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DEVELOPMENT OF RAPID MEASUREMENT OF OXIDATION OF LOW-DENSITY LIPOPROTEIN AND ITS APPLICATION IN DIABETIC PATIENTS

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RESEARCH ARTICLE

ABSTRACT. WE HAVE DEVELOPED a new procedure for the rapid measurement of the oxidation of low-density lipoprotein (LDL) using 2,2'-azobis-4-methoxy-2,4-dimethylvaleronitorile (V-70) and compared the formation of conjugated diene (CD) mediated by V-70 with that by Cu^{2+}. The LDL for the V-70-mediated oxidation procedure was isolated from fresh plasma by vertical centrifugation and immediately used, while that for the Cu^{2+}–mediated oxidation procedure was obtained after extensive (20 hours) dialysis. The lag times did not differ significantly ($P < 0.02$) between the two procedures, both of which enabled efficient determination of the inhibitory effect of antioxidants on LDL oxidation. Using the V-70-mediated LDL oxidation procedure, the present study showed that the lag time in Type 2 diabetic patients was significantly shorter than control subjects, whereas correlated with fasting plasma glucose and HbA1c levels. Thus, the V-70-mediated LDL oxidation procedure appeared to be simple, rapid, and clinically applicable for measuring the susceptibility of LDL to oxidation.

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1. INTRODUCTION

The atherogenic role of hypercholesterolemia lent numerous clinical trials designed to test the hypothesis that a decrease in cholesterol level leads to a reduction in morbidity and mortality from cardiovascular disease [1]. Most of these studies have demonstrated a decrease in ischemic cardiac events as well as a reduction in the mortality rate due to cardiovascular disease in some studies [2]. It has been suggested that not only the elevation of serum levels of cholesterol and low-density lipoprotein (LDL), but oxidation of LDL is also responsible for the formation of foam cells [3]. The most compelling evidence for the involvement of oxidized lipoproteins in atherogenesis is the findings that atherosclerosis is markedly ameliorated in experimental animals by the antioxidative drugs (such as probucol) [4-7] and vitamins [8] via their inhibition of LDL oxidation. Although there are many antioxidative compounds, including a gemfibrozil metabolite (M1) [9] and a acetylsaltirate metabolite (gentigenic acid) [10], they have not been established on antiatherogenic effect in vivo. Although it is not clear whether oxidized LDL levels directly correlated with atherosclerosis [11-13], there is a relationship between susceptibility of LDL to oxidation and cardiovascular disease [14,15].

Numerous methods have been developed to measure LDL oxidation [16]. For example, measurement of the formation of conjugated diene (CD) is widely used to detect LDL oxidation because its simplicity and sensitivity [17], although this procedure required considerable time to separate and dialyze LDL [18], and was interfered with various factors in these process.

To circumvent such problems, we combined separation of LDL by a vertical rotor technique [19] with detection of CD resulting from 2,2'-azobis-4-methyl-2,4-dimethylvaleronitrile (V-70)-mediated oxidation of LDL [20,21]. In this experiment, we developed V-70-mediated LDL oxidation procedure and studied whether susceptibility of LDL to oxidation is increased in patients with diabetes as previously demonstrated [22,23].

2. MATERIALS, SUBJECTS, AND METHODS

2.1. MATERIALS

2,2'-Azobis-4-methyl-2,4-dimethylvaleronitrile (V-70) was obtained from Wako Pure Chemical Industries (Osaka, Japan). 5-(4-hydroxy-2,5-dimethylphenoxy)-2,2-dimethypentanoic acid (a gemfibrozil metabolite, M1) [9] and probucol [24] were provided by Waner-Lamber K. K. (Tokyo, Japan) and Daiichi Pharmaceutic Co. (Tokyo, Japan), respectively. Other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). V-70 was dissolved in acetonirile to 400 mM and $CuSO_4$ was done in sterile water to 1 mM. M1 and probucol were dissolved in ethanol to 1 mM before use.

2.2. STUDY SUBJECTS

Blood samples were collected from 23 Japanese subjects after an overnight fasting, which included 15 patients (11 men, 4 women, aged from 38 to 79) with Type 2 diabetes without hyperlipidemia, and 8 healthy control subjects (5 men, 3 women, aged from 21 to 52).

2.3. METHODS

The LDL was isolated by centrifugation (65,000 rpm for 90 min at 4ºC) in a vertical rotor (Hitachi RP67-VF) according to the modified method described by Chung et al. [19], and the isolated LDL was used immediately for the V-70-mediated LDL oxidation. The LDL was dialyzed for 20 hrs against phosphate buffered saline (PBS, pH 7.4) after isolation by preparative ultracentrifugation and was then used for the Cu^{2+}-mediated LDL oxidation. Protein concentrations were determined by the method of Lowry et al. [25]. For V-70-mediated LDL oxidation, LDL protein concentrations of 50, 70 and 100 μg/mL and V-70 concentrations of 200 and 400 μM were tested. On the other hand, for Cu^{2+}-mediated LDL oxidation, concentrations of LDL and Cu^{2+} were used at 100 μg/mL and 1.66 μM, respectively [21]. CD formation was monitored at 234 nm for 300 min, with Shimazu UV-160A spectrophotometer

(Kyoto, Japan), as reported earlier [24]. CD curves were constructed according to the absorbance data obtained in the continuous monitoring of the samples and the lag time values were calculated. To evaluate the inhibitory effect of antioxidants on the oxidation of LDL, M1 and probucol were used. FPG, HbA1c, C-peptide immunoreactivity (CPR) in serum and urine, plasma total cholesterol and triglyceride, and M value were measured. Hyperinsulinemic euglycemic clamp technique was performed by DeFronzo et al. [26] as M value was calculated.

2.4. STATISTICAL METHODS

The results were presented as means ± S.D. The differences between the groups were analyzed by using the Student's t-test.

3. RESULTS

Optimal concentrations of LDL and V-70 are shown in FIG. 1. From the lag times, LDL protein (at 70 μg/mL) and V-70 concentration (at 200 μM) were considered the optimal concentrations for the ex vivo studies. On the other hand, it was found that CD curve with a very short lag time could be obtained if the concentrations of LDL and V-70 were increased to 100 μg/mL and 400 μM, respectively. Thus, we used these concentrations of LDL and V-70 to test the effects of antioxidants on the oxidation of LDL.

Measurements of the lag time were shown in FIG. 2. A typical absorbance curve of CD formation consists of an initial and a rapid propagation phase. After the slopes of these two phases were determined, their intersection represents the lag time. For V-70 curve, a LDL protein concentration of 70 μg/mL and a V-70 concentration of 200 μM were used. In the Cu^{2+}-mediated LDL oxidation experiments, concentrations of LDL and Cu^{2+} were 100 μg/mL and 1.66 μM, respectively. As shown in FIG. 2, the slope of the rapid propagation phase of Cu^{2+}–mediated LDL oxidation was steeper than

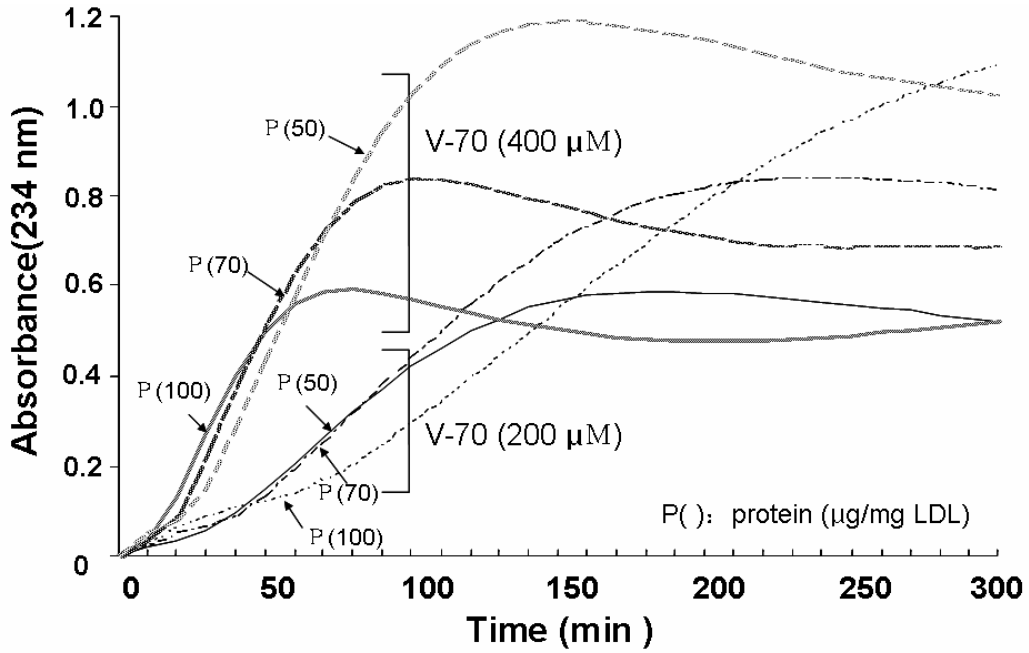

FIGURE 1. EFFECT OF DIFFERENT CONCENTRATIONS OF LDL AND V-70 ON THE V-70- MEDIATED LDL OXIDATION MONITORING BY CONJUGATED DIENE ABSORPTION. The LDL protein concentrations were 50, 70 or 100 μg/mL, and the V-70 concentrations were 200 or 400 μM. The V-70-mediated LDL oxidation was monitored for conjugated diene formation at 234 nm for 300 min.

that of the V-70 curve. However, this did not present a problem for determining the lag time. Using LDL isolated from the blood of the control (non-diabetic) subjects, the V-70- and Cu^{2+}–mediated LDL oxidation procedure yielded a lag time of 68 ± 16.5 min and 66.6 ± 19.9 min, respectively (FIG. 3). A significant positive correlation ($P < 0.002$) was observed between the lag times obtained with the two methods.

To assess the effects of antioxidants on LDL oxidation, V-70- and Cu^{2+}–mediated LDL oxidation were studied. Both M1 (at 2.5, 5.0 and 10 μM) and probucol (at 5 and 10 μM) inhibited dose-dependently the oxidation of LDL with both assay methods (FIG. 4).

As measured by V-70-mediated LDL oxidation in the control and Type 2 diabetic subjects, the lag times from Type 2 diabetic patients were significantly lower ($P < 0.004$) than those from the conrol subjects (FIG. 5). In Type 2 diabetic patients, a negative correlation was observed between values of lag time and those of FPG and HbA1c (FIG. 6a, 6b). However, there is not correlated between values of lag time and those of U-CPR and M (FIG. 6c, 6d). No correlation was also found between values of lag time and those of total cholesterol, triglyceride, S-CPR, % of body fat, or body mass index (data not shown).

4. DISCUSSION

A key initial step in the atherosclerotic process is the conversion of macrophages to foam cells [27-29]. Several forms of modified LDL have shown to initiate the formation of foam cells [30], among which oxidized LDL (ox-LDL) is considered to play a privotal role in the initiation and progression of atherosclerosis [3,11-13]. To evaluate the susuceptability of LDL to oxidation, the formation of CD induced by Cu^{2+} is routinely measured. This procedure is simple, sensitive and specific [16,17], but time-consuming. Furthermore, the reaction is

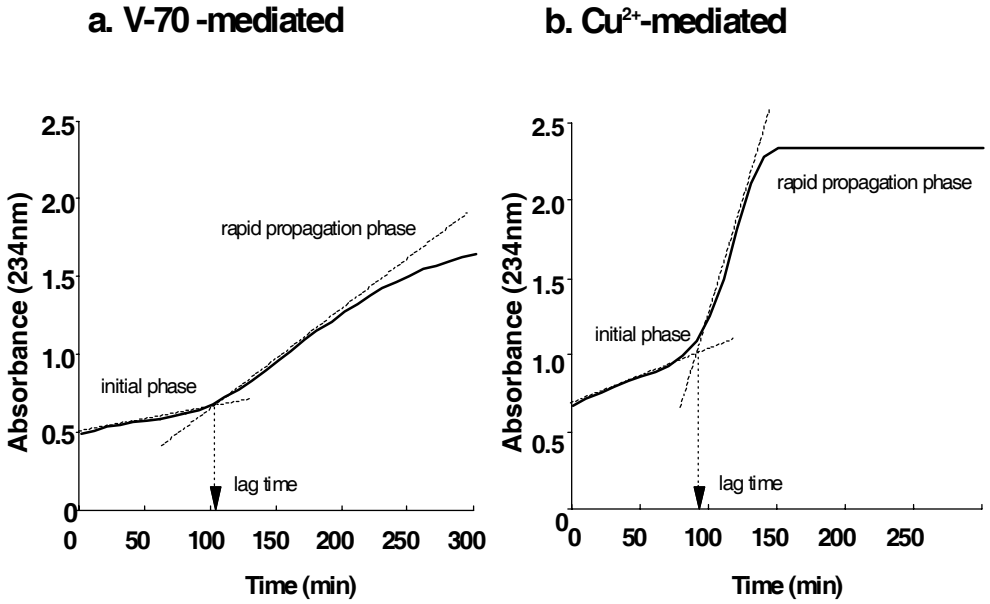

FIGURE 2. DETERMINATION OF THE LAG TIME FROM CURVES FOR THE OXIDATION OF LDL MEDIATED BY V-70 AND Cu^{2+}. For the V-70- mediated LDL oxidation (panel a), LDL protein concentration was 70 μg/mL and V-70 concentration was 200 μM. For Cu^{2+}-mediated LDL oxidation (panel b), the LDL protein concentration was 100 μg/mL and Cu^{2+} concentration was 1.66 μM. Reaction mixtures were monitored at 234 nm for 300 min to detect the formation of conjugated diene. The slopes of the initial and rapid propagation phases were constructed and their intersection point, representing the lag time, was determined.

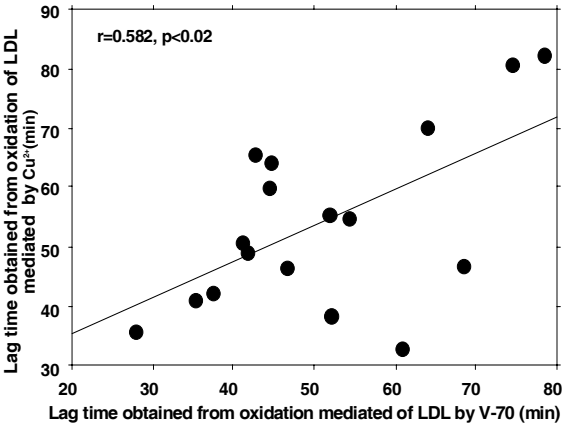

FIGURE 3. RELATIONSHIP BETWEEN LAG TIME FROM V-70- AND Cu^{2+}-MEDIATED LDL OXIDATION. Lag times from the experiments on the V-70- and Cu^{2+}-mediated LDL oxidation were determined as described in FIG. 2. There was a significant positive correlation between these values ($P < 0.02$).

influenced by the process of trace metals [31]. The rate of the chain reaction in the Cu^{2+}–mediated oxidation of methyl linoleate is directly proportional to the concentration of methyl linoleate hydroperoxide [32], with little oxidation being observed when this hydroperoxide is reduced. To circumvent such drawback, a new method to induce LDL oxidation independent of metal ions has been developed which yields azo compounds (diazenes) [33], to generate free radicals at a constant rate and specific site. Since a widely used oxidation-inducer, the hydrophilic compound, 2,2'-azobis-2-amidinopropane dihydrochrolide (AAPH), as well as the lipophilic azo compound, 2,2'-azobis-2,4-dimethylveleronitrile, absorbs strongly at 234 nm, they would interfere with the detection of CD [34]. It has been reported that V-70 induced oxidation of LDL without interference with CD absorbance at 234 nm. It has been suggested that Cu^{2+} binds to

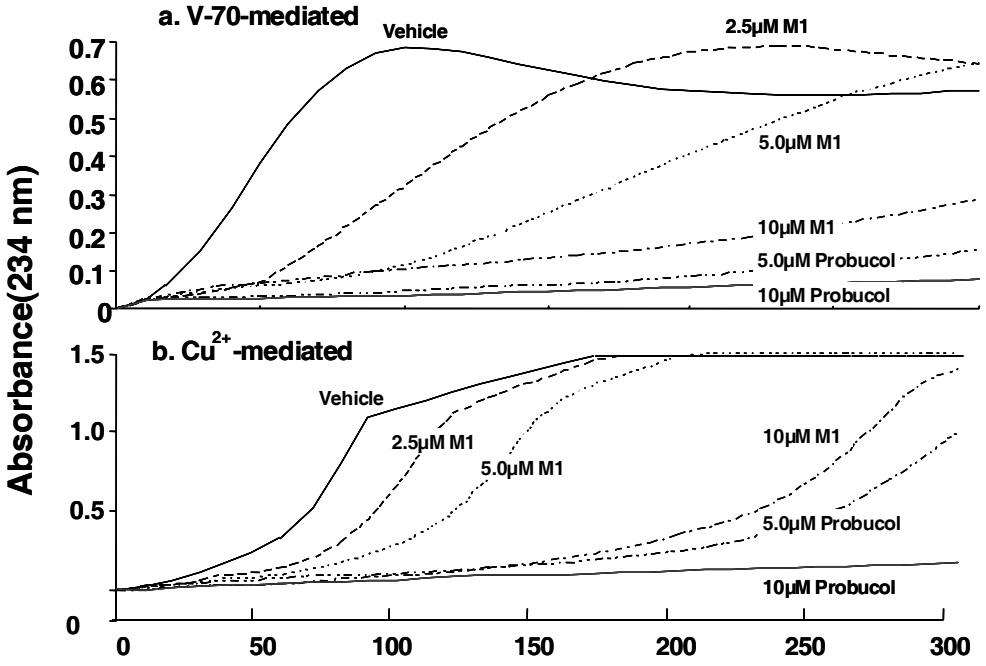

FIGURE 4. EFFECT OF ANTIOXIDANTS ON V-70- AND Cu^{2+}-MEDIATED LDL OXIDATION. For the V-70-mediated LDL oxidation (panel a), LDL protein concentration of 100 μg/ml and Cu^{2+} concentration of 1.66 μM were used. Reactions were monitored as described in FIG. 2, except that M1 (2.5, 5.0 or 10 μM, final concentrations) or probucol (5 or 10 μM, final concentrations) was added to the reaction mixture.

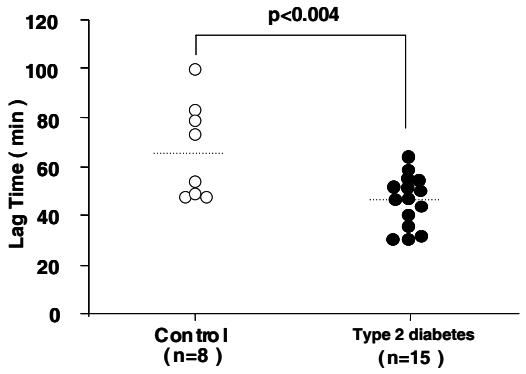

FIGURE 5. CORRELATION OF LAG TIMES OBTAINED IN TYPE 2 DIABETIC PATIENTS VS. CONTROL SUBJECTS. Lag times from V-70-mediated LDL oxidation were determined in the Type 2 diabetic patients and the control subjects as described in FIG. 2. The dotted line represents the mean. There was a highly significant difference in lag times between the two groups ($P < 0.04$).

LDL and breaks down lipid hydroperoxides by a Haber-Weiss reaction to yield peroxyl and alkoxyl radicals at or near the LDL surface [35], which both V-70 and AAPH generate free radicals at different sites to induce a similar oxidation of LDL [36].

The LDL used in the Cu^{2+}-mediated LDL oxidation procedure requires extensive and time-consuming dialysis before its use. In the present V-70-mediated LDL oxidation procedure, LDL was ready for use immediately after separation by ultracentrifugation. Under the optimal conditions for V-70-mediated LDL oxidation in terms of the concentrations of LDL and V-70, the V-70-mediated LDL oxidation procedure gave similar lag times to those of the Cu^{2+}-mediated LDL oxidation procedure. Using both procedures, we would assess the inhibitory effects of antioxidants (probucol,

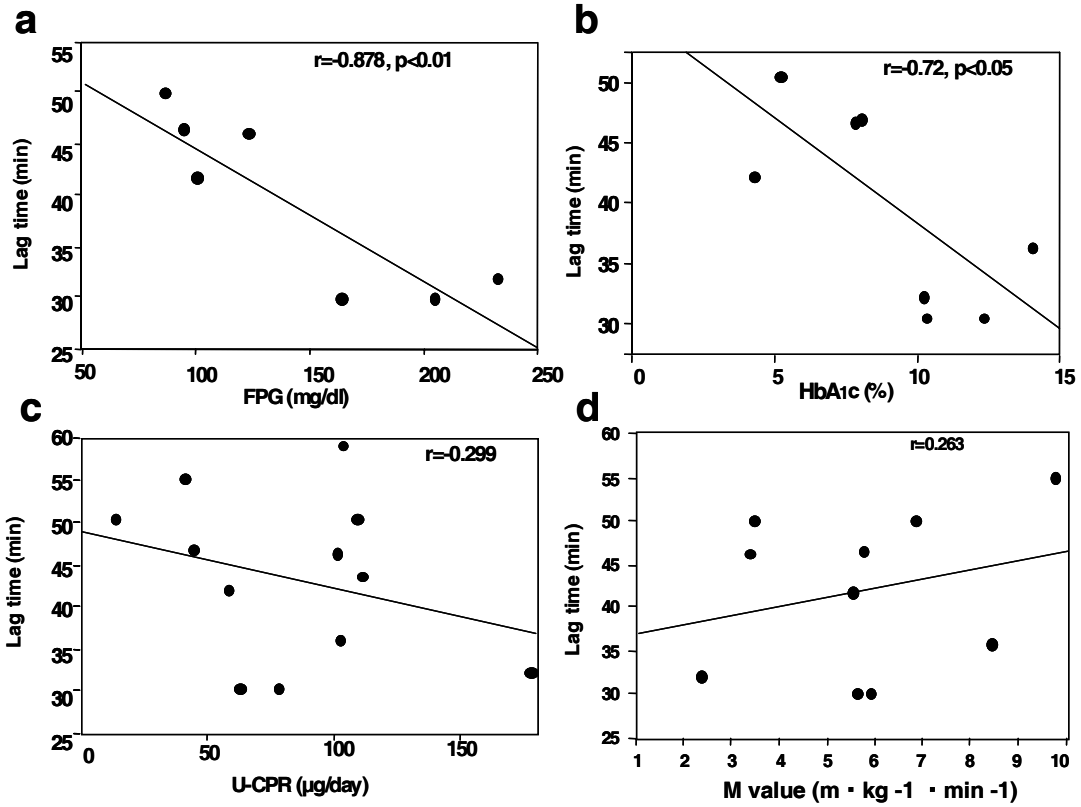

FIGURE 6. CORRELATION OF LAG TIMES WITH PARAMETERS IN TYPE 2 DIABETIC PATIENTS. Lag times showed a negative correlation with FPG (panel a), HbA1c levels (panel b), but no correlation with values for U-CPR (panel c) or M value (panel d).

M1) on LDL oxidation by prolongation of lag times. This inhibition of LDL oxidation was in dose-dependent manner for both M1 and probucol (FIG. 4). In particular, 10 μM probucol completely eliminated the oxidation of LDL in each procedure. Thus, our study demonstrated that the V-70- and Cu^{2+}-mediated LDL oxidation procedures yield almost identical results.

Previous studies have shown the susceptibility of LDL to oxidation is increased in diabetic patients [22,23]. It has been shown that glucose enhances the lipid peroxidation of LDL [37], and glycosylated LDL is more oxidized readily than nonglycosylated LDL [38]. Using the V-70-mediated LDL oxidation procedure, we confirmed that patients with Type 2 diabetes had siginificantly shorter lag times than healthy volunteers. These data suggest that the LDL from the Type 2 diabetic patients was more susceptibility to oxidation, a finding consistent with a previous report [22]. The present results that the lag times obtained from the V-70-mediated LDL oxidation procedure was negatively correlated with serum levels of FPG and HbA1c, which was also in agreement with previous reports [37,38]. However, the present result of no correlation between the lag times and S-CPR, U-CPR or M values conflicts with previous reports showing that insulin resistance [39,40] and/or hyperinsulinemia may enhance the lipid peroxidation of LDL [41].

In conclusion, the present study showed that the lag times obtained from both V-70- and Cu^{2+}-mediated LDL oxidation were about similar, and the time required to measure LDL oxidation by CD formation of V-70-mediated LDL oxidation was reduced by half of that of Cu^{2+}-mediated LDL oxidation. Thus, the present new method is simpler and faster than the Cu^{2+}- mediated LDL oxidation technique. Using this method, we showed that patients with Type 2 diabetes exhibited shorter lag times than the control subjects, which suggests increased susceptibility of their LDL to oxidation in diabetes negatively correlated with the FPG and HbA1c.

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