Nitric oxide released from zwitterionic polyamine/NO adducts inhibits Cu^{2+}-induced low density lipoprotein oxidation

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Abstract The effects of nitric oxide (NO) released from zwitterionic polyamine/NO adducts on Cu^{2+}-induced low density lipoprotein (LDL) oxidation were studied. When each of the two kinds of NO releasing zwitterionic polyamine/NO adducts (NOC5 and NOC7) was incubated at 5 μM with isolated human LDL (0.25 mg/ml) and Cu^{2+}, the formation of thiobarbituric acid reactive substances (TBARS) was inhibited. The duration of inhibition by NOC7 (20 min) and NOC5 (100 min) corresponded to the NO generation lives of respective zwitterionic polyamine/NO adducts. The duration of inhibition was dependent on the amount of NOC5 added (2.5–20 μM). Repeated additions of 5 μM NOC5 at 100 min intervals worked as inhibitor in the same manner. NOC5 broke to inhibit at any process of the Cu^{2+}-induced LDL oxidation reaction. Fragmentation of apolipoprotein B derived from Cu^{2+}-induced LDL oxidation was also prevented by the addition of NOC5. These results clearly indicate that NO inhibits the oxidative modification of LDL induced by Cu^{2+}. NO releasing zwitterionic polyamine/NO adducts are good reagents for NO studies.

Key words: Nitric oxide; Zwitterionic polyamine/NO adducts; Antioxidation; Low density lipoprotein

1. Introduction

Oxidation of low density lipoprotein (LDL) has been reported to be an important process in the development of atherosclerosis [1]. Macrophages target the oxidatively modified LDL for uptake and are characterized as foam cells [1]. Now, endothelium cells are considered to be involved in LDL oxidation [1] and nitric oxide (NO) itself may be an endothelium derived relaxing factor [2]. Endothelium cells and macrophages are known to accelerate LDL oxidation [3–6]. Endothelium cells, macrophages, neutrophils and neuronal cells generate superoxide (O_2^-) and NO, which can combine to form peroxynitrite anion ($ONOO^-$) [7,8]. Synthesized peroxynitrite was shown to induce lipid peroxidation of liposomes [9]. The sydnonimine, SIN-1, which is known to liberate both O_2^- and NO during autoxidation, could initiate lipid peroxidation in human LDL [10] and synthesized peroxynitrite resulted in an increase in electrophoretic mobility of apolipoprotein B in LDL [11]. In contrast, the extent of cell mediated oxidative modification of LDL and the generation of high uptake LDL was depressed in macrophages active in NO synthesis [12]. Lipid peroxidation of LDL, as measured by thiobarbituric acid reactive substances (TBARS), was reduced in interferon-γ and tumor necrosis factor-α stimulated macrophages to increase the production of NO [13]. Jessup and Dean also reported that NO synthesis of macrophages appeared to mediate the suppression of LDL oxidation [14,15].

Recently, new NO releasing zwitterions have been developed [16]. These reagents are known to release NO and amines by decomposing by themselves, and do not generate any other radicals [16]. On the other hand, macrophages and other cells which generate NO are well known to produce other reactive oxygen species such as O_2^-. Therefore, these zwitterions are useful reagents for studying the effect of NO itself on oxidation of LDL. The above facts led us to study the effect of NO on cell free oxidation systems. In the present study, we investigated the effect of NO released from NO releasing zwitterions (NOC5 and NOC7) on Cu^{2+} induced LDL oxidation by the determination of TBARS and the fragmentation of apolipoprotein in LDL. Our results indicate that NO releasing zwitterions inhibited Cu^{2+} induced oxidation of LDL and these antioxidative effects depended on the range of NO generation life of the zwitterions used.

2. Materials and methods

2.1. Human LDL preparation

Human plasma was obtained from volunteers according to the Declaration of Helsinki. Human LDL (d=1.005–1.065) was prepared by the method of Havel et al. [17] and its purity was monitored by two-dimensional electrophoresis [18]. Isolated LDL was dialyzed against 20 mM sodium phosphate buffer, pH 7.2, containing 0.15 M sodium chloride (PBS) and 2 μM EDTA, overnight at 4°C in the dark. Before use, the LDL solution was diluted with 0.1 M sodium phosphate buffer, pH 7.2, containing 0.15 M sodium chloride and 0.2 μM EDTA (PBS-EDTA). The protein concentration of LDL was measured with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA) using bovine serum albumin as a standard. Zwitterionic polyamine/NO adducts for releasing NO were as follows: 1-hydroxy-2-oxo-3-(3-aminopropyl)-3-isopropyl-1-triazene (NOC5) and 1-hydroxy-2-oxo-3-(*N*-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC7) (Dojin Chemicals Co., Ltd., Kumamoto, Japan). Epigallocatechin gallate (EGCG) was obtained from Kurita Kogyo Ltd. (Tokyo, Japan). Butylated hydroxytoluene (BHT) and all the other chemicals used were special grade (Wako Pure Chemicals, Tokyo, Japan).

2.2. LDL oxidation

LDL (0.25 mg/ml in PBS-EDTA) was incubated at 37°C in the presence of 5 μM $CuSO_4$ and the effects of various zwitterionic NO/polyamine adducts were examined.

2.3. Measurement of LDL oxidation

A 100 μl aliquot obtained from the reaction mixture every 20 min was added to 10 μl of 10 mM EDTA to stop oxidation. Lipid peroxidation was estimated fluorometrically as TBARS [19] and/or measured by absorbance at 234 nm to detect conjugated diene [20]. In TBARS analysis, malondialdehyde (MDA) formed from 1,1,3,3-tetraethoxypropane was used as a reference standard and the results are expressed as nmol equivalents of MDA. We repeated this series of experiments two or three times, and the results showed almost the same tendency. Typical results are shown in the figures.

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2.4. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE for investigating fragmentation of apolipoprotein B in LDL by oxidation was performed according to the method of Laemmli [21] using a 4–12% gradient gel. The reaction was stopped by adding 20 μl of 10 mM EDTA to 200 μl reaction mixture. LDL protein was precipitated by adding 200 μl of 50% trichloroacetic acid, and recovered by centrifugation at 10000×*g* for 15 min. The precipitated protein was washed three times with 200 μl of 10 mM phosphate buffer, pH 7.2, and lyophilized. The lyophilized sample was dissolved with 15 μl of 10 mM Tris-HCl buffer, pH 7.2, containing 2% SDS and 8 M urea, and 10 μg of protein was applied to SDS-PAGE.

3. Results

Fig. 1 shows the effects of NOC5 and NOC7 on Cu^{2+}-induced TBARS formation in LDL. As other antioxidative reagents, 1.5 μM EGCG and 3 μM BHT were also examined. A clear prolongation in lag time of accelerative TBARS formation was observed. The lag times were as follows: NOC7 20 min, NOC5 100 min, EGCG 260 min. BHT continued to inhibit TBARS formation under the experimental conditions. When NOC5 was preincubated in PBS-EDTA for 3 h or more at 37°C and then added to LDL oxidation mixture, the inhibitory effect of TBARS formation was not obtained (data not shown). The inhibitory effects of 0.5, 2.5, 5.0, 10.0 and 20.0 μM NOC5 on Cu^{2+} induced TBARS formation are shown in Fig. 2. The lag times to initiate accelerating TBARS formation in the presence of 0.5, 2.5, 5.0, 10.0 and 20.0 μM NOC5 were 20 min, 80 min, 100 min, 120 min and 180 min, respectively. The lag times were dependent on the concentrations of NOC5 added. Yet, the lag times were not linearly prolonged in relation to NOC5 concentration, especially at higher concentrations, because of the half-life of NOC5. The changes in Cu^{2+} concentration (5–15 μM) had no effect on inhibition of TBARS formation by NOC5 (data not shown). The results indicate that the chelating effect of NO to Cu^{2+} and/or trace amounts of transition metals such as iron was not related to this inhibiting action. The Cu^{2+} induced formation of conjugated diene in LDL was also inhibited by the addition of NOC5 (data not shown).

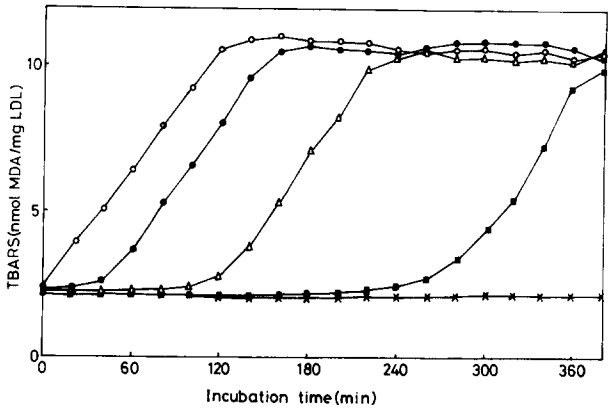

Fig. 1. Inhibitory effect of zwitterionic NO/polyamine adducts on Cu^{2+} induced TBARS formation in LDL. LDL (0.25 mg/ml) was dissolved in PBS-EDTA and was incubated with 5 μM $CuSO_4$ at 37°C (○), in the presence of 5 μM NOC5 (△), 5 μM NOC7 (●), 1.5 μM EGCG (■) or 3 μM BHT (×). TBARS formation was measured as described in Section 2.

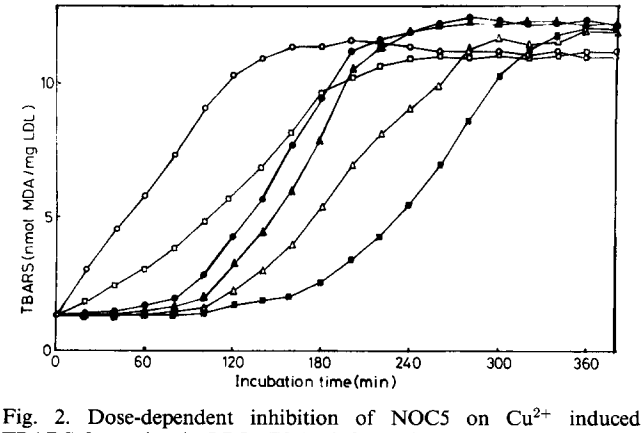

Fig. 2. Dose-dependent inhibition of NOC5 on Cu^{2+} induced TBARS formation in LDL. TBARS formation was measured as described in Fig. 1 with no NOC5 (○), 0.5 (□), 2.5 (●), 5.0 (▲), 10.0 (△) or 20.0 μM NOC5 (■).

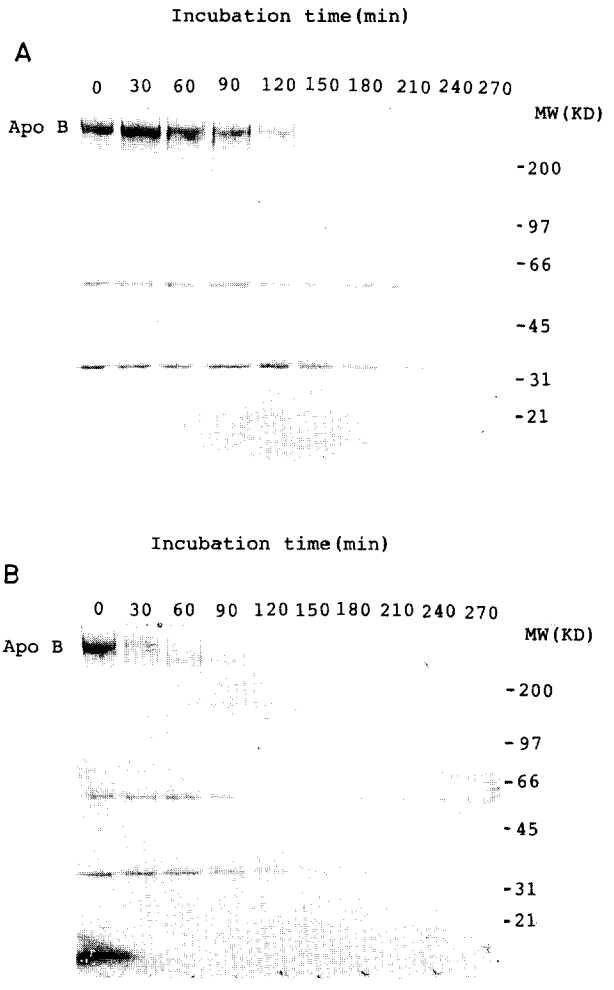

Fig. 3. Cu^{2+} induced apolipoprotein B fragmentation in the presence of 5 μM NOC5. LDL (0.25 mg/ml in PBS-EDTA) solution was incubated with 5 μM $CuSO_4$ at 37°C for 0–300 min in the presence or absence of 5 μM NOC5. After the incubation, 20 μl of 10 mM EDTA was added to 200 μl of reaction mixture. Proteins were recovered by adding 200 μl of 50% trichloroacetic acid solution and washed to lyophilize as described in Section 2. 10 μg of protein was applied to SDS-PAGE (4–12% gradient gel). A: With 5 μM NOC5. B: Without NOC5.

During the time of inhibition of TBARS formation in LDL by NOC5 addition, the fragmentation of apolipoprotein B was also inhibited as shown in Fig. 3A. With the gradual oxidative acceleration over 120 min, the original apolipopro-

tein B band had the tendency to diminish and disappear. In contrast, as shown in Fig. 3B, without NOC5 addition, the fragmentation was apparently accelerated to diminish after 30 min and disappear over 60 min.

Fig. 4 shows the inhibition of Cu^{2+} induced TBARS formation in the presence of 5 μM NOC5; it was prolonged with each successive addition of 5 μM NOC5 (final concentration) every 100 min. Four additions of NOC5 every 100 min inhibited TBARS formation continuously for a total of about 420 min. As shown in Fig. 2, the inhibitory effect of 20 μM NOC5 on Cu^{2+} induced TBARS formation continued for about 180 min. In contrast, the inhibitory time was prolonged about 2.6-fold by four NOC5 additions (corresponding to a total of 20 μM NOC5) every 100 min. The results further confirm that the generation life of NOC5 to release NO was critical to the inhibitory action, because of the short life of released NO from the zwitterionic polyamine/NO adducts used. The inhibitory effect of NOC5 was also obtained in the same manner at any time during the process of burst Cu^{2+} induced TBARS formation in LDL as shown in Fig. 5.

4. Discussion

The purpose of this study was to observe the possible role of NO generated from NO-releasing zwitterions on Cu^{2+} induced LDL oxidation. Depending on the life time of the zwitterions used, the oxidation of LDL measured was inhibited (half-life at 22°C and pH 7.4 in phosphate buffer: NOC7 10.1 min, NOC5 93.0 min) [16]. Separate additions of NOC5 were more effective than the corresponding amount of NOC5 added at once (Figs. 2 and 4) and preincubated NOC5 (3 h or more) was without effect (data not shown). These facts indicate that the zwitterions have an inhibitory effect on LDL oxidation depending on the life time of the NO released. Endothelial cells and macrophages have been reported to accelerate LDL oxidation [3–6]. Various free radicals, such as peroxynitrite anion produced from O_2^- and NO [7–9] or other radicals such as O_2^- or OH^- [22] produced by these cells,

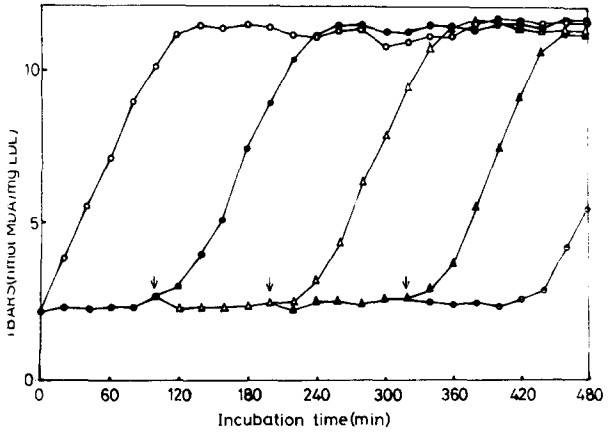

Fig. 4. Effect of successive additions of NOC5 on Cu^{2+} induced TBARS formation in LDL. After 10 ml of LDL (0.25 mg/ml in PBS-EDTA) was incubated with 5 μM $CuSO_4$ in the presence of 5 μM NOC5 for 100 min, a 2.0 ml aliquot was removed. The remaining solution was further incubated with further addition of NOC5 (final concentration 5 μM). The same procedure was repeated 3 times every 100 min (indicated with arrows). TBARS formation was measured as described in Section 2.

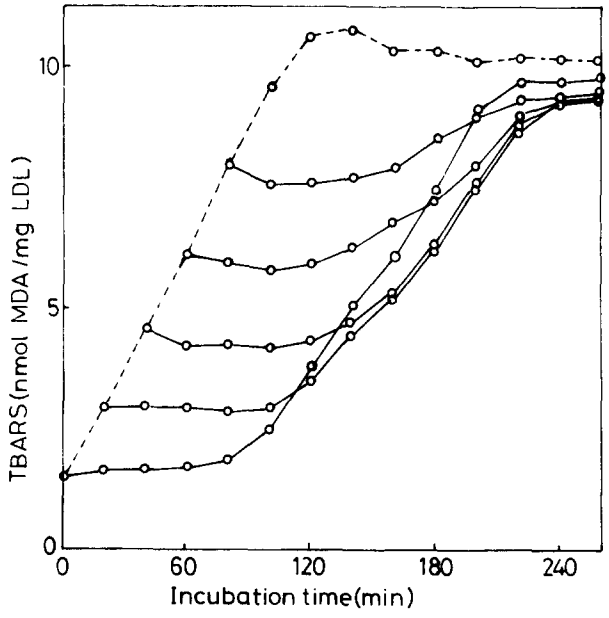

Fig. 5. The inhibitory effect of NOC5 addition on processes of burst Cu^{2+} induced TBARS formation in LDL. After 20 ml of LDL (0.25 mg/ml in PBS-EDTA) solution was incubated with 5 μM $CuSO_4$, a 2.0 ml aliquot was removed and the remaining solution was further incubated to measure TBARS formation. To the removed aliquot, 10 μmol of NOC5 was added (final concentration 5 μM NOC5) and the TBARS formation was further measured as shown. The process was repeated 5 times every 20 min. TBARS formation was measured as described in Section 2.

might be involved as stimulators of LDL oxidation. On the other hand, macrophages stimulated by interferon-γ and tumor necrosis factor-α, which increased NO generation, were reported to depress LDL oxidation [13–15]. The mechanism underlying the suppressive activity of NO was suggested to be as follows [14]. (1) NO affects the activity of a number of heme and iron-sulfur enzymes such as guanylate cyclase, several enzymes of respiratory metabolism and lipoxygenase. (2) NO forms stable chelates with inorganic iron, suggesting that NO secretion could lead to changes in the ability of the culture medium to support lipid oxidation. NO might act in a similar way on intracellular stores of iron and other transition metals.

We used a cell free experimental system and zwitterionic polyamine/NO adducts, which release NO and amine by decomposing by themselves and do not generate any other radicals [16]. Therefore, we need not consider the effect of NO on the above mentioned enzymes. Sufficient amounts of Cu^{2+} (5–15 μM) did not change the inhibitory effect of 5 μM NOC5 (data not shown). The facts indicate that chelating activity of NO with Cu^{2+} is not essential for the inhibitory effect. In our experiments, EDTA was used during the isolation procedure and in the reaction system. However, the participation of contaminating trace amounts of transition metals such as iron is not completely overlooked but may not be essential. As NO is a weak reducing agent [12,23], it has the possibility to react with related free radicals to reduce and inhibit the peroxidative chain reaction. Further studies to clarify the suppressive activity of NO on Cu^{2+} induced lipid peroxidation need to be done.

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