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Original article

Detection and inhibition of lipid-derived radicals in low-density lipoprotein

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ABSTRACT

Oxidized low density lipoprotein (Ox-LDL) is implicated in a variety of oxidative diseases. To clarify the mechanisms involved and facilitate the investigation of therapeutics, we previously developed a detection method for lipid-derived radicals using the fluorescent probe 2,2,6-trimethyl-6-pentyl-4-(4-nitrobenzo[1,2,5]oxadiazol-7-ylamino)piperidine-1-oxyl (NBD-Pen). In this study, NBD-Pen was used to detect lipid-derived radicals in Ox-LDL from *in vitro* and *in vivo* samples using an iron overloaded mouse model. By following the timeline of lipid radical generation using this method, the iron overloaded mice could be successfully treated with the antioxidant Trolox, resulting in successful lowering of the plasma lipid peroxidation, aspartate transaminase and alanine transaminase levels. Furthermore, using a combination therapy of the chelating agent deferoxamine (DFX) and Trolox, liver injury and oxidative stress markers were also reduced in iron overloaded mice. The NBD-Pen method is highly sensitive as well as selective and is suitable for targeting minimally modified LDL compared with other existing methods.

1. Introduction

Oxidized low-density lipoprotein (Ox-LDL) plays an important role in the pathogenesis of atherosclerosis [1,2]. Recently, the existence of an enhanced plasma concentration of ox-LDL has been confirmed in many oxidative diseases including thalassemia [3] and diabetes [4]. These patients also have a high risk of atherosclerosis incidence [5,6]. Hence, to identify disease causing agents and a biomarker of oxidative disease damage, research of Ox-LDL has become increasingly important. The development of a detection method targeting Ox-LDL is also required to prevent complications and propose therapeutic methods to treat these diseases. Currently, an enzyme-linked immunosorbent assay (ELISA) method [7] and agarose gel electrophoresis [8] are primarily used to detect oxidized LDL in clinical samples. Although the LDL oxidation pathway is roughly divided into two stages [9], lipid oxidation and protein modification, these detection methods only target protein-modified ox-LDL. LDL with only a small amount of modified proteins and oxidized lipids is called minimally modified LDL (MM-LDL) [9,10]. This oxidized LDL also induces a pro-arteriosclerotic effect [11–13] because of an oxidized lipid in MM-LDL [14]. Taking this into consideration, to clarify the mechanisms involved in oxidative diseases and facilitate the investigation of effective therapeutics, the development of a detection method targeting not only protein modification but also lipid peroxidation in LDL is quite important.

Herein, we focused on lipid-derived radicals, which are initial products and key molecules involved in complex lipid peroxidation reactions [15]. Targeting lipid-derived radicals, common molecules in the LDL oxidation pathway, should provide a highly sensitive detection method for Ox-LDL measurement. Recently, we developed the highly sensitive and selective fluorescent probe 2,2,6-trimethyl-6-pentyl-4-(4-nitrobenzo[1,2,5]oxadiazol-7-ylamino)piperidine-1-oxyl (NBD-Pen) for lipid-derived radicals (Fig. 1a) [16], and succeeded in using it to detect lipid-derived radicals in the liver and plasma of hepatocellular carcinoma mice models.

Hence, in this study, we aimed to detect lipid-derived radicals in LDL from *in vitro* and *in vivo* samples using an iron-overloaded mouse model. Moreover, we examined the effect of treating iron-overloaded mice with an antioxidant based on detection of lipid radical generation using this method. Furthermore, we proposed a combination therapy using an antioxidant and iron chelate to reduce liver injury in iron-overloaded mice.

2. Materials and methods

2.1. Chemicals

The starting materials, reagents, and solvent from commercial suppliers were used without further purification. All solvents were

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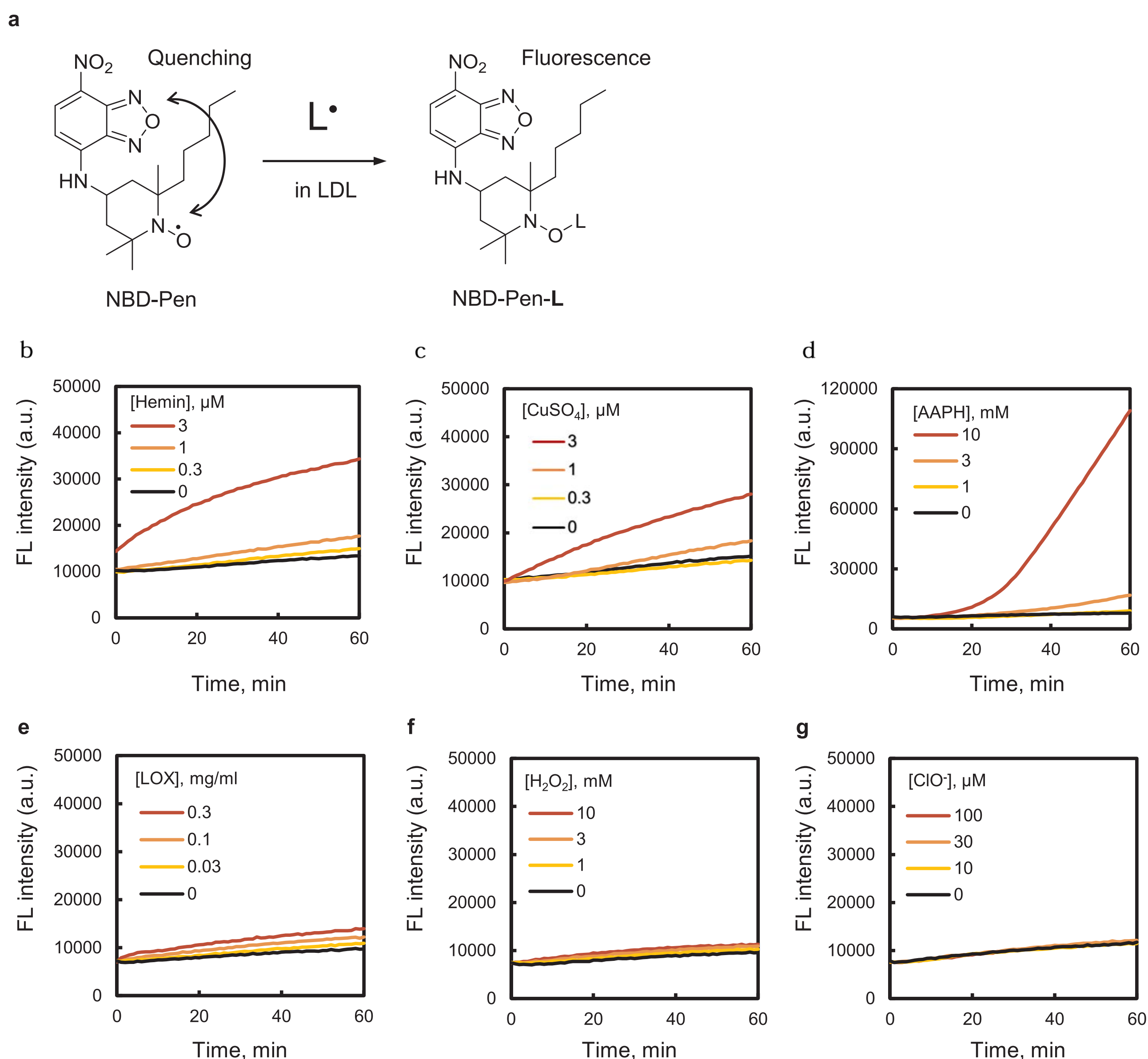

Fig. 1. Fluorescent detection of lipid peroxidation by various oxidants. (a) Summary of lipid-derived radical fluorescence detection probe NBD-Pen. (b)-(g) Time profile of fluorescent intensity (λ_{ex}: 470 nm, λ_{em}: 530 nm) of 10 μM NBD-Pen with 20 μg protein/ml LDL and (b) 0–3 μM hemin (c) 0–3 μM $CuSO_4$, (d) 0–10 mM AAPH, (e) 0–0.3 mg/ml LOX, (f) 0–10 mM H_2O_2, (g) 0–100 μM NaClO.

purified prior to use. Wakogel® C‒200 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used for column chromatography. TLC analysis was performed using precoated silica plates and detected with iodine vapor. The low-density lipoprotein (LDL) in human plasma for the *in vitro* experiments was purchased from Lee Biosolutions Inc. (MO, USA). The agarose gel was prepared using Agarose H14 "TAKARA" (TAKARA Bio. Inc., Shiga, Japan).

2.2. Synthesis

2,2,6-Trimethyl-6-pentyl-4- (4-nitrobenzo[1,2,5]oxaduazol-7-ylamino)piperidine-1-oxyl (NBD-Pen)

NBD-Pen was synthesized according to a previously described method [16].

2.3. Optical measurement

The absorbance and fluorescent intensities were measured using an Enspire Instrument (PerkinElmer Japan Co. Ltd. Kanagawa, Japan).

2.4. Determination of protein concentration in LDL

We measured protein concentration by Lowry protein assay according to a previously described method [17].

2.5. Fluorescence detection of lipid-derived radicals in LDL by NBD-Pen

The fluorescence intensity (λ_{ex}: 470 nm, λ_{em}: 530 nm) was monitored at 37 °C for 1 h after addition of 10 μM NBD-Pen and 0–3 μM hemin (TCI Co. Ltd., Tokyo, Japan) to 20 μg protein/ml LDL. We also evaluated in the same way the addition of reaction initiators (0–30 μM $CuSO_4$, 0–30 mM H_2O_2, 0–300 μM NaClO (Wako Pure Chemical Industries, Ltd.) or 0–1.0 mg/ml lipoxygenase (LOX) (Sigma-Aldrich Japan Co., Tokyo, Japan)) instead of 10 μM NBD-Pen and 0–3.0 μM hemin. Furthermore, we investigated the addition of 30 μM Trolox (EMD Millipore Co., Billerica, MA, USA) to 20 μg protein/ml LDL.

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