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Ferritin enhances production of DNA strand breaks by 6-hydroxydopamine, ascorbic acid and H_2O_2 in CCC PM2 bacteriophage DNA

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Damage of CCC PM2 DNA by 6-hydroxydopamine (6-OHDA) and ascorbic acid (AA), compounds that are both able to release iron from ferritin, was significantly enhanced in the presence of ferritin. H_2O_2, a product of 6-OHDA autoxidation, did not induce DNA strand breaks in the absence of ferritin and only to a minor extent in the presence of ferritin. DNA damage by 6-OHDA and AA could be reduced by the hydroxyl radical scavenger mannitol, the iron chelator desferrioxamine, and, partly, by a combination of superoxide dismutase and catalase. These inhibitory effects were clearly less pronounced in the presence of ferritin. Ferritin obviously played an important role as a source of iron in the pro-oxidative processes of 6-OHDA and AA. These features might be of importance in cancer therapy since many tumor cells contain elevated ferritin levels.

Keywords: ascorbic acid, DNA damage, ferritin, hydrogen peroxide, 6-hydroxydopamine

Introduction

It was previously shown in various *in vitro* studies that stored iron can be effectively released from ferritin by reducing agents such as ascorbic acid (AA; Boyer & McCleary 1987, O'Connell & Peters 1987), *o*-diphenols (Boyer *et al.* 1988) or 6-hydroxydopamine (6-OHDA; Monteiro & Winterbourn 1989, Lode *et al.* 1990). Peroxidation of liposomes by 6-OHDA, polyhydroxypyrimidines (Monteiro & Winterbourn 1989 a, b) and adriamycin (Winterbourn *et al.* 1991) was remarkedly enhanced in the presence of ferritin, indicating that mobilized iron promoted free radical reactions. There are also many indications that free iron (Theil 1987, Loeb *et al.* 1988, Schneider *et al.* 1988), iron contained in asbestos (Jackson *et al.* 1987) or iron bound to DNA (Blakely *et al.* 1990) is involved in reactions producing DNA damage in isolated DNA—probably by catalyzing the formation of hydroxyl radicals (·OH) from superoxide anions ($\cdot O_2^-$) or H_2O_2 in a Fenton-like reaction. The recent observation that many tumor cells (including neuroblastoma) contain elevated levels of ferritin (Iancu 1989) led to the question of its possible biological implications in cancer therapy. The approach to the problem in the present study is to investigate the influence of ferritin on oxidative DNA damage in an *in vitro* system. CCC PM2 bacteriophage DNA was chosen as a sensitive biological target that was exposed to 6-OHDA, AA and H_2O_2. This choice of substances resulted from experiences with neuroblastoma cells where the 6-OHDA/AA system was originally suggested and previously applied for purging bone marrow from neuroblastoma cells prior to autologous bone marrow transplantation (Reynolds *et al.* 1982). 6-OHDA, which may also be formed during dopamine metabolism in neuroblastoma cells (Slivka & Cohen 1985), rapidly autoxidizes in the presence of iron generating cytotoxic H_2O_2 and 6-OHDA-quinone. Since AA is able to reduce 6-OHDA-quinone, it might thus promote additional production of

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H_2O_2 in 6-OHDA autoxidation (Heikkila & Cohen 1972).

Materials and methods

Chemicals

Horse spleen ferritin (about 12% iron saturated) and EDTA were from Serva (Heidelberg, Germany). CCC PM2 DNA was obtained from Boehringer (Mannheim, Germany). L-AA, 6-OHDA, ethidium bromide, D-mannitol, catalase and superoxide dismutase (SOD) were from Sigma (St Louis, MO, USA). Desferrioxamine (Desferal) was from Ciba Pharma (Wehr, Germany). Tripotassium phosphate was obtained from Merck (Darmstadt, Germany). Reagents for bicinchoninic acid (BCA) protein determination were obtained from Pierce (Rockford, IL, USA).

Measurement of DNA Damage

DNA strand breaks were determined according to a modification of the fluorescence assay of Lown (1984) as performed by Jackson *et al.* (1987).

CCC PM2 DNA (10 μl, 0.25 $\mu g\,\mu l^{-1}$) was incubated with 30 μl phosphate buffered saline (PBS) containing ferritin and the agents/combinations (pH 7.4) under investigation. The reaction mixtures were then incubated at room temperature for 1 h. After addition of 200 μl fluorescence assay solution (FAS: 0.5 $\mu g\,ml^{-1}$ ethidium bromide, 0.5 mM EDTA, 20 mM tripotassium phosphate, pH 11.8) the samples were heated at 96 °C for 4 min, placed on ice and equilibrated to 25 °C. Fluorescence was measured after addition of 750 μl FAS at room temperature using a fluorescence spectrophotometer (model MPF-3; Perkin-Elmer GmbH, Ueberlingen, Germany) with an excitation wavelength of 525 nm and an emission wavelength of 600 nm. The percentage of double-stranded DNA (% dsDNA) was calculated by comparing the fluorescence of the samples to that of a reference with 10 μl CCC PM2 DNA (0.25 $\mu g\,\mu l^{-1}$) and 30 μl PBS which was equivalent to 100% dsDNA.

Dialysis of ferritin, SOD and Catalase

Ferritin (10 $\mu g\,\mu l^{-1}$), SOD (2500 U ml^{-1}) and catalase (16000 U ml^{-1}) were dialyzed at 4 °C against PBS without Ca^{2+} and Mg^{2+} for 24 h. Protein concentration was determined before and after dialysis.

Results

Effects of dialysis of SOD, catalase and ferritin on the induction of DNA strand breaks (Table 1)

The first experiments using 250 U ml^{-1} SOD and 1600 U ml^{-1} catalase without further additives led to the unexpected formation of DNA strand breaks. These effects could no longer be observed after dialysis and were obviously due to impurities of transition metals in the commercial preparations.

Table 1. Influence of dialysis of ferritin (1 μM), SOD (250 U ml^{-1}) and catalase (1600 U ml^{-1}) on DNA strand break formation

Substance	Samples	
	before dialysis (% dsDNA)	after dialysis (% dsDNA)
Ferritin	93 ± 8 (6)	100 ± 5 (6)
SOD	78 ± 16 (3)	97 ± 4 (3)
Catalase	40 ± 3 (3)	108 ± 13 (3)

Values are means ± SE. The number of experiments is given in parentheses.

If not otherwise indicated, ferritin was applied in a final concentration of 1 μM. At this concentration ferritin induced only minimal DNA damage, which could also be prevented by dialysis.

Influence of ferritin on DNA damage by 6-OHDA, AA and H_2O_2 (Figure 1)

6-OHDA was found to be a strong DNA damaging agent. Its action could be increased by the addition of ferritin, especially in samples with low 6-OHDA concentrations. 6-OHDA-induced DNA damage (at a concentration of 0.1 mM) was additionally enhanced in the presence of 1 mM AA by 45.8 ± 9.3% ($n = 6$).

AA alone showed a concentration-dependent DNA damaging activity. DNA cleavage was also clearly enhanced in the presence of ferritin.

In contrast, H_2O_2—even at concentrations as high as 1 mM—had almost no effect on the integrity of exposed CCC PM2 DNA in the absence of ferritin. However, the addition of 1 μM ferritin significantly promoted DNA damage by H_2O_2. The combination of 0.1 mM H_2O_2 with 1 mM AA resulted in an over-additive increase of DNA strand break formation by 73.2 ± 9.8% ($n = 8$).

The extent of DNA damage by 6-OHDA and AA increased with the concentration of added ferritin. This effect was less pronounced in samples with H_2O_2 (Figure 2).

Inhibition of DNA damage by mannitol, desferrioxamine, SOD and catalase in the absence or presence of ferritin (Table 2)

The addition of 50 mM mannitol, an ·OH scavenger, to 1 mM AA resulted in an increased reduction of DNA damage in the absence of ferritin, whereas in

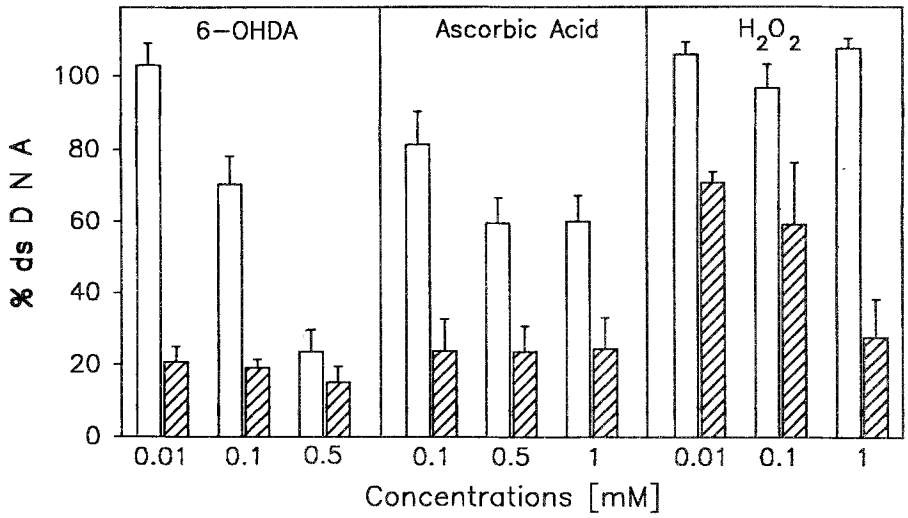

Figure 1. DNA damage by different concentrations of 6-OHDA, AA and H_2O_2 in the absence (□) or presence (▨) of 1 μM ferritin (mean ± SD, $n > 5$).

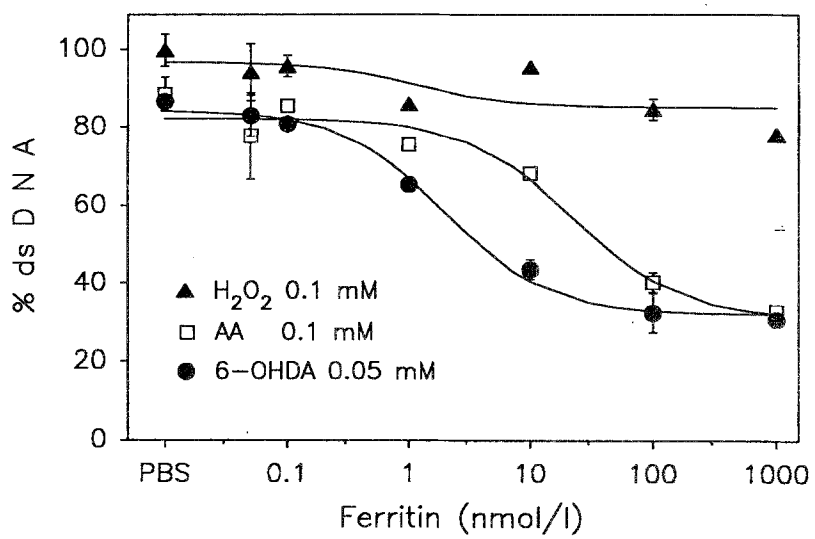

Figure 2. Influence of ferritin concentration (nmol l^{-1}; PBS: sample without ferritin) on DNA damage by 0.05 mM 6-OHDA, 0.1 mM AA and 0.1 mM H_2O_2. Mean ± SD, $n = 3$ (only error bars greater than symbols are drawn), regression curves.

the presence of ferritin no inhibitory effect could be registered. Similarly, 6-OHDA (0.1 mM)-induced DNA cleavage was clearly decreased by the addition of mannitol in the absence of ferritin and less significantly in the presence of ferritin.

The iron chelator desferrioxamine (0.1 mM) also showed inhibitory effects on the formation of DNA strand breaks by 6-OHDA and AA which were clearly less expressed in the presence of ferritin.

A combination of SOD (250 U ml^{-1}) and catalase (1600 U ml^{-1}) did not influence 6-OHDA-induced DNA cleavage either in the absence or in the presence of ferritin. However, this combination reduced DNA strand breaks by AA (1 mM) equally well in samples with or without ferritin.

Discussion

The present study showed that ferritin was obviously an important source of iron that allowed (in the case of H_2O_2) or significantly enhanced (in the case of 6-OHDA and AA) the formation of DNA strand breaks in a concentration dependent way. These results underline its role as a store of catalytic iron which has to be released before promoting pro-oxidative reactions. In previous studies it could be

Table 2. Effect of 50 mM mannitol, 0.1 mM desferrioxamine and a combination of 250 U ml^{-1} SOD and 1600 U ml^{-1} catalase on DNA damage by 1 mM AA and 0.1 mM 6-OHDA in the absence or presence of 1 μM ferritin

Substance/ concentration	Samples	
	without ferritin (% dsDNA)	with ferritin (% dsDNA)
Mannitol 50 mM	107.8 ± 2.0 (5)	110.2 ± 0.4 (5)
AA 1 mM	60.0 ± 7.3 (11)	24.4 ± 8.7 (9)
AA + mannitol	93.3 ± 5.3 (6)[a]	23.3 ± 8.4 (7)
6-OHDA 0.1 mM	70.5 ± 7.7 (13)	19.1 ± 2.4 (9)
6-OHDA + mannitol	90.4 ± 2.5 (10)[a]	29.3 ± 11.8(7)
DFO 0.1 mM	95.8 ± 9.3 (5)	92.7 ± 3.6 (5)
AA 1 mM	60.0 ± 7.3 (11)	24.4 ± 8.7 (9)
AA + DFO	85.6 ± 8.9 (5)[a]	37.3 ± 9.2 (5)[a]
6-OHDA 0.1 mM	70.5 ± 7.7 (13)	19.1 ± 2.4 (9)
6-OHDA + DFO	91.2 ± 8.5 (5)[a]	34.9 ± 3.9 (5)[a]
SOD/catalase	102.3 ± 9.9 (3)	91.3 ± 3.7 (3)
AA 1 mM	56.5 ± 6.2 (3)	25.1 ± 1.4 (3)
AA + SOD/ catalase	79.3 ± 10.8 (3)	43.8 ± 2.9 (3)[a]
6-OHDA 0.1 mM	30.4 ± 1.0 (3)	24.3 ± 6.8 (3)
6-OHDA + SOD/ catalase	31.8 ± 1.0 (3)	24.0 ± 8.8 (3)

Values are means ± SE. The number of experiments is given in parentheses. Samples with SOD/catalase only contained 25% (0.0625 μg μl^{-1}) of CCC PM2 DNA.
[a]Means of the samples without or with mannitol, DFO or SOD/catalase are different at the 0.05 level in the independent *t*-test.

demonstrated that many substances, among them 6-OHDA and AA, were able to release iron from ferritin.

However, DNA strand breaks caused by 6-OHDA and AA were observed even in the absence of ferritin. Traces of iron in the reaction mixture or DNA bound ferric iron probably acted—after being reduced to Fe^{2+} by 6-OHDA or AA—as a catalyst in the oxidative reactions, forming hydroxyl radicals from autoxidation products like H_2O_2 or $\cdot O_2^-$ (Aronovitch *et al.* 1987, Kobayashi *et al.* 1988). This suggestion is supported by the inhibitory effects of the iron chelator desferrioxamine on the extent of DNA damage (Table 2).

The well known participation of reactive oxygen compounds in DNA damage was confirmed by the partial inhibition of damage by the ·OH scavenger mannitol or by the effects of the combination of SOD and catalase. Since DNA damage could not be completely eliminated, further DNA damaging intermediates such as the 6-OHDA-semiquinone radical or the ascorbyl radical may have participated in this process.

With regard to therapeutic applications, it may be important to note that in the presence of ferritin mannitol, desferrioxamine and SOD/catalase were unable to inhibit DNA damage effectively, suggesting that cellular defense mechanisms against reactive oxygen compounds might be insufficient in tumor cells with high levels of ferritin.

In addition to its DNA damaging effects, AA, however, proved to be a redox-cycling agent in combination with 6-OHDA (Heikkila & Cohen 1972, Sachs & Jonsson 1975) and enhanced DNA cleavage. The less reactive AA probably reduced oxidized 6-OHDA, thus restoring it for the further formation of reactive oxygen compounds. Additionally, AA facilitated DNA cleavage by H_2O_2 in the ferritin-free samples where H_2O_2 was totally ineffective (Bruchelt *et al.* 1991). This combination was more effective than AA alone, indicating that AA acted as a pro-oxidant under these conditions. Since AA might reduce ferric iron (from ferritin, impurities in the reaction mixture or DNA-bound iron), it could promote the formation of hydroxyl radicals in the Fenton reaction ($H_2O_2 + Fe^{2+} \rightarrow \cdot OH + OH^- + Fe^{3+}$). The similar generation of a catalytic cycle has already been described for a $Fe(EDTA)^{2-}/H_2O_2$ system (Woolley *et al.* 1987) and an ascorbate/Fe system (Schneider *et al.* 1988).

Reactions that may occur during the autoxidation of 6-OHDA in the presence of AA and ferritin are summarized in Figure 3. Traces of iron released from ferritin (by 6-OHDA-semiquinone radicals, ascorbyl radicals or superoxide anions) may start or

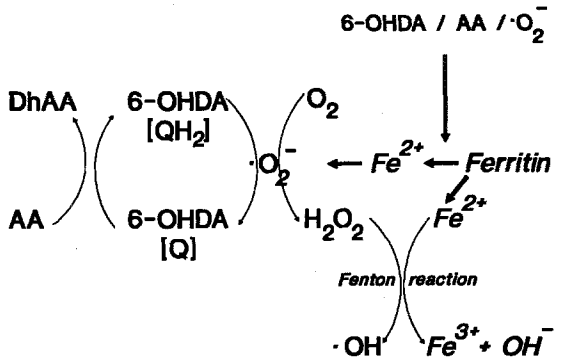

Figure 3. Influence of released iron from ferritin and of AA on the formation of reactive oxygen species in the autoxidation of 6-OHDA. DhAA, dehydroascorbic acid; QH_2, hydroquinone; Q, quinone of 6-OHDA.

promote 6-OHDA autoxidation, leading to more radicals and superoxide anions, which may enhance iron release, and to the production of H_2O_2, an educt of DNA-damaging hydroxyl radicals in the Fenton reaction.

In summary, ferritin could be a potential intracellular source of toxic iron. Since ferritin is often elevated in tumor cells it could possibly play an important role in damaging DNA or other biomolecules by substances such as 6-OHDA or AA that can (i) release iron from ferritin and (ii) produce reactive oxygen species. These characteristics might also be applicable to the action of several antitumor agents, e.g. adriamycin or daunomycin (Samokyszyn *et al.* 1988). Recent investigations with neuroblastoma cells actually showed the ferritin-dependent cytotoxicity for 6-OHDA and its combination with AA (Bruchelt *et al.* 1991). Further studies are being designed to clarify the role of ferritin *in vivo*.

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