Low Concentration of Oxidized Low Density Lipoprotein Suppresses Platelet Reactivity *in vitro*: An Intracellular Study

Duen-Suey Chou[a,b], George Hsiao[b], Ming-Yi Shen[a], Tsorng-Harn Fong[a], Chien-Huang Lin[a], Tzeng-Fu Chen[b], and Joen-Rong Sheu[a,b,*]

Graduate Institutes of [a]Medical Sciences and [b]Pharmacology, Taipei Medical University, Taipei 110, Taiwan

ABSTRACT: The intracellular mechanisms underlying oxidized low density lipoprotein (oxLDL)-signaling pathways in platelets remain obscure and findings have been controversial. Therefore, we examined the influence of oxLDL in washed human platelets. In this study, oxLDL concentration-dependently (20–100 μg/mL) inhibited platelet aggregation in human platelets stimulated by collagen (1 μg/mL) and arachidonic acid (60 μM), but not by thrombin (0.02 U/mL). The activity of oxLDL was greater at 24 h in inhibiting platelet aggregation than at 12 h. At 24 h, oxLDL concentration-dependently inhibited intracellular Ca^{2+} mobilization and thromboxane B_2 formation in human platelets stimulated by collagen. In addition, at 24 h oxLDL (40 and 80 μg/mL) significantly increased the formation of cyclic AMP, but not cyclic GMP or nitrate. In an ESR study, 24 h-oxLDL (40 μg/mL) markedly reduced the ESR signal intensity of hydroxyl radicals ($OH^{\cdot-}$) in both collagen (2 μg/mL)-activated platelets and Fenton reaction (H_2O_2 + Fe^{2+}). The inhibitory effect of oxLDL may induce radical–radical termination reactions by oxLDL-derived lipid radical interactions with free radicals (such as hydroxyl radicals) released from activated platelets, with a resultant lowering of intracellular Ca^{2+} mobilization, followed by inhibition of thromboxane A_2 formation, thereby leading to increased cyclic AMP formation and finally inhibited platelet aggregation. This study provides new insights concerning the effect of oxLDL in platelet aggregation.

Paper no. L9421 in *Lipids 39,* 433–440 (May 2004).

The oxidation theory of atherosclerosis proposes that formation of oxidized LDL (oxLDL) in the subendothelial space of artery walls represents a causative event for atherogenesis. Evidence from oxidized lipoproteins has been detected in atherosclerotic lesions (1), and oxLDL exhibits various proatherogenic activities (2). Macrophage cholesterol accumulation and foam cell formation is a hallmark of atherogenesis (3). Platelets interact with plasma lipoprotein as well as with arterial wall macrophages that play an important role in atherogenesis (4). Plasma lipoproteins have been shown to affect platelet activity *in vitro* and *in vivo*. The susceptibility of platelets to aggregation *in vitro* on stimulation by aggregating agents, such as ADP and collagen, has been shown to increase in most, but not all, studies of platelet-rich plasma (PRP) from hypercholesterolemic individuals (5).

Oxidation of LDL may be one of the main factors involved in the initial development of atherosclerotic lesions (1). LDL can be oxidized within the arterial wall by macrophages; this process, which favors the accumulation of LDL in the intima of the arterial wall, is thought to be a possible explanation for the progression of arteriosclerotic lesions. Endothelial cells also induce oxLDL formation, but in turn may be damaged by oxLDL (1). LDL has been suggested to have platelet-activating properties such as decreasing the threshold for stimulation by aggregation agents and inducing platelet aggregation (6,7). However, evidence regarding the induction of platelet activation by LDL is controversial. Several studies have reported the inhibition of agonist-induced platelet aggregation by native LDL (nLDL) (8,9). In others, platelet-activating effects of either lower nLDL (10–50 μg/mL) (10) or higher concentrations of LDL (more than 1–2 mg/mL) have been described (7). Furthermore, platelet-activating activity was found to reside in oxLDL rather than in nLDL (11). oxLDL plays an important role in the pathogenesis of atherosclerosis; it also has been reported to enhance platelet activation in some (6,12), but not all, studies (13,14). Vlasova *et al.* (14) found that highly oxidized LDL not only failed to activate platelet aggregation but also inhibited ADP-induced aggregation. However, they also found that mildly oxidized LDL diminished the time-dependent decrease in platelet aggregability in PRP (15). Such discrepancies among reported oxLDL–platelet interactions may depend on methodological variations in the isolation, oxidization, and dosage of lipoproteins employed, which might result in nonhomogeneous oxidation (8).

In this study, we found that oxLDL (LDL oxidized with copper), at lower concentrations (40–80 μg/mL), significantly inhibited agonist-induced platelet aggregation. However, the detailed intracellular mechanisms underlying oxLDL–platelet interaction still remain obscure. We therefore systemically examined the influence of oxLDL in washed human platelets and utilized the findings to characterize the mechanisms involved in this influence.

MATERIALS AND METHODS

Materials. Collagen (Type I, bovine achilles tendon), arachidonic acid (AA), sodium citrate, luciferin-luciferase,

*To whom correspondence should be addressed at Graduate Institute of Medical Sciences, Taipei Medical University, No. 250, Wu-Hsing St., Taipei 110, Taiwan. E-mail: sheujr@tmu.edu.tw

Abbreviations: 12h-oxLDL, 12-h oxidized LDL; 24h-oxLDL, 24-h oxidized LDL; AA, arachidonic acid; $[Ca^{2+}]i$, concentration of inorganic calcium ion; DEPMPO, 5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide; DTS, dense tubular system; EIA, enzyme immunoassay; nLDL, native LDL; oxLDL, oxidized LDL; PGE_1, prostaglandin E_1; PLA_2, phospholipase A_2; PRP, platelet-rich plasma; TxA_2 and TxB_2, thromboxanes A_2 and B_2.

indomethacin, prostaglandin E_1 (PGE_1), nitroglycerin, apyrase, catalase, thrombin, BSA, and heparin were purchased from Sigma Chemical (St. Louis, MO). Fura 2-AM was purchased from Molecular Probe (Eugene, OR). DEPMPO (5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide) was purchased from OXIS (Portland, OR). Cyclic AMP, cyclic GMP, and thromboxane B_2 (TxB_2) enzyme immunoassay (EIA) kits were purchased from Cayman (Ann Arbor, MI).

Preparation of human platelet suspensions. Human platelet suspensions were prepared as previously described (16). In this study, human volunteers gave informed consent. In brief, blood was collected from healthy human volunteers who had taken no medicine during the preceding 2 wk and then mixed with an acid/citrate/glucose anticoagulant (9:1 blood/anticoagulant, vol/vol). After centrifugation at $120 \times g$ for 10 min at room temperature, the supernatant (PRP) was supplemented with PGE_1 (0.5 μM) and heparin (6.4 IU/mL), then incubated for 10 min at 30°C, and centrifuged at $500 \times g$ for 10 min. The washed platelets were finally suspended in Tyrode's solution containing BSA (3.5 mg/mL) and adjusted to a concentration of 4.5×10^8 platelets/mL. The final concentration of Ca^{2+} in Tyrode's solution was 1 mM.

Isolation of human plasma LDL. Human LDL (at a density of 1.109–1.063) was isolated from fresh plasma of fasted normolipidemic human volunteers collected in EDTA (2 mM) by sequential density gradient ultracentrifugation as described by Dousset *et al.* (17). In brief, plasma density was adjusted to 1.02 g/mL using a NaCl/KBr solution for immediate separation of VLDL and intermediate density lipoproteins, and then adjusted to 1.063 g/mL for the separation of LDL. The LDL was further purified by resuspension in the appropriate NaCl/KBr solution (1.063 g/mL) and ultracentrifuged. The LDL was then dialyzed for 24 h at 4°C against three changes of nitrogen-gassed 0.02 M Tris buffer to remove the EDTA and KBr. After dialysis, LDL was stored for not more than 7 d under nitrogen at 4°C in the dark. Total protein concentration was measured by the Lowry method using BSA as the standard (18).

Oxidation of LDL. After adjustment of the LDL concentration to 0.5 mg/mL (expressed as total LDL concentration), lipoprotein preparations were dialyzed against 100 vol of 10 mM sodium phosphate buffer (containing 150 mM NaCl, pH 7.4) for 18 h, at 4°C in the dark. Oxidation was initiated by addition of $CuSO_4$ (5 μM) for 12 and 24 h at 37°C, respectively, and was stopped after the addition of EDTA (20 μM). Before the functional studies, oxLDL was filtered through a Sephadex PD-10 column (Pharmacia, Uppsala, Sweden) to remove EDTA and copper and was reconcentrated at 4°C using Centricon 3000 filters (Amicon, Bedford, MA) according to the manufacturer's instructions. Oxidation was confirmed by the TBARS assay (19). Tetramethoxypropane was used as a standard, and the results were expressed as nanomoles of malondialdehyde equivalents per milligram protein of LDL. The extent of aldehyde-modified lysine in oxidized LDL (adjusted to 0.1 mg LDL protein/mL) was monitored by determining the fluorescence intensity (excitation at 350 nm, emission at 420 nm) (20). The 12-h (12h-oxLDL) and 24-h (24h-oxLDL) lipoproteins were used in all of the functional studies within 48 h of preparation.

Platelet aggregation. The turbidimetric method was applied to measure platelet aggregation (19), using a Lumi-Aggregometer (Payton, Ontario, Canada). Platelet suspensions (4.5×10^8 platelets/mL, 0.4 mL) were prewarmed to 37°C for 2 min (stirring at 1200 rpm) in a silicone-treated glass cuvette. oxLDL (20–100 μg/mL) was added 3 min before the addition of platelet-aggregation inducers. The reaction was allowed to proceed for at least 6 min, and the extent of aggregation was expressed in light-transmission units. When measuring ATP release, 20 μL of the luciferin/luciferase mixture was added 1 min before the addition of the agonists, and ATP release was compared with that of the control.

Measurement of platelet [Ca^{2+}]i mobilization by Fura 2-AM fluorescence. Citrated whole blood was centrifuged at $120 \times g$ for 10 min. The supernatant was protected from light and incubated with Fura 2-AM (5 μM) at 37°C for 1 h. Human platelets were then prepared as described above. Finally, the external Ca^{2+} concentration of the platelet suspensions was adjusted to 1 mM. The rise in [Ca^{2+}]i was measured using a fluorescence spectrophotometer (CAF 110; JASCO, Tokyo, Japan) at excitation wavelengths of 340 and 380 nm, and an emission wavelength of 500 nm. [Ca^{2+}]i was calculated from the fluorescence, using 224 nM as the Ca^{2+}-Fura 2 dissociation constant (21).

Measurement of TxB_2 formation. Washed human platelet suspensions (4.5×10^8/mL) were preincubated for 3 min in the absence or presence of oxLDL (40 and 80 μg/mL) before the addition of collagen (1 μg/mL). Six minutes after the addition of the agonist, 2 mM EDTA and 50 μM indomethacin were added to the reaction suspensions. The vials were then centrifuged for 3 min at $15{,}000 \times g$. The TxB_2 levels of the supernatants were measured using an EIA kit (Cayman) according to the instructions of the manufacturer.

Estimation of platelet cyclic AMP and cyclic GMP formations. The method of Karniguian *et al.* (22) was followed. In brief, platelet suspensions were warmed to 37°C for 1 min, then either PGE_1 (10 μM), nitroglycerin (10 μM), or oxLDL (40 and 80 μg/mL) was added and incubated for 6 min. The incubation was stopped, and the solution was immediately boiled for 5 min. After cooling to 4°C, the precipitated protein was collected as sediment after centrifugation. Fifty microliters of supernatant was used to determine the cyclic AMP and cyclic GMP contents by EIA kits (Cayman) following acetylation of the samples as described by the manufacturer.

Estimation of nitrate in human platelet suspensions. Platelet suspensions (1×10^9/mL) were preincubated with collagen (1 μg/mL) or oxLDL (40 and 80 μg/mL) for 6 min followed by centrifugation ($17{,}500 \times g$) for 5 min. The supernatants were deproteinized by incubation with 95% ethanol at 4°C for 30 min. Samples were then centrifuged for a further 7 min at $15{,}000 \times g$. It should be noted that the nitrate concentrations in the platelet suspensions reported in this study actu-

ally represent the total of both nitrite and nitrate concentrations in the platelet suspensions. The amount of nitrate in the platelet suspensions (10 μL) was measured by adding a reducing agent (0.8% VCl_3 in 1 M HCl) to the purge vessel to convert nitrate to NO, which was stripped from the platelet suspensions by a helium purge gas. The NO was then drawn into a Sievers Nitric Oxide Analyzer (280 NOA; Sievers Instruments, Boulder, CO). Nitrate concentrations were calculated by comparison with standard solutions of sodium nitrate.

Measurement of free radicals in platelet suspensions by ESR spectrometry. The ESR method used a Bruker EMX ESR spectrometer as described previously (23). In brief, platelet suspensions (4.5×10^8 platelets/mL, 0.4 mL) were prewarmed to 37°C for 2 min, and then oxLDL (40 μg/mL) or catalase (1000 U/mL) was added 3 min before the addition of collagen (2 μg/mL). The reaction was allowed to proceed for 1 min, followed by the addition of 100 mM DEPMPO for the ESR study. In addition, Fe^{2+} (1 μM) was preincubated with H_2O_2 (10 μM) in Tyrode's solution, followed by the addition of 24h-oxLDL (40 and 80 μg/mL) and further incubated with 50 mM DEPMPO for the Fenton reaction. ESR spectra were recorded on a Bruker EMX ESR spectrometer using a flat quartz cell designed for aqueous solutions. Conditions of ESR spectrometry were as follows: 20 mW power at 9.78 GHz, 1 G modulation, and 100 G scanning for 42 s, with 10 scans accumulated.

Statistical analysis. The experimental results are expressed as the means ± SEM and are accompanied by the number of observations. Data were assessed using ANOVA. If this analysis indicated significant differences among the group means, then each group was compared using the Newman–Keuls method. A P value <0.05 was considered statistically significant.

RESULTS

Effect of oxLDL on platelet aggregation in human platelet suspensions. The 12h- and 24h-oxLDL preparations were oxidized under identical conditions. Under these conditions, the concentrations of 12h- and 24h-oxLDL after incubation were 50.3 ± 0.6 and 51.4 ± 1.8 nmol of MDA/mg protein, respectively. The extents of aldehyde-modified lysine in 12h- and 24h-oxLDL were about 11.0 ± 1.1 and 13.1 ± 1.4, respectively. In this study, we found that the 12h- and 24h-oxLDL, but not nLDL, concentration-dependently (20–100 μg/mL) inhibited platelet aggregation stimulated by collagen (1 μg/mL) (Figs. 1, 2) and AA (60 μM) (Fig. 3) in human platelet suspensions. Furthermore, both types of oxLDL inhibited the ATP-release reaction when stimulated by agonists (i.e., collagen) (Fig. 1). The IC_{50} values of the 12h- and 24h-oxLDL for platelet aggregation induced by collagen were estimated to be about 46.3 ± 1.1 and 55.2 ± 1.0 μg/mL, respectively (Fig. 2). The 24h-oxLDL exhibited more potent activity than 12h-oxLDL in inhibiting platelet aggregation stimulated by agonists. On the other hand, neither type of oxLDL significantly inhibited thrombin (60 μM)-induced platelet aggregation (Fig. 3). Additionally, neither 12h- nor

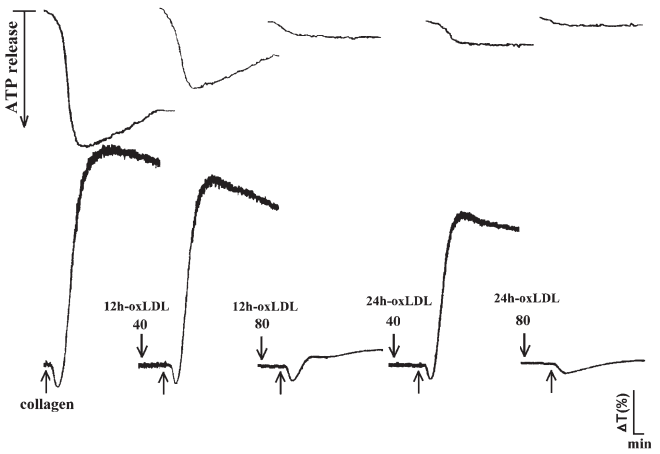

FIG. 1. Tracing curves of oxidized LDL (oxLDL) on collagen (1 μg/mL)-induced platelet aggregation in washed human platelets. Platelets were preincubated with 12h- and 24h-oxLDL (40 and 80 μg/mL) for 3 min. Collagen was then added to trigger aggregation (lower tracings) and ATP release (upper tracings). Profiles are representative examples of five similar experiments. ΔT(%), percent change in transmission.

24h-oxLDL significantly induced spontaneous platelet aggregation in the absence of agonists (data not shown). In the following studies, we used 24h-oxLDL as a tool to further explore the mechanisms of oxLDL in platelet aggregation.

Effect of 24h-oxLDL on [Ca^{2+}]i mobilization. Free cytoplasmic Ca^{2+} concentrations in human platelets were measured by the Fura 2-AM loading method. As shown in Figure 4, collagen (1 μg/mL) evoked a marked increase in [Ca^{2+}]i mobilization. 24h-oxLDL (40 and 80 μg/mL) concentration-dependently inhibited the collagen-evoked increase in [Ca^{2+}]i by about 91 and 96%, respectively. This suggests that oxLDL exerts an inhibitory effect on [Ca^{2+}]i mobilization in human platelets stimulated by collagen.

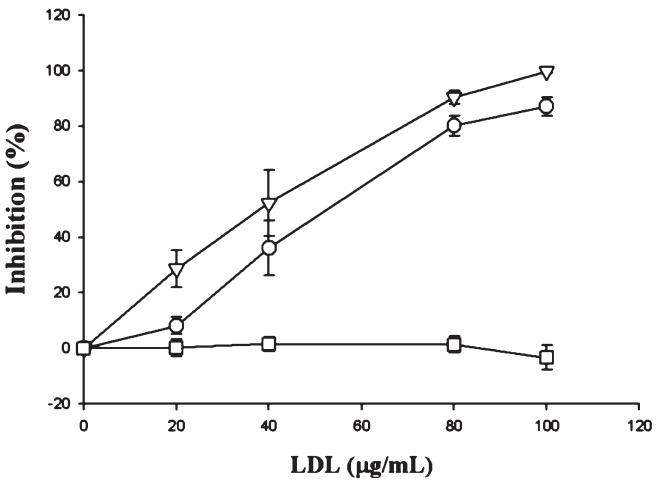

FIG. 2. Concentration-inhibition curves of LDL on collagen (1 μg/mL)-induced platelet aggregation in washed human platelets. Platelets were preincubated with various concentrations (20–100 μg/mL) of 12h-oxLDL (○), 24h-oxLDL (▽), and native LDL (nLDL) (□) for 3 min, followed by the addition of collagen to trigger platelet aggregation. Data are presented as a percentage of the control (means ± SEM, n = 5). For other abbreviation see Figure 1.

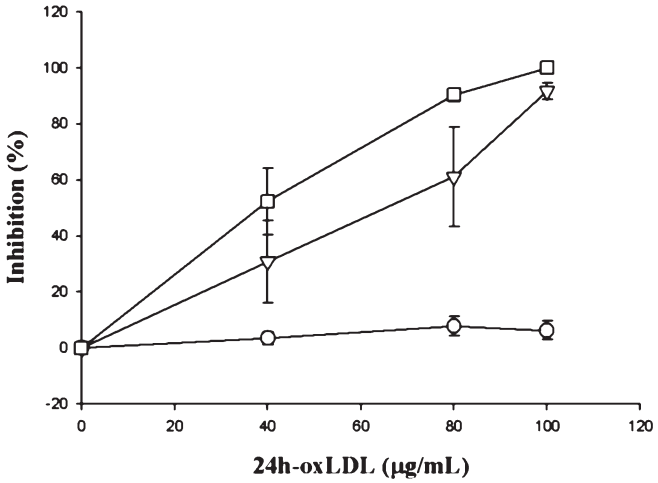

FIG. 3. Concentration-inhibition curves of 24h-oxLDL on thrombin (0.02 U/mL, ○)-, arachidonic acid (60 μM, ▽)-, and collagen (1 μg/mL, □)-induced platelet aggregation in washed human platelets. Platelets were preincubated with 24h-oxLDL (40–100 μg/mL) for 3 min. Agonists were then added to trigger platelet aggregation. Data are presented as a percentage of the control (means ± SEM, $n = 5$).

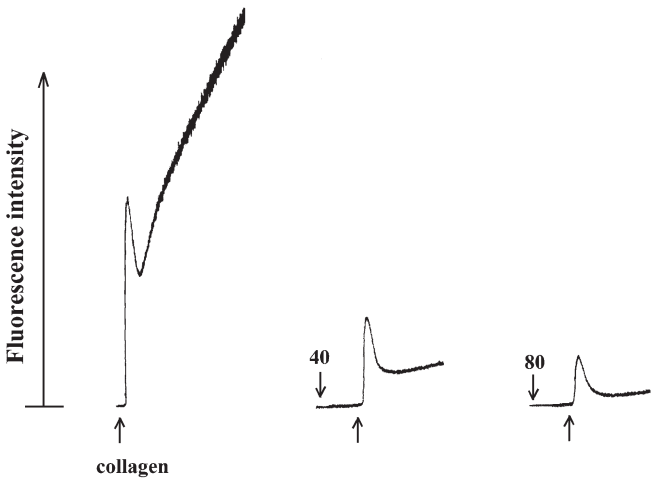

FIG. 4. Effect of 24h-oxLDL on collagen-induced intracellular Ca^{2+} mobilization in Fura 2-AM-loaded human platelets. Platelet suspensions were preincubated with Fura 2-AM (5 μM), followed by the addition of collagen (1 μg/mL) in the absence or presence of 24h-oxLDL (40 and 80 μg/mL), which was added 3 min prior to the addition of collagen. Profiles are representative examples of four similar experiments. For abbreviation see Figure 1.

Effect of 24h-oxLDL on TxB_2 formation. As shown in Table 1, resting platelets produced relatively little TxB_2 compared with collagen-activated platelets. PGE_1 (10 μM) inhibited TxB_2 formation in collagen-activated platelets by 82% (data not shown). Furthermore, results obtained using various concentrations of 24h-oxLDL indicated that 24h-oxLDL markedly inhibited TxB_2 formations in platelets stimulated by collagen (1 μg/mL). At 80 μg/mL, 24h-oxLDL almost completely inhibited TxB_2 formation (Table 1). These results suggest that oxLDL exerts an inhibitory effect on TxA_2 formation.

TABLE 1
Effect of Oxidized LDL (oxLDL) on Thromboxane B_2 Formation in Washed Human Platelets[a]

	Concentration	Thromboxane B_2[b] (ng/mL)
Resting		10.8 ± 5.3
Collagen (μg/mL)	1	80.5 ± 17.2**
24h-oxLDL (μg/mL)	40	47.9 ± 11.2#
	80	9.5 ± 3.0##

[a]Platelet suspensions were preincubated with 24h-oxLDL (40 and 80 μg/mL) for 3 min at 37°C and then collagen (1 μg/mL) was added to trigger thromboxane B_2 formation.
[b]Data are presented as the means ± SEM ($n = 4$). **$P < 0.01$ as compared with the resting group; #$P < 0.05$ and ##$P < 0.01$ as compared with the collagen group.

Effect of 24h-oxLDL on cyclic AMP, cyclic GMP, and nitrate formations in washed human platelets. The level of cyclic AMP in unstimulated platelets was low (2.1 ± 0.5 pmol/10^9 platelets, $n = 4$). Addition of PGE_1 (10 μM) increased the cyclic AMP level to 18.9 ± 3.9 pmol/10^9 platelets ($n = 4$). When platelet suspensions were preincubated with various concentrations of 24h-oxLDL (40 and 80 μg/mL) for 3 min at 37°C, we found that 24h-oxLDL increased cyclic AMP levels to 4.9 ± 0.2 and 5.4 ± 0.8 pmol/10^9 platelets, respectively (Table 2). We also performed similar studies measuring the cyclic GMP response. The level of cyclic GMP in unstimulated platelets was very low, but when nitroglycerin (10 μM) was added to the platelet suspensions, the cyclic GMP level increased from the resting level to 3.6 ± 0.4 pmol/10^9 platelet (Table 2). However, addition of 24h-oxLDL (40 and 80 μg/mL) resulted in no significant increase in platelet cyclic GMP levels (0.9 ± 0.2 and 0.8 ± 0.1 pmol/10^9 platelets, $n = 4$).

On the other hand, NO was quantified using a sensitive and specific ozone redox–chemiluminescence detector. As shown in Table 2, collagen (1 μg/mL) caused about a 2.6-fold rise in nitrate formation, compared with that in resting platelets. In the presence of 24h-oxLDL (40 and 80 μg/mL), nitrate production did not significantly increase after incubation with platelets for 6 min (Table 2). Furthermore, nitrate production did not increase even after prolongation of the incubation time to 30 min (data not shown). These results imply that the antiplatelet activity of oxLDL may act partially through stimulation of cyclic AMP formation in human platelets.

Free radical-scavenging activity of 24h-oxLDL in collagen-activated platelets. The rate of free radical-scavenging activity is defined by the following equation: inhibition rate = 1 – signal height (24h-oxLDL)/signal height (control) (20). In this study, a typical ESR signal of the hydroxyl radical (OH^-) was induced by collagen (2 μg/mL) in human platelets (Fig. 5B). 24h-oxLDL (40 μg/mL) and catalase (1000 U/mL) suppressed hydroxyl radical formation by about 43 and 76% ($n = 4$), respectively (Figs. 5C, 5D). On the other hand, 24h-oxLDL (40 and 80 μg/mL) also concentration-dependently inhibited the nonenzymatic hydroxyl radical generation in the Fenton reaction ($H_2O_2 + Fe^{2+}$) by about 71 and 91% (Figs. 5F, 5G) compared with the control group (Fig. 5E). This observation provides *in vitro* evidence of the free radical-scavenging activity of oxLDL in both activated platelets and the Fenton reaction.

TABLE 2
Effect of 24-h Oxidized LDL (24h-oxLDL) on Cyclic AMP, Cyclic GMP, and Nitrate Formation in Washed Human Platelets[a]

	Concentration	Cyclic AMP[b] (pmol/10^9 platelets)	Cyclic GMP[b] (pmol/10^9 platelets)	Nitrate[b] (μM)
Resting		2.1 ± 0.5	0.6 ± 0.1	3.2 ± 0.6
PGE_1 (μM)	10	18.9 ± 3.9**	—	—
NTG (μM)	10	—	3.6 ± 0.4***	—
Collagen (μg/mL)	2	—	—	8.3 ± 1.1**
24h-oxLDL (μg/mL)	40	4.9 ± 0.2**	0.9 ± 0.2	4.3 ± 0.2
	80	5.4 ± 0.8*	0.8 ± 0.1	4.9 ± 0.7

[a]Platelet suspensions were preincubated with 24h-oxLDL (40 and 80 μg/mL) at 37°C. Addition of prostaglandin E_1 (PGE_1), nitroglycerin (NTG), and collagen in platelet suspensions served as positive controls of cyclic AMP, cyclic GMP, and nitrate, respectively.
[b]Data are presented as the means ± SEM (n = 4). *P < 0.05, **P < 0.01, and ***P < 0.001 as compared with the resting groups.

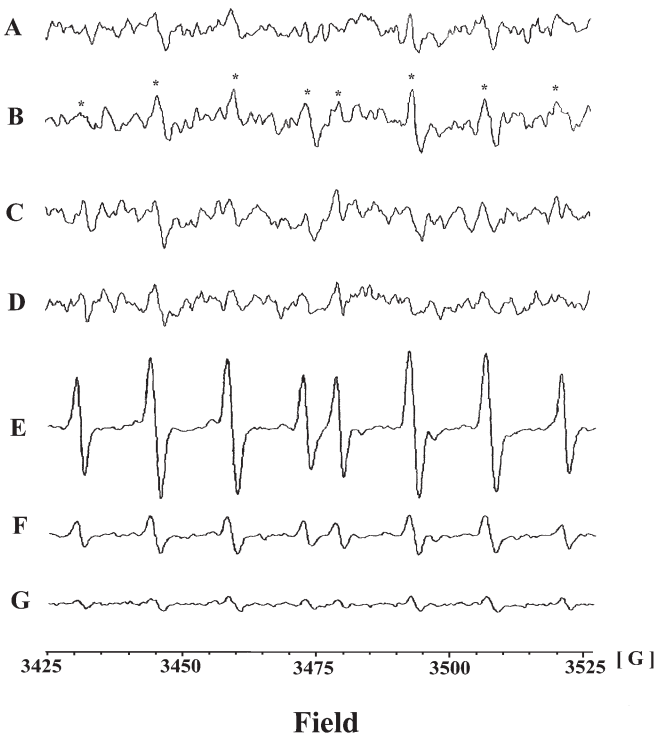

FIG. 5. ESR spectra of 24h-oxLDL in the inhibition of hydroxyl radical ($OH^{\cdot}$) formation in collagen-activated platelets and Fenton reaction. Platelet suspensions (4.5×10^8 platelets/mL, 0.4 mL) were preincubated with (A) PBS without activation (resting) or with (B) PBS, (C) 24h-oxLDL (40 μg/mL), and (D) catalase (1000 U/mL) for 3 min; then collagen (2 μg/mL) was added to trigger platelet aggregation as described in the Materials and Methods section. Furthermore, hydroxyl radical was generated in the Fenton reaction (H_2O_2 + Fe^{2+}) incubation with (E) PBS, (F) 24h-oxLDL (40 μg/mL), and (G) 24h-oxLDL (80 μg/mL) for 3 min, followed by the addition of 50 mM DEPMPO (5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide) for ESR. The reaction was allowed to proceed for 5 min, followed by the addition of DEPMPO (100 mM) for ESR experiments. The spectrum is a representative example of four similar experiments.

DISCUSSION

The principal objective of this study was to describe the intracellular mechanisms involved in the inhibition of agonist-induced human platelet aggregation by oxLDL. This inhibitory effect of oxLDL was demonstrable with the use of the agonists collagen and AA but not with thrombin. The inhibition was directly proportional to the concentrations of oxLDL used. In this study, both platelet aggregation and the ATP-release reaction induced by agonists (i.e., collagen) appeared to be affected by the presence of oxLDL. Therefore, this implies that oxLDL may partially affect Ca^{2+} release from intracellular Ca^{2+} storage sites [i.e., the dense tubular system (DTS) or dense bodies] (Fig. 4), and this is in accord with the concept that intracellular Ca^{2+} release is responsible for the ATP-release reaction (21).

TxA_2 is an important mediator of the release reaction and aggregation of platelets (Fig. 6) (24). Collagen-induced formation of TxB_2, a stable metabolite of TxA_2, was concentration-dependently inhibited by oxLDL (40 and 80 μg/mL) (Table 1). It has been demonstrated that phosphoinositide breakdown can induce TxA_2 formation *via* free AA release by DG lipase or by endogenous phospholipase A_2 (PLA_2) from membrane phospholipids (Fig. 6) (25). Thus, it seems likely that TxB_2 formation plays a role in mediating the inhibitory effect of oxLDL on human platelets.

Activation of human platelets is inhibited by two intracellular pathways regulated by either cyclic AMP or cyclic GMP (26). The importance of cyclic AMP in modulating platelet reactivity is well established (22). In addition to inhibiting most platelet responses, elevated levels of cyclic AMP decrease intracellular Ca^{2+} concentrations by the uptake of Ca^{2+} into the DTS, which negatively affects the action of protein kinase C (Fig. 6) (26). Elevated platelet TxA_2 levels reportedly inhibited platelet membrane-associated adenylate cyclase, which lowers cyclic AMP levels in platelets (27). On the other hand, signaling by cyclic GMP somehow interferes with the agonist-stimulated phosphoinositide turnover that creates Ca^{2+}-mobilizing second messengers (28). In this study, we found that oxLDL did not induce NO formation in human platelets. This result is in accord with results of the cyclic GMP study, because the NO being produced is biologically active, since most cellular actions of NO occur *via* stimulation of intracellular guanylate cyclase, leading to an increase in cyclic GMP (28). Therefore, the inhibitory effects of oxLDL on collagen-induced platelet aggregation seem to

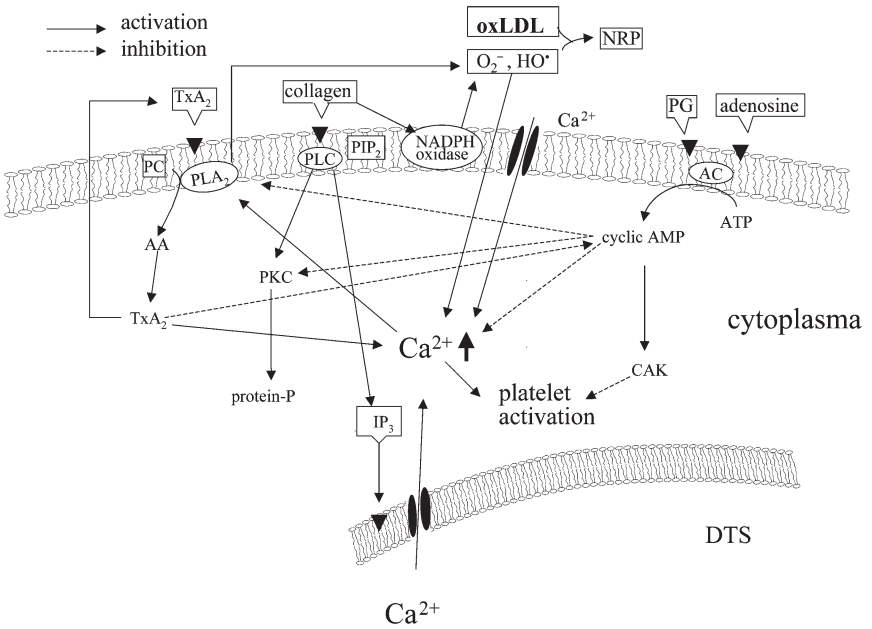

FIG. 6. Schematic hypothesis of the intracellular mechanisms of oxLDL on platelet aggregation. Agonists can activate several phospholipases, including phospholipase C (PLC) and phospholipase A_2 (PLA_2). Collagen or arachidonic acid (AA) stimulates the release of O_2^- and OH^- from platelets. oxLDL can react with O_2^- and OH^- to form nonradical products (NRP). Products of the action of PLC on phosphatidylinositol 4,5-bisphosphate (PIP_2) include 1,2-DAG and inositol 1,4,5-trisphosphate (IP_3). DAG stimulates protein kinase C (PKC), followed by phosphorylation of a 47-kDa protein (protein-P). IP_3 induces the release of Ca^{2+} from dense tubular systems (DTS). The major metabolite of AA in platelets is thromboxane A_2 (TxA_2). Cyclic AMP, cyclic 3′,5′-adenosine monophosphate; AC, adenylate cyclase; CAK, cyclic AMP-activated cyclic AMP-dependent protein kinase; for other abbreviation see Figure 1.

be mediated, at least partially, by an increase in cyclic AMP levels in human platelets.

Modification of LDL *in vitro* is usually performed by incubation of LDL with traces of transition metals (copper or iron). Kalyanaraman *et al.* (29) showed direct evidence for the formation of the α-tocopheroxyl radical and lipid radicals during the oxidation of LDL using the spin trap technique. Schneider *et al.* (30) also detected lipid radical formation in the Cu^{2+}-induced oxidation of LDL. Thus, it has been suggested that the oxidation of lipid present in LDL particles proceeds *via* a radical mechanism (30,31).

Lipid peroxidation is initiated by abstraction of bisallylic hydrogen (LH) from lipids by the peroxyl radical (ROO^-), thereby generating a lipid radical (L^-) and a peroxide (ROOH). In the presence of O_2, lipid peroxyl radicals (LOO^-) are formed, which stimulate lipid peroxidation (LOOH) *via* a chain reaction that produces many molecules of LOOH per ROO^- (32). Thomas and Stocker (32) found that these lipid radicals react with other radical oxidants, including the α-tocopheroxyl radical, Cu^{2+}, or the hydroxyl radical (OH^-), to form nonradical products (by the radical–radical termination reaction) (32). Furthermore, reactive oxygen species act as second messengers during the initial phase of platelet activation processes (33). Mirabelli *et al.* (34) showed an increase in cytosolic Ca^{2+} concentration upon platelet exposure to oxidative stress (Fig. 6). Platelets primed by exposure to subthreshold concentrations of AA or collagen are known to be activated by nanomolar levels of hydrogen peroxide, and this effect is mediated by hydroxyl radicals formed in an extracellular Fenton-like reaction (35). It is also evident that some of the hydrogen peroxide produced by platelets is converted into hydroxyl radicals, as platelet aggregation can be inhibited by hydroxyl radical scavengers (36). A recent study demonstrated that collagen-induced platelet aggregation is associated with O_2^- and OH^- formation, which is dependent on AA release and metabolism (Fig. 6) (37). In addition, the study also found that AA, but not ADP or thrombin, stimulates the release of O_2^- and OH^- from platelets (37). Our findings are also consistent with this observation, because the release of hydroxyl radicals by collagen-stimulated platelets was reduced to the resting level by oxLDL (Fig. 5).

In conclusion, the observations of this study suggest that oxLDL inhibits collagen- and AA-induced human platelet aggregation. The inhibitory effect of oxLDL may induce the radical–radical termination reaction by the interaction of oxLDL-derived lipid radicals with the free radicals (such as hydroxyl radicals) released from activated platelets, with a resultant lowering of intracellular Ca^{2+} mobilization, followed by inhibition of PLA_2 activation and of TxA_2 formation, thereby leading to increased cyclic AMP formation, and finally to further inhibition of $[Ca^{2+}]i$ mobilization and platelet aggregation. This study provides new insights concerning the effects of oxLDL on platelet aggregation.

ACKNOWLEDGMENT

This work was supported by a grant from the National Science Council of Taiwan (NSC 92-2320-B-038-026).

REFERENCES

1. Leeuwenburgh, C., Hardy, M.M., Hazen, S.L., Wagner, P., Ohishi, S., Steinbrecher, U.P., and Heinecke, J.W. (1997) Reactive Nitrogen Intermediates PromoteLow-Density Lipoprotein Oxidation in Human Atherosclerotic Intima, *J. Biol. Chem. 272*, 1433–1436.
2. Berliner, J.A., and Heinecke, J.W. (1996) The Role of Oxidized Lipoproteins in Atherogenesis, *Free Radic. Biol. Med. 20*, 707–727.
3. Gerrity, R.G. (1981) The Role of the Monocyte in Atherogenesis. I. Transition of Bloom-Borne Monocyte into Foam Cells in Fatty Lesions, *Am. J. Pathol. 103*, 181–185.
4. Weksler, B.B., and Nachman, R.L. (1981) Platelets and Atherosclerosis, *Am. J. Med. 71*, 331–333.
5. Tremoli, E., Colli, S., Maderna, P., Baldassarre, D., and Di Minno, G. (1993) Hypercholesterolemia and Platelets, *Semin. Thromb. Hemost. 19*, 115–121.
6. Ardlie, N.G., Selley, M.L., and Simons, L.A. (1989) Platelet Activation by Oxidatively Modified Low-Density Lipoproteins, *Atherosclerosis 76*, 117–124.
7. Andrews, H.E., Aitken, J.W., Hassall, D.G., Skinner, V.O., and Bruckdorfer, K.R. (1987) Intracellular Mechanisms in the Activation of Human Platelets by Low-Density Lipoproteins, *Biochem. J. 242*, 559–564.
8. Takahashi, Y., Chiba, H., Matsuno, K., Akita, H., Hui, S.P., Nagasaka, H., Nakamura, H., Kobayashi, K., Tandon, N.N., and Jamieson, G.A. (1996) Native Lipoproteins Inhibit Platelet Activation Induced by Oxidized Lipoproteins, *Biochem. Biophys. Res. Commun. 222*, 453–458.
9. Katzman, P.L., Bose, R., Walker, S., Perry, Y., and Bolli, P. (1991) Temperature-Dependence of LDL Binding and Activation of Human Platelets, *Thromb. Res. 64*, 503–508.
10. Block, L.H., Knorr, M., Vogt, E., Locher, R., Vetter, W., Groscurth, P., Qiao, B.Y., Pometta, D., James, R., Regenass, M., *et al.* (1988) Low-Density Lipoprotein Causes General Cellular Activation with Increased Phosphatidyl Inositol Turnover and Lipoprotein Catabolism, *Proc. Natl. Acad. Sci. USA 85*, 885–889.
11. Weidtmann, A., Scheithe, R., Hrboticky, N., Pietsch, A., Lorenz, R., and Siess, W. (1995) Mildly Oxidized LDL Induces Platelet Aggregation Through Activation of Phospholipase A_2, *Arterioscler. Thromb. Vasc. Biol. 15*, 1131–1138.
12. Katzman, P.L., Bose, R., Henry, S., McLean, D.L., Walker, S., Fyfe, C., Perry, Y., Mymin, D., and Bolli, P. (1994) Serum Lipid Profile Determines Platelet Reactivity to Native and Modified LDL-Cholesterol in Humans, *Thromb. Haemost. 71*, 627–632.
13. Pedreno, J., de Castellarnau, C., Cullare, C., Ortin, R., Sanchez, J.L., Llopart, R., and Gonzalez-Sastre, F. (1994) Platelet Receptor Recognizes with Same Apparent Affinity Both Oxidative and Native LDL, *Arterioscler. Thromb. Vasc. Biol. 14*, 401–408.
14. Vlasova, I.I., Azizova, O.A., and Lopukhin, Y.M. (1997) Inhibitor Analysis of LDL Induced Platelet Aggregation, *Biochemistry (Moscow) 62*, 307–311.
15. Vlasova, I.I. (2000) The Effect of Oxidatively Modified Low-Density Lipoproteins on Platelet Aggregability and Membrane Fluidity, *Platelets 11,* 406–414.
16. Sheu, J.R., Lee, C.R., Lin, C.C., Kan, Y.C., Lin, C.H., Hung, W.C., Lee, Y.M., and Yen, M.H. (1999) The Antiplatelet Activity of PMC, a Potent α-Tocopherol Analogue, Is Mediated Through Inhibition of Cyclooxygenase, *Br. J. Pharmacol. 127*, 1206–1212.
17. Dousset, N., Ferretti, G., Taus, M., Valdiguie, P., and Curatola, G. (1994) Fluorescence Analysis of Lipoprotein Peroxidation, *Methods Enzymol. 233*, 459–469.
18. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein Measurement with the Folin Phenol Reagent, *J. Biol. Chem. 193,* 265–275.
19. Sheu, J.R., Lee, C.R., Hsiao, G., Hung, W.C., Lee, Y.M., Chen, Y.C., and Yen, M.H. (1999) Comparison of the Relative Activities of α-Tocopherol and PMC on Platelet Aggregation and Antioxidative Activity, *Life Sci. 65*, 197–206.
20. Steinbrecher, U.P. (1987) Oxidation of Human Low-Density Lipoprotein Results in Derivatization of Lysine Residues of Apolipoprotein B by Lipid Peroxide Decomposition Products, *J. Biol. Chem. 262*, 3603–3608.
21. Sheu, J.R., Lee, C.R., Lin, C.H., Hsiao, G., Ko, W.C., Chen, Y.C., and Yen, M.H. (2000) Mechanisms Involved in the Antiplatelet Activity of *Staphylococcus aureus* Lipoteichoic Acid in Human Platelets, *Thromb. Haemost. 83*, 777–784.
22. Karniguian, A., Legrand, Y.J., and Caen, J.P. (1982) Prostaglandins: Specific Inhibition of Platelet Adhesion to Collagen and Relationship with Cyclic AMP Level, *Prostaglandins 23*, 437–457.
23. Hsiao, G., Shen, M.Y., Lin, K.H., Chou, C.Y., Tzu, N.H., Lin, C.H., Chou, D.S., Chen, T.F., and Sheu, J.R. (2003) Inhibitory Activity of Kinetin on Free Radical Formation of Activated Platelets *in vitro* and on Thrombus Formation *in vivo*, *Eur. J. Pharmacol. 465*, 281–287.
24. Hornby, E.J. (1982) Evidence That Prostaglandin Endoperoxides Can Induce Platelet Aggregation in the Absence of Thromboxane A_2 Production, *Biochem. Pharmacol. 31*, 1158–1160.
25. McKean, M.L., Smith, J.B., and Silver, W.J. (1981) Formation of Lysophosphatidylcholine in Human Platelets in Response to Thrombin, *J. Biol. Chem. 256*, 1522–1524.
26. Walter, U., Eigenthaler, M., Geiger, J., and Reinhard, M. (1993) Role of Cyclic Nucleotide-Dependent Protein Kinases and Their Common Substrate VASP in the Regulation of Human Platelets, *Adv. Exp. Med. Biol. 344*, 237–249.
27. Chen, S.Y., Yu, B.J., Liang, Y.Q., and Lin, W.D. (1990) Platelet Aggregation, Platelet cAMP Levels and Thromboxane B_2 Synthesis in Patients with Diabetes Mellitus, *Clin. Med. J. 103*, 312–318.
28. McDonald, L.J., and Murad, F. (1996) Nitric Oxide and Cyclic GMP Signaling, *Proc. Soc. Exp. Biol. Med. 211*, 1–6.
29. Kalyanaraman, B., Antholine, W.E., and Parthasarathy, S. (1990) Oxidation of Low-Density Lipoprotein by Cu^{2+} and Lipoxygenase: An Electron Spin Resonance Study, *Biochim. Biophys. Acta 1035*, 286–292.
30. Schneider, M., Jentzsch, A.M., Trommer, W.E., and Biesalski, H.K. (1998) EPR Kinetic Studies of the LDL Oxidation Process Driven by Free Radicals, *Free Radic. Res. 28*, 451–458.
31. Witting, P.K., Willhite, C.A., Davies, M.J., and Stocker, R. (1999) Lipid Oxidation in Human Low-Density Lipoprotein Induced by Metmyoglobin/H_2O_2: Involvement of α-Tocopheroxyl and Phosphatidylcholine Alkoxyl Radicals, *Chem. Res. Toxicol. 12*, 1173–1181.
32. Thomas, S.R., and Stocker, R. (2000) Molecular Action of Vitamin E in Lipoprotein Oxidation: Implications for Atherosclerosis, *Free Radic. Biol. Med. 28*, 1795–1805.
33. Iuliano, L., Colavita, A.R., Leo, R., Pratico, D., and Violi, F. (1997) Oxygen Free Radicals and Platelet Activation, *Free Radic. Biol. Med. 22*, 999–1006.
34. Mirabelli, F., Salis, A., Vairetti, M., Bellomo, G., Thor, H., and Orrenius, S. (1989) Cytoskeletal Alterations in Human Platelets Exposed to Oxidative Stress Are Mediated by Oxidative and Ca^{2+} Dependent Mechanism, *Arch. Biochem. Biophys. 270*, 478–488.
35. Pietraforte, D., Turco, L., Azzini, E., and Minetti, M. (2002) Online EPR Study of Free Radicals Induced by Peroxidase/H_2O_2

in Human Low-Density Lipoprotein, *Biochim. Biophys. Acta 1583*, 176–184.
36. Leo, R., Ghiselli, A., Iuliano, L., and Violi, F. (1995) Detection of Hydroxyl Radicals by the Salicylate *Bis*-Hydroxylation During Arachidonic Acid-Dependent Platelet Activation, *Thromb. Haemost. 73*, A347.
37. Caccese, D., Pratico, D., Ghiselli, A., Natoli, S., Pignatelli, P., Sanguigni, V., Iuliano, L., and Violi, F. (2000) Superoxide Anion and Hydroxyl Radical Release by Collagen-Induced Platelet Aggregation—Role of Arachidonic Acid Metabolism, *Thromb. Haemost. 83*, 485–490.

[Received December 12, 2003; accepted July 13, 2004]