# Synthetic melanin and ferric ions promote superoxide anion-mediated lipid peroxidation

A. Sotomatsu, M. Tanaka*, S. Hirai

*Department of Neurology, Gunma University School of Medicine, Showa-machi, Maebashi, Gunma 371, Japan*

## Abstract

In this study, we demonstrate that synthetic dopa-melanin produced superoxide anions and promoted the peroxidative cleavage of phospholipids in the presence of $Fe^{3+}$–ADP complexes. SOD significantly suppressed this lipid peroxidation, while catalase or sodium benzoate did not. During the reaction, MCLA-dependent chemiluminescence was detected, and this was suppressed to the control level by the addition of SOD. Melanin has been postulated to be toxic to tissues because of its interaction with redox-active paramagnetic metal ions, and these findings suggest that superoxide anion-mediated lipid peroxidation is induced by melanin in the presence of iron.

*Key words:* Melanin; Iron; Lipid peroxidation; Superoxide anion

## 1. Introduction

Melanin is known to be a group of redox polymers and to exhibit a very high affinity for metal ions [1]. Many investigations have been carried out on diverse aspects of the interactions between melanin and iron in vitro. Melanin is reported to have a protective role in some lipid peroxidation models [2,3], but the interaction of melanin and iron has been suggested to be harmful because of possible oxygen radical formation [4]. These contradictory findings may be caused by the properties of melanin as an iron-chelator and a redox polymer [1,5]. In the present study, we clearly demonstrate that synthetic dopa-melanin and ferric iron produce membrane lipid peroxidation in phospholipid liposomes and that this reaction is mediated by superoxide anions with no intervention of hydroxyl radicals.

## 2. Experimental

L-3,4-Dihydroxyphenylalanine (dopa), adenosine diphosphate sodium salt (ADP), Cu-Zn superoxide dismutase (SOD) derived from bovine erythrocytes, bovine liver catalase, bovine serum albumin (BSA) free of fatty acids and globulins, and deferoxamine mesylate were purchased from Sigma. 2-Methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo[1,2-a]pyrazin-3-one (MCLA) was obtained from Tokyo Kasei (Japan). Synthetic melanin was prepared by autooxidation of dopa by the method of Das et al. [6]. The wet melanin was lyophilized to obtain

the dry weight and was then resuspended in the reaction mixture at various concentrations. Phospholipid (free of free fatty acids and cholesterol) was prepared from rat liver microsomes and used to prepare liposomes according to the method described previously [7]. $Fe^{3+}$–ADP complexes were prepared by the method described previously [7].

The formation of thiobarbituric acid-reactive substances (TBARS) was determined as described previously [8,9], and phosphatidylethanolamine hydroperoxides (PEOOH) were determined by the HPLC method of Yamamoto et al. [10]. Superoxide anion generation was determined by the MCLA-dependent chemiluminescence method [11,12] using a BRL-301 Luminescence Reader (Aloka, Japan), and was recorded as photon counts per min [13]. Reaction mixtures contained 10 $\mu$M MCLA with or without dopa-melanin, $Fe^{3+}$–ADP complexes, and SOD in a total volume of 1 ml. The incubation mixture was agitated in a liquid scintillation vial (Wheaton) by rotation. After the components had been pre-incubated for 5 min, the reaction was initiated by the addition of $Fe^{3+}$–ADP complexes. All experiments were carried out in 0.1 M Tris-HCl buffer at pH 7.4 and 37°C.

## 3. Results and discussion

Synthetic dopa-melanin promoted liposomal phospholipid peroxidation in the presence of $Fe^{3+}$–ADP complexes. When the phospholipid liposomes were incubated with $Fe^{3+}$ (50 $\mu$M)–ADP (0.83 mM) complexes alone, a small amount of TBARS was formed (2 nmol/ml at 30 min and 5 nmol/ml at 120 min). When dopa-melanin was added to this system at various concentrations, TBARS formation increases dose-dependently to a maximum at 100 $\mu$g/ml of dopa-melanin (Fig. 1A), after which it dwindled away to the basal level at higher dopa-melanin concentrations. At more than 1 mg/ml, dopa-melanin completely inhibited TBARS formation (data not shown). However, an excess of melanin, more than 4 mg/ml, affected the TBARS assay. When the concentra-

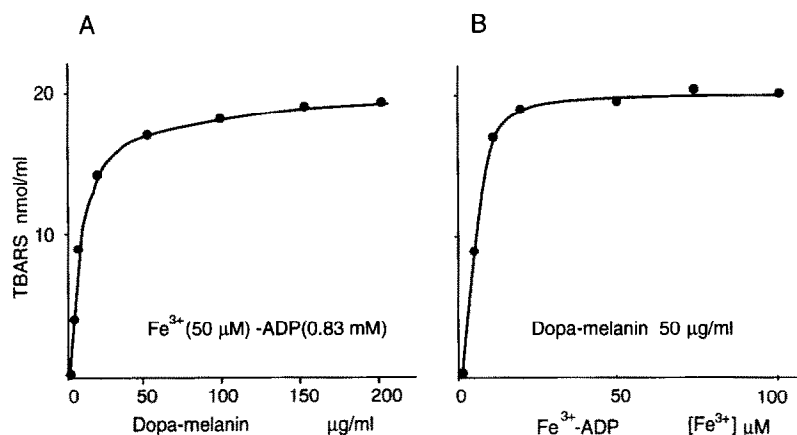* Corresponding author. Fax: (81) (272) 34-9354.

Fig. 1. The dose-dependent effect of dopa-melanin (A) and $Fe^{3+}$-ADP complexes (B) on membrane phospholipid peroxidation monitored by TBARS formation. The incubation mixture contained phospholipid liposomes ($0.85\,\mu$mol of lipid phosphorous/ml) in 0.1 M Tris-HCl buffer at pH 7.4. TBARS values obtained after 30 min of incubation were corrected for those in control systems which contained the liposomes and $Fe^{3+}$-ADP complexes. Addition of dopa-melanin at more than 500 µg/ml suppressed TBARS formation and it was completely inhibited at more than 1 mg/ml (data not shown).

tion of dopa-melanin was fixed at 50 µg/ml, TBARS formation increased with an increase in the $Fe^{3+}$-ADP complex concentration (Fig. 1B).

In experiments to examine possible effects of several reagents on phospholipid peroxidation induced by dopa-melanin and $Fe^{3+}$-ADP complexes, we employed a system containing phospholipid liposomes and dopa-melanin (50 µg/ml). Of the scavengers tested, deferoxamine mesylate (a powerful iron chelator) completely inhibited the TBARS formation. In contrast, catalase (a hydrogen peroxide scavenging enzyme), sodium benzoate (a hydroxyl radical scavenger), or sodium azide (a singlet oxygen scavenger) had little or no effect on the lipid peroxidation (Fig. 2). However, SOD (a superoxide anion scavenging enzyme) suppressed TBARS formation to about 30% of that without SOD. Heat-treated SOD or BSA at a protein level equivalent to 0.5 $\mu$M SOD (16.5 µg of protein/ml) had no effect on TBARS formation. When the time course of phospholipid peroxidation in liposomes induced by dopa-melanin and $Fe^{3+}$-ADP complexes was monitored by assessing the formation of TBARS (Fig. 3) and PEOOH (Fig. 4), it was found that SOD effectively suppressed lipid peroxidation, indicating that superoxide anions were involved in the reaction.

When dopa-melanin or $Fe^{3+}$-ADP complexes alone were added to the MCLA-dependent chemiluminescence assay system, the resulting luminescence was essentially equal to the basal level observed with MCLA alone (Fig. 5). In contrast, dopa-melanin in the presence of $Fe^{3+}$-ADP complexes produced prominent luminescence, which was completely abolished by SOD and not influenced by sodium azide (data not shown). SOD also reduced the basal photon count slightly because of its preventive effect on the autooxidation of MCLA. These findings confirmed that superoxide anions produced by the interaction of dopa-melanin and $Fe^{3+}$-ADP com-

plexes had a role in lipid peroxidation. All these experiments were repeated at least 3 times independently and gave essentially the same results each time.

In the present study, we clearly demonstrated that dopa-melanin promoted phospholipid peroxidation in liposomes in the presence of iron. The production of superoxide anions in this reaction was suggested by the suppressive effect of SOD. MCLA-dependent chemiluminescence [11–13] was not quantitative in this experiment because of considerable absorbance by melanin, but it also demonstrated superoxide anion production since MCLA is a luminescence probe that is highly sensi-
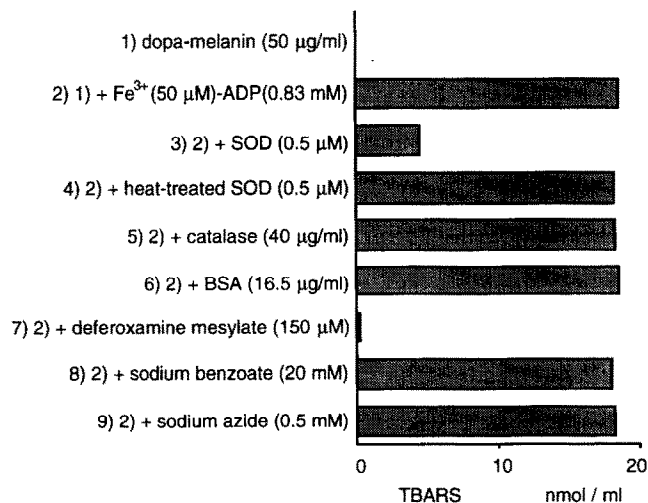


Fig. 2. The effect of various scavengers on TBARS formation after 30 min of incubation in the presence of dopa-melanin and $Fe^{3+}$-ADP complexes. The chief components of the reaction mixture were the same as in Fig. 1. Final concentrations of the additives are indicated in parenthesis. Dopa-melanin alone produced no TBARS. SOD effectively suppressed and deferoxamine caused nearly complete inhibition of TBARS formation. TBARS values were also corrected as described in Fig. 1.
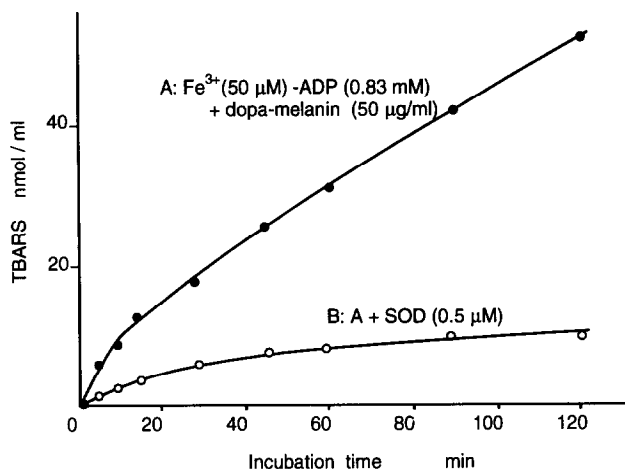
Fig. 3. Time–course of TBARS formation induced by dopa-melanin and $Fe^{3+}$–ADP complexes. The experimental conditions were the same as in Fig. 2, except for the incubation time. TBARS formation was significantly suppressed by SOD.
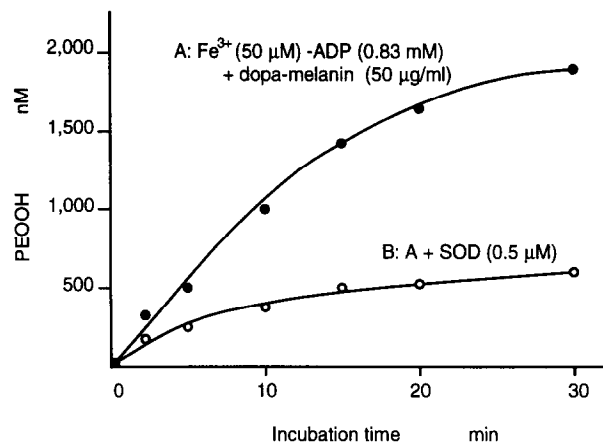


Fig. 4. Time–course of lipid peroxide (PEOOH) formation by dopa-melanin and $Fe^{3+}$–ADP complexes and its suppression by SOD. The experimental conditions were the same as in Fig. 3. SOD also effectively inhibited PEOOH formation, giving direct evidence that lipid peroxidation was mediated by superoxide anions.

tive and specific to superoxide anions and singlet oxygen [12]. In our experiment, SOD completely eliminated the chemiluminescence, but sodium azide did not. SOD also effectively reduced TBARS and PEOOH formation.

Pilas et al. [5] have reported the effects of melanin on the iron-catalyzed decomposition of hydrogen peroxide to hydroxyl radicals and hydroxyl ions. In this reaction, ferric ions are reduced to ferrous ions by melanin which then decompose hydrogen peroxides into hydroxyl radicals and hydroxyl ions. However, they also reported that an excess of melanin over ferric ions resulted in the suppression of hydroxyl radical formation because of the iron-chelating effects of melanin. Moreover, hydroxyl radical formation depends on the iron chelators used in the reaction. For instance, ferric ion–EDTA complexes produce a large amount of hydroxyl radicals, because melanin is able to reduce ferric ions chelated by EDTA and hence accelerates a Fenton-like process. On the other hand, when ferric ions are complexed with ADP, which is probably a better model for loosely bound iron in cells, melanin may inhibit hydroxyl radical production. In our experiments, ADP was employed as an iron chelator. Sodium benzoate, a direct hydroxyl radical scavenger, had no effect on TBARS formation. In addition, catalase (which should prevent hydroxyl radical generation from hydrogen peroxide in the Fenton reaction) did not reduce lipid peroxidation and sodium azide (a singlet oxygen scavenger) also had no effect. Therefore, lipid peroxidation was apparently not mediated by hydroxyl radicals or singlet oxygens under our experimental conditions. This is consistent with some examples of lipid peroxidation mediated by superoxide anions and iron previously documented [14,15]. A highly reactive compound may also be formed, i.e. an iron-dopa-melanin complex like the iron–dopa complex pro-

duced during lipid peroxidation induced by iron and dopa [9,16].

Some investigators have postulated that melanin has an injurious effect in vivo on the basis of the demonstration of melanin and iron-induced membrane lipid peroxidation in vitro [17]. On the other hand, melanin has been reported to suppress iron-dependent lipid peroxidation under some conditions [2,3]. Indeed, an excess of dopa-melanin inhibited TBARS formation in the present study. These contradictory findings may be understandable on the basis of the property of melanin as an iron-chelator and a redox polymer [5]. There appear to be close similarities with the lipid peroxidation induced by $Fe^{3+}$–ADP complexes and dopa [9].
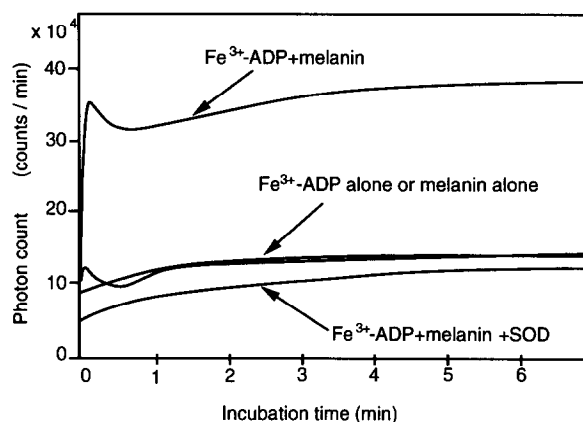


Fig. 5. MCLA-dependent chemiluminescence produced by dopa-melanin and $Fe^{3+}$–ADP complexes. The reaction mixture contained 10 $\mu$M MCLA in 0.1 M Tris-HCl buffer with or without dopa-melanin (50 $\mu$g/ml), $Fe^{3+}$(50 $\mu$M)–ADP(0.83 mM) complexes and 0.5 $\mu$M SOD. The chemiluminescence yielded by the addition of dopa-melanin or $Fe^{3+}$–ADP complexes was nearly equal to the basal photon counts produced by addition of MCLA alone.

In conclusion, melanin, present intracellularly under physiological conditions or extracellularly under pathological conditions, is potentially cytotoxic, and could produce superoxide anions in concert with iron that was inappropriately stored or present in excess, e.g. as occurs in the substantia nigra of parkinsonian brains [4,18].

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