Inhibitory Effects of Pentamethine Trinuclear Cyanine Dyes on ADP/Fe$^{3+}$-Induced Lipid Peroxidation in Rat Liver Mitochondria: Changes in the Mode of Action with the Hydrophobic Nature of the Dyes

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The effects of various pentamethine trinuclear cyanine dyes, each of which has three alkyl chains, on ADP/Fe$^{3+}$-induced lipid peroxidation in rat liver mitochondria were examined. Although the dye having the shortest $-C_{n}H_{2n+1}$ chains (tri-S-C$_{6}$(5)) did not show any appreciable effect, the dyes having $-C_{n}H_{4}$ (tri-S-C$_{6}$(5)), $-C_{2}H_{4}$ (tri-S-C$_{6}$(5)), and $-C_{2}H_{4}$ (tri-S-C$_{6}$(5)) chains significantly inhibited lipid peroxidation, the most potent inhibitory effect being observed with tri-S-C$_{6}$(5). The mode of antiperoxidation effect of the dyes was dependent on the length of the alkyl chains. The relatively hydrophilic dye tri-S-C$_{6}$(5) was suggested to scavenge radicals more efficiently at or near the membrane surface rather than in the interior of the lipid membrane, whereas the more hydrophobic dye tri-S-C$_{6}$(5) was suggested to scavenge radicals efficiently in the membrane rather than at or near the membrane surface. The hydrophilic/hydrophobic balance of the dye was found to regulate the site of action of the dyes.

Key words: cyanine dye; antioxidant; ADP/Fe$^{3+}$-induced lipid peroxidation; mitochondria; hydrophobicity

Photosensitizing pentamethine trinuclear cyanine dyes show various biological activities, such as antimicrobial activity, antioxidant activity, and uncoupling of oxidative phosphorylation. However, their effects are dependent on their chemical properties. The divalent cationic pentamethine trinuclear cyanine dyes consist of a pentapoleylene chain, 3 thiazolium rings, and 3 homologous alkyl chains ($-C_{n}H_{2n+1}$) attached to each thiazolium ring, but the length of the alkyl chain differs, and they are shown by tri-S-C$_{n}$(5) (see Chart). Nakagawa et al. reported that the scavenging of various active oxygen species by various pentamethine trinuclear cyanine dyes in the aqueous solution proceeded more rapidly with the dye having shorter alkyl chains; the activity increased in the order of tri-S-C$_{6}$(5)<tri-S-C$_{6}$(5)<tri-S-C$_{6}$(5) (for chemical structures, see Chart). As we have studied the mechanism of the anti-lipid peroxidation of various chemicals in liposomes and mitochondrial membranes, it is of interest to examine whether the effects of these cyanine dyes on lipid peroxidation are dependent on their alkyl chain length. Therefore, we examined the effects of several cyanine dyes on the ADP/Fe$^{3+}$-induced lipid peroxidation in rat liver mitochondrial membrane.

MATERIALS AND METHODS

Materials Cyanine dyes were obtained from Nihon Kanko-Shikisou Kenkyusho (Japan Photosensitizer Laboratories, Ltd. (Okayama, Japan), 2,6-Di-r-buty-$p$-cresol (BHT) and $\alpha$-tocopherol were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Other reagents were of the highest grade commercially available.

Preparation of Rat Liver Mitochondria Mitochondria were prepared from the liver of male Wistar rats by differential centrifugation as described by Myers and Slater. The amount of mitochondrial protein was determined by the Biure method with bovine serum albumin as a standard.

Lipid Peroxidation Assay Lipid peroxidation in the mitochondrial membrane was examined at 25°C and pH 7.4 unless otherwise noted. We determined the effects of cyanine dyes in two ways, first by determining the amount of lipid peroxides as an amount of thiobarbituric acid-reactive substances (TBARS), and second, by monitoring oxygen consumption due to lipid peroxidation. In all cases, lipid peroxidation was started by the addition of ADP (final concentration, 1 mM) and FeSO$_{4}$ (0.1 mM) to the mitochondrial suspension at 0.7 mg protein/ml. The amount of TBARS in terms of malondialdehyde (MDA) using 1,1,3,3-tetraethoxypropane as a standard was determined as described by Okawa et al. The peroxidation-related oxygen consumption in incubation medium consisting of 175 mM KCl and 10 mM Tris- HCl buffer (pH 7.4) in a total volume of 2.53 ml was monitored using a Clark-type oxygen probe (Yellow Spring, YSI 5331) as described previously. The saturated concentration of O$_{2}$ in the incubation medium was assumed to be 258 $\mu$M at 25°C.

**Chart**. Chemical Structures of $\alpha$-Tocopherol, BHT and Cyanine Dyes

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RESULTS

Effects of Cyanine Dyes on TBARS Formation In this study, we examined the effects on the lipid peroxidation of the four divalent cationic pentamethine trinuclear cyanine dyes: tri-S-C₅(5), tri-S-C₄(5), tri-S-C₃(5) and tri-S-C₂(5). We also examined the effects of the well known anti-lipid peroxidation reagents α-tocopherol and BHT. First, we determined the effects of the cyanine dyes on the lipid peroxidation of mitochondrial membrane in terms of TBARS formation 10 min after the addition of ADP/Fe⁺⁺ at pH 7.4 and 25°C.

For the quantitative evaluation of their antiperoxidation effects, the amount of TBARS with dye relative to that without cyanine dyes was determined as a function of their concentration, and these results are shown in Fig. 1. Although the dye with the shortest chain of tri-S-C₅(5) (n=2) did not show any appreciable effect, the dyes with longer chain lengths of tri-S-C₆(5) (n=4) and tri-S-C₅(5) (n=7) significantly inhibited TBARS formation (Fig. 1). The 50% inhibition was observed at 20 μM with tri-S-C₅(5) and at 3 μM with tri-S-C₅(5). Contrary to our expectation, the effect of the dye with the longest alkyl chain tri-S-C₅(5) (n=12) was about the same as that of tri-S-C₅(5). However, its effect was not complete, the highest inhibitory effect being about 80% at 60 μM, and the antiperoxidation activity was decreased at more than 60 μM (Fig. 1). The concentration necessary for 50% inhibition of the peroxidation (IC₅₀), as summarized in Table 1, increased in the order of BHT<tri-S-C₅(5)<tri-S-C₄(5)<tri-S-C₃(5)<α-tocopherol<tri-S-C₂(5).

Effects of Cyanine Dyes on Peroxidation-Related Oxygen Consumption Next, we monitored the time-dependent change in the peroxidation-related oxygen concentration of the rat liver mitochondrial suspension at pH 7.4 and 25°C. As shown in Fig. 2, the oxygen concentration of the mitochondrial suspension in the absence of the test compound decreased slowly for a few minutes after the addition of ADP (final concentration, 1 mM) and Fe⁺⁺ (0.1 mM), then it decreased rapidly. As little oxygen consumption took place without ADP and Fe⁺⁺, the first slow oxygen consumption and the subsequent rapid oxygen consumption were regarded to be due to the generation of active oxygen species derived from ADP/Fe⁺⁺, such as ADP-Fe⁺⁺•O₂⁻, and following the peroxidation radical chain reaction of membrane lipids induced by the active oxygen species, respectively.14-18 In this paper, we referred to the period of the first slow oxygen consumption as lag-time.

The well known antiperoxidation reagents, α-tocopherol and BHT, significantly affected the oxygen consumption in the mitochondrial suspension in different ways. α-Tocopherol decreased the rate of rapid oxygen consumption, but it did not show any effect on lag-time (Fig. 2A). In contrast, BHT prolonged the lag-time, but the rate of oxygen consumption after lag-time remained almost the same (Fig. 2B).

Next, we examined the effect of cyanine dyes on the peroxidation-related oxygen consumption induced by ADP/Fe⁺⁺ in rat liver mitochondria (Fig. 3). Although tri-S-C₅(5) was essentially ineffective, tri-S-C₄(5) and tri-S-C₃(5) remarkably inhibited the oxygen consumption (Figs. 3A—C). It is noteworthy that these two cyanine dyes both prolonged the lag-time and decreased the rate of rapid oxygen consumption in contrast to the effects of α-tocopherol and BHT (see Fig. 2). However, the effect of tri-S-C₂(5) was different from those of tri-S-C₅(5) and tri-S-C₄(5), as shown in Fig. 3D. This dye only inhibited the rate of oxygen consumption after lag-time, like α-tocopherol (Fig. 2A), and it was ineffective on lag-time.

DISCUSSION

In this study, we examined the effect on ADP/Fe⁺⁺-induced lipid peroxidation of rat liver mitochondrial membrane of various pentamethine trinuclear cyanine dyes. The chemical structures of these divalent cationic cyanine dyes were only different in terms of the length of 3 homologous alkyl
chains attached to each of 3 thiazolium rings. Although the dye having \(-C_9H_2\) chains (tri-S-C\(_5\)) did not show any appreciable antiperoxidation activity, the dyes having longer alkyl chains, tri-S-C\(_4\) and tri-S-C\(_3\), inhibited lipid peroxidation significantly, the activity being greater with tri-S-C\(_4\) (Fig. 1 and Table 1). Tri-S-C\(_3\) having the longest alkyl chains showed potent antiperoxidation activity, but its effect was lower than that of tri-S-C\(_4\). It is noteworthy that the inhibitory effects of tri-S-C\(_4\), tri-S-C\(_3\), and tri-S-C\(_2\) were less than that of BHT, but much greater than that of \(\alpha\)-tocopherol. Of the cyanine dyes examined, tri-S-C\(_3\) was found to be the most potent antiperoxidation reagent, at least in rat liver mitochondria, its IC\(_{50}\) being 3 \(\mu\)M (Table 1).

As the hydrophobicity represented by the logarithmic form of the partition coefficient in the \(n\)-octanol-H\(_2\)O system (log \(P_{oct}\)) is increased about 0.5 by the introduction of a \(-CH_2-\) group, and as each cyanine dye used in this study has 3 alkyl chains, the log \(P_{oct}\) of tri-S-C\(_4\) should be 4.5 greater than that of tri-S-C\(_3\). It has generally been observed with various membrane-acting compounds that their biological activity (BR) in the membrane increases one order of magnitude by an increment of log \(P_{oct}\) of one. According to this, the difference between the log \(1/IC_{50}\) of tri-S-C\(_4\) and that of tri-S-C\(_3\) should be 4.50. However, it was only 0.82 (Table 1). This value is extremely lower than what was expected. Therefore, it is apparent that the effects of cyanine dyes were not dependent simply on their hydrophobicity.

Lipid peroxidation induced by ADP/Fe\(^{2+}\) is thought to consist of two major stages, and they are well observed in the process of peroxidation-related oxygen consumption. The first stage is the generation and accumulation of the ADP/Fe\(^{2+}\)-derived active oxygen species such as ADP-Fe\(^{3+}\)-O\(_2\)\(^-\) in the vicinity of the membrane surface, and the rate of oxygen consumption in this period (lag-time) is slow but steady. Then, in the second stage, the subsequent propagation of a radical chain reaction due to the attack of the active oxygens against unsaturated acyl chains in the membrane interior results in rapid oxygen consumption. Therefore, the process of lipid peroxidation and the effect of the test compound are able to be monitored by the time-dependent oxygen consumption induced by ADP/Fe\(^{2+}\) (Figs. 2 and 3).

The typical radical scavenger BHT only prolonged the lag-time in lipid peroxidation (Fig. 2). Therefore, it can be assumed that BHT scavenges active oxygen species at or near the membrane surface. Possibly, its major site is located at or near the membrane surface. In contrast, as \(\alpha\)-tocopherol inhibited the propagative radical chain reaction without affecting lag-time, the major action site of \(\alpha\)-tocopherol could be the membrane interior. Our result is consistent with previous reports on the location of the chromanol moiety of \(\alpha\)-tocopherol at a position beneath the membrane surface.

Accordingly, the different effects of cyanine dyes could be explained by the different locations of their alkyl chains, because the location of the radical trapping site pentapolyene chain that is commonly present in the cyanine dyes should be dependent on the length of the alkyl chains. As tri-S-C\(_4\) and tri-S-C\(_3\) both significantly prolong the lag-time and inhibit the propagative radical chain reaction (Figs. 3B and C), they scavenge radicals both at/near the membrane surface and in the interior of the membrane. It is noteworthy that the effect on lag-time was more significant with tri-S-C\(_4\), and that the inhibition of the peroxidative radical chain reaction was greater with tri-S-C\(_3\). These differences could be because the more hydrophilic tri-S-C\(_4\) is located at the position close to the membrane surface, whereas the more hydrophobic tri-S-C\(_3\) is located in the interior of the membrane. The hydrophobic and hydrophilic natures of tri-S-C\(_4\) are well balanced, so that it is located at a position favorable for scavenging radicals both at the surface and in the interior of the membrane.

The most hydrophobic cyanine dye, tri-S-C\(_5\), may not bind to the lipid membrane, thus causing it to be ineffective in inhibiting lipid peroxidation (Figs. 1 and 3A). In contrast, tri-S-C\(_4\), which inhibited only the rate of rapid oxygen consumption after lag-time, is thought to be located deeply in the membrane, thus it is not able to scavenge active oxygen species at or near the membrane surface (Fig. 3D). The reason the antiperoxidation effect of tri-S-C\(_4\) decreased at concentrations higher than 60 \(\mu\)M is not clear at present (Figs. 1 and 3D). However, it is possible that the assembly of the dye molecules takes place to form micelles at more than 60 \(\mu\)M, and this assembly is unfavorable for wide and homogeneous distribution of the dye in the membrane.

In conclusion, we found that cyanine dyes are very useful antiperoxidation reagents in the biological membrane system. The dyes are available according to the required purpose, either scavenging radicals at the surface or in the membrane, or both. The hydrophobic/hydrophilic nature of the dye well regulates its action site on the membrane. This study will be useful for developing a potent antiperoxidation reagent in lipid membranes.

REFERENCES

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