-benzenetriols investigated were very effective in iron release. The initial rates increased from 1,2,4-benzenetriol to Topa and 6-OHDA (Table 1). In contrast, 5-OHDA (3,4,5-trihydroxyphenethylamine) showed distinctly lower initial rates (1.89 ± 0.53 nmol Fe/min, $n = 3$). These data indicate that hydroxyl groups in the positions 1,2 and 4 of the aromatic compound in the molecules are most important for a marked iron release from ferritin.

TABLE I
Initial rates of iron release from ferritin by 6-OHDA and related compounds (concentrations used: 10^{-4} mol/l) under aerobic conditions and reduction potentials (E in mV)

Substance	Initial rate[a] (nmol Fe/min)	E[b] (mV)
1,2,4-Benzenetriols		
6-OHDA	156.8 ± 14.7(8)	−90
Topa	55.0 ± 5.2 (3)	+90
1,2,4-Benzenetriol	41.2 ± 5.2(3)	
Benzenediols		
L-Epinephrine	0.4 ± 0.3(3)	
L-Norepinephrine	0.3 ± 0.1(3)	
Dopamine	0.5 ± 0.03(3)	+240
Dopa	0.1 ± 0.02(3)	+300
Protocatechuic acid	0.1 ± 0.0(3)	
Catechol	0.1 ± 0.02(3)	
Resorcinol	0.0 ± 0.0(3)	
Hydroquinone	0.02 ± 0.0(3)	

[a]Mean ± SD; number of experiments are shown in parentheses.
[b]Data from Ref[3].

DISCUSSION

Our studies have shown that 6-OHDA is able to release large amounts of iron from ferritin. Under aerobic conditions superoxide anion may participate in iron release. However, as shown in Figure 3, an increased release of iron was observed under anaerobic conditions, an effect that was recently reported for other compounds.[10,11] For 6-OHDA and related 1,2,4-benzenetriols we propose that decreased iron release in the presence of oxygen is caused by (a) the accelerated formation 6-OHDA quinones in the presence of superoxide anion[15] and H_2O_2, and (b) a partial oxidation of ferrous iron into the ferric form by H_2O_2.[10]

Analysis of 6-OHDA analogues showed the importance of three hydroxyl groups in the positions 1,2,4 in the aromatic ring system for an effective release of iron from ferritin. Furthermore, we suggest that the reduction potential (Table 1) of the 1,2,4-benzenetriols contribute to the high initial rates of iron release in comparison to the benzenediols.

The influence of SOD, catalase and oxygen-free buffer on iron release by other 1,2,4-benzenetriols was found similar to that of 6-OHDA (data not shown).

The iron release effects of 6-OHDA could be enhanced in the presence of ascorbic acid. Since the combination of 6-OHDA and ascorbic acid has been proposed as a purging system for removal of neuroblastoma cells from bone marrow prior to autologous bone marrow transplantation[8] and since neuroblastoma cells contain iron-rich ferritin,[9] this approach should be especially suitable for effective formation of cytotoxic reactive oxygen species. Unfortunately, only a small number of neuroblastoma cells selectively take up catecholamines,[19] so that the use of 6-OHDA as a specific purging agent is of limited value. However, since radiation therapy[18] and many chemotherapeutical agents[11] are able to release iron from ferritin, the fact that many neuroblastoma cells accumulate large amounts of ferritin might be of general therapeutic interest.

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